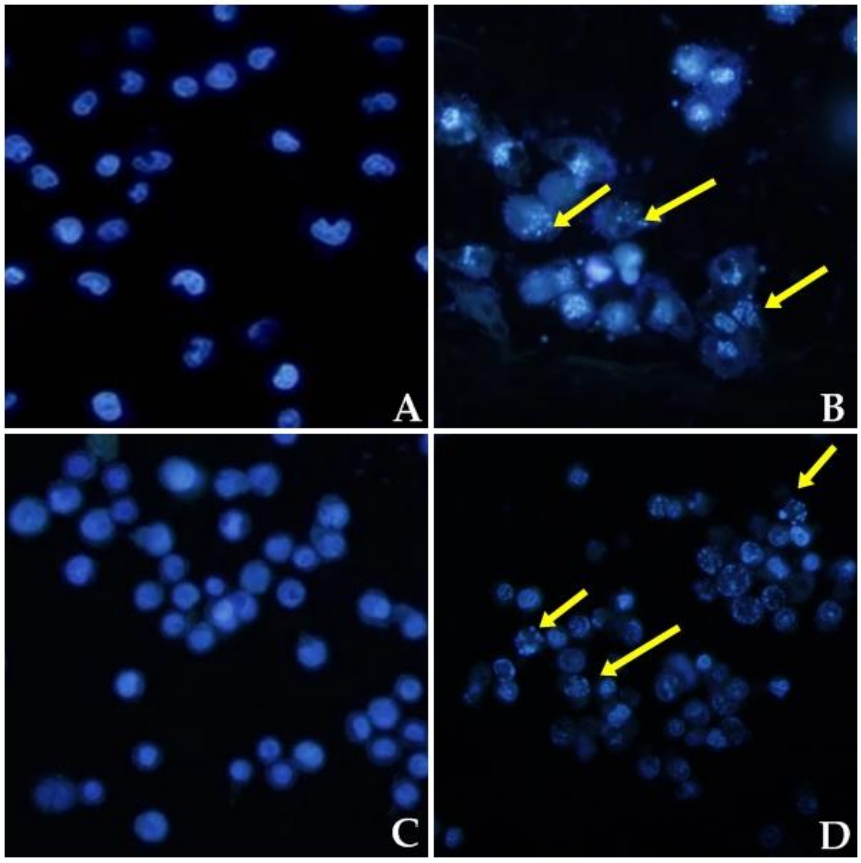
Applied Ecology and Environmental Research

International Scientific Journal



VOLUME 18 * NUMBER 6 * 2020

http://www.aloki.hu
ISSN 1589 1623 / ISSN 1785 0037
DOI: http://dx.doi.org/10.15666/aeer

IMPROVMENT OF GROWTH AND QUALITY AND REGULATION OF THE ANTIOXIDANT SYSTEM AND LIPID PEROXIDATION IN CHINESE CABBAGE (*Brassica pekinensis* (Lour.) Rupr.) BY EXOGENOUS SODIUM SELENITE

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(Received 15th Dec 2019; accepted 26th Mar 2020)

Abstract. Selenium (Se) is a necessary mineral nutrient for humans. In order to study the effects of exogenous sodium selenite on Chinese cabbage, an important food in people's daily life, the present study was conducted with four applications with different concentrations of sodium selenite, 0 (CK), 50 (Se1), 150 (Se2), 250 (Se3) mg L^{-1}. The results showed that the concentrations of Se, Fe and Zn in Chinese cabbage increased due to the Se application while the fresh weight and dry weight were affected beneficially by the treatment of Se just like chlorophyll contents. Foliar application of sodium selenite also remarkably increased the Vc, proline, protein and sugar contents. Furthermore, the foliar application of Se remarkably enhanced antioxidant enzyme activities of POD, SOD, CAT, APX and GR. In conclusion, exogenous sodium selenite would improve the growth and quality and enhance antioxidant resistance of Chinese cabbage.
Keywords: *chlorophyll, vegetable, selenium, crop quality, antioxidant enzymes*

Introduction

Selenium (Se) is one of the essential trace elements for human and animal growth. 72% of the Chinese counties (cities) are selenium deficient or low selenium areas, and two-thirds of the population suffer from selenium malnutrition or selenium deficiency (Tan et al., 2002; Xia et al., 2005). Previous research studies have proved that proper selenium supplementation is an effective measure to enhance human health, prevent diseases and prolong life (Rayman, 2000; Yang et al., 2017). Plants are the main source of selenium for humans and animals, thus the production of selenium - rich plants is an effective way to supplement human selenium.

As an important vegetable for the life of Chinese, Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.) is an indispensable delicious vegetable on the dining table of urban and rural residents in autumn and winter. Chinese cabbage not only possesses high contents of dry substances, vitamin C (Vc), protein and soluble sugar, but also has low acidity and crude fiber (Zhang et al., 2002; Su et al., 2018). Chinese cabbage also had a long history of cultivation and a large cultivated area in China (De Medici et al., 2019; Mi et al., 2019). Some previous studies have shown the beneficial effect of exogenous Se on Chinese cabbage performances. For example, the study of Wu et al. (2018) revealed that foliar application of Se selectively induced glutathione reductase (GR) and dehydroascorbic acid reductase (DHAR) in Chinese cabbage tissues and mitigated the cadmium toxicity. Dai et al. (2019) also used a year field experiment to find an interaction effect between soil application of Se and Zinc (Zn) on Chinese cabbage quality and antioxidant system through a pot experiment. However, the foliar field application of Se on Chinese cabbage was rarely reported the utilization rate of

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microelements would be much higher in foliar application than in soil application (Farooq et al., 2018).

Therefore, in order to improve the scientific database on factors affecting the quality and yield of Chinese cabbage and enhance the application of Se in Chinese cabbage production, present field experiment was conducted in Shandong province, China, in 2019 with two hypotheses, that are (1) foliar application of selenite would improve the growth and development of Chinese cabbage and (2) foliar application of selenite would promote the quality of Chinese cabbage.

Materials and Methods

Experimental details

The field experiment was conducted in Heze city of Shandong province, China. The experimental soil is sandy loam soil and the pH value was 6.7, and with 25.99 g kg^{-1} total nitrogen, 2.44 g kg^{-1} available phosphorus and 16.90 mg kg^{-1} available potassium. Four concentrations of sodium selenite were applied as follows: overhead sprinkling with 0, 50, 150, and 250 $mg \cdot L^{-1}$ and these treatments were known as CK, Se1, Se2, Se3, respectively. The treatments were arranged in a randomized complete block design (RCBD) in triplicate. One day before harvest, three representative plants of each treatment were collected for the bio-chemical indexes including nutritional components, antioxidative enzymes, chlorophyll, malondialdehyde, Se, Fe and Zn. At harvest, ten random Chinese cabbage plants were collected from each treatment to measure the fresh weight and dry weight.

Estimation of antioxidative enzymes, malondialdehyde and chlorophyll

The activities of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and the malondialdehyde (MDA) were measured according to the methods described by Luo et al. (2018). The chlorophyll a, chlorophyll b and carotenoid contents were estimated by the methods of He et al. (2019). The activities of ascorbate peroxidase (APX) and glutathione reductase (GR) were determinate according to Dai et al. (2015).

Estimation of nutritional components of Chinese Cabbage

The protein and proline contents were determinate by the methods of Li et al. (2016) using Coomassie brilliant blue G-250 and ninhydrin colorimetry, respectively. The content of vitamin C (Vc) was estimated through titration with 2,6-dichlorophenolindophenol and the sugar content was measured by anthrone method according to Ma et al. (2014).

Determination of Se, Fe, Zn contents

The plant samples were ground into powder form and digested (0.1 g) with 1.5 ml of concentrated HNO_3 and 0.5 ml 30% H_2O_2 in a digestive stove at 180°C for 1.5 h. Total Se, Fe and Zn content in samples were determined by using atomic absorption spectrometer (Z2300, HITACHI, Japan)-HFS (HFS-3, HITACHI, Japan).

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Statistical analysis

Data were analyzed on Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) while differences among means were separated by using least significant difference (LSD) test at 5% probability level. Graphical representation was conducted via Sigma Plot 14.0 (Systat Software Inc., California, USA).

Results

Biomass

Foliar application of sodium selenite had impacts on fresh weight and dry weight of Chinese cabbage (*Figure 1*). Compared with CK, Se1 treatment significantly increased the fresh weight and dry weight by 36.26 and 47.70%, respectively. However, there was no remarkable difference observed among CK, Se2 and Se3 in both fresh weight and dry weight of Chinese cabbage.

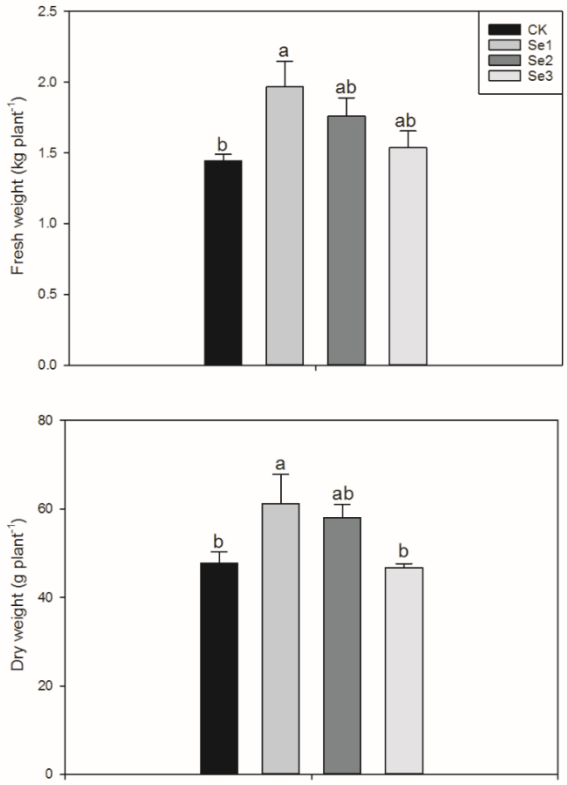

Figure 1. *Effects of exogenous sodium selenite on fresh weight and dry weight in Chinese cabbage. Capped bars represent S.E. of three replicates. Means sharing a common letter do not differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

Antioxidative enzymes and lipid peroxidation

Foliar application of sodium selenite regulated the activities of POD, SOD, CAT, GR, APX and contents of MDA in Chinese cabbage (*Figure 2*). Compared to CK, Se1, Se2 and Se3 treatments significantly increased POD activities by 103.36%, 56.83% and 16.63%, respectively. For SOD activity, 31.86% and 12.67% higher activities were

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recorded in Se1 and Se2 treatments than in CK whiles there was no remarkable difference between CK and Se3. Compared to CK, Se1, Se2 and Se treatments significantly increased CAT activities by 104.32%, 51.57% and 13.10%, respectively. For APX activities, 190.78%, 101.59% and 39.15% higher activities were recorded in Se1, Se2 and Se3 treatments compared with CK, respectively. Compared to CK, Se1, Se2 and Se3 treatments significantly increased GR activities by 120.71%, 63.71% and 20.95%, respectively. For the contents of MDA, Se1, Se2 and Se3 treatments significantly increased MDA contents compared with CK whilst there was no remarkable difference among Se1, Se2 and Se3 in MDA contents.

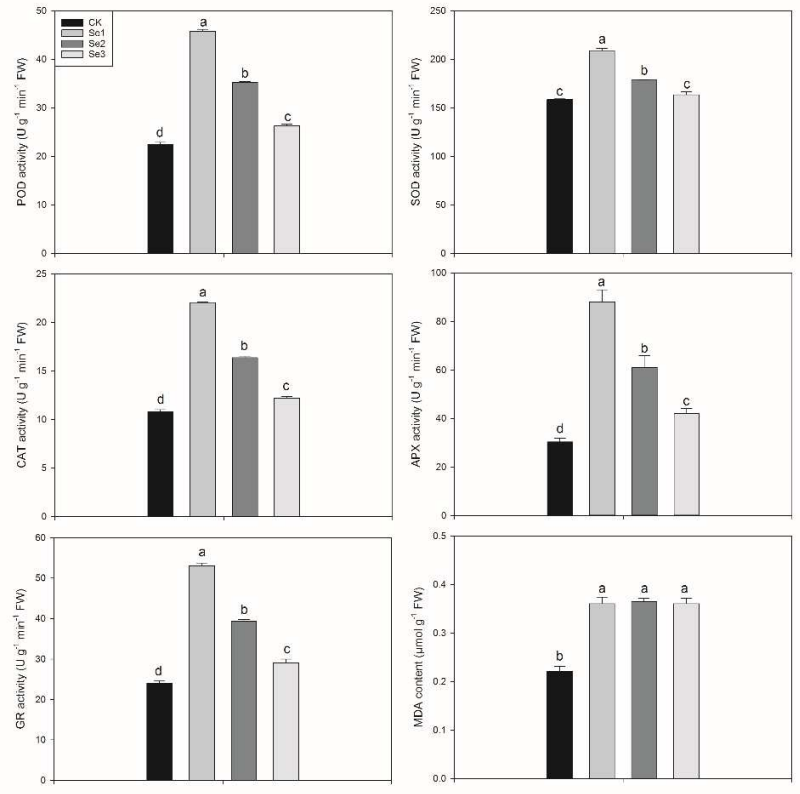

Figure 2. *Effects of exogenous sodium selenite on activities of POD, SOD, CAT, GR, APX and contents of MDA in Chinese cabbage. Capped bars represent S.E. of three replicates. Means sharing a common letter do not differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

Chlorophyll content

As shown in *Figure 3*, foliar application of sodium selenite significantly affected the contents of chlorophyll a, chlorophyll b and carotenoids in Chinese cabbage. Compared with CK, Se1, Se2 and Se3 treatments all significantly increased the chlorophyll a content while the highest content was recorded in Se1 treatment. Similar trends were also found in both chlorophyll b and carotenoids contents.

Nutritional components

Foliar application of sodium selenite regulated the contents of proline, protein, Vc and sugar in Chinese cabbage (*Figure 4*). Compared to CK, Se1 and Se2 significantly

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increased proline content by 40.16% and 26.18%, respectively. For Vc content, 27.07%, 17.56% and 10.46% higher contents were recorded in Se1, Se2 and Se3 treatments than in CK, respectively. Compared to CK, Se1, Se2 and Se treatments significantly increased protein content by 48.43%, 31.08% and 18.12%, respectively. For sugar content, 65.97%, 45.00% and 25.44% higher values were recorded in Se1, Se2 and Se3 treatments compared with CK.

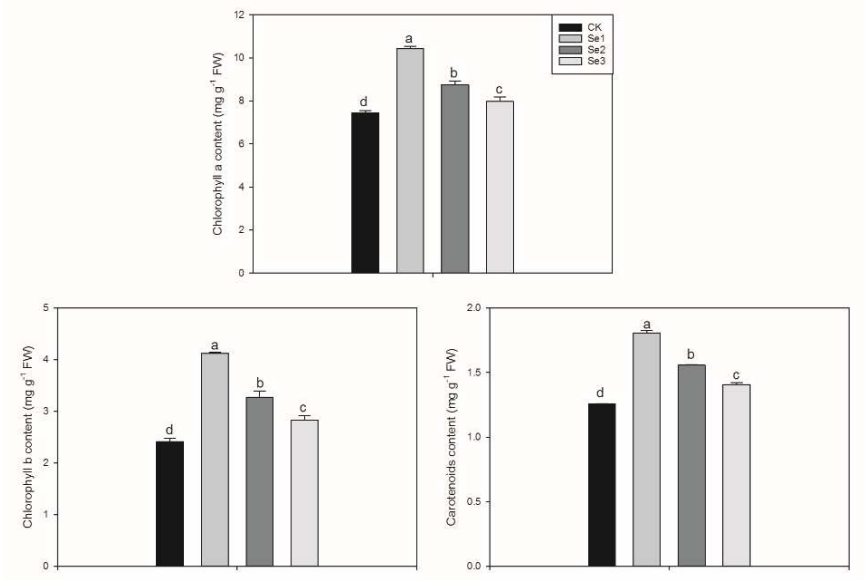

Figure 3. *Effects of exogenous sodium selenite on contents of chlorophyll a, chlorophyll b and carotenoids in Chinese cabbage. Capped bars represent S.E. of three replicates. Means sharing a common letter do not differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

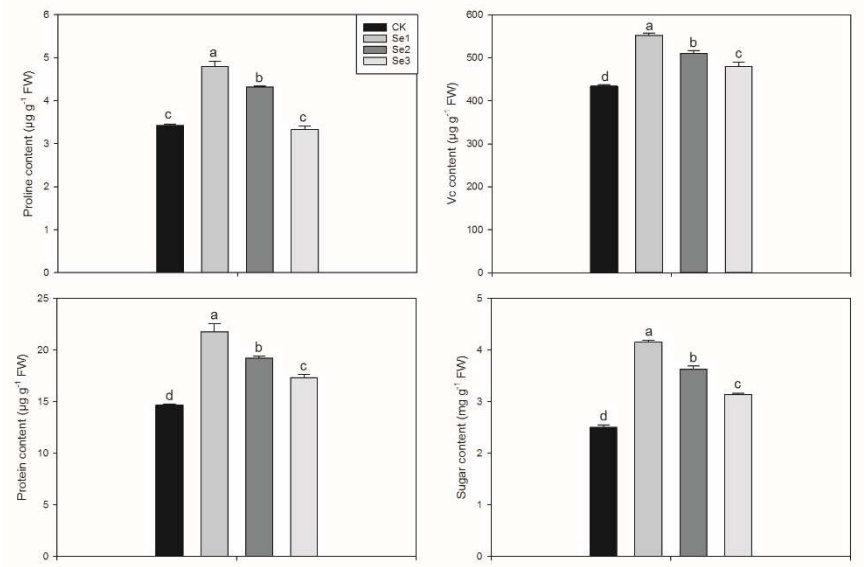

Figure 4. *Effects of exogenous sodium selenite on contents of proline, protein, Vc and sugar in Chinese cabbage. Capped bars represent S.E. of three replicates. Means sharing a common letter do not differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

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Se, Fe and Zn content

As shown in *Figure 5*, foliar application of sodium selenite has some impacts on Se, Fe and Zn concentrations in Chinese cabbage. The Se content increased with the increment of Se applied concentrations and the highest content was recorded in Se3 treatment. However, the Fe content showed a different trend compared to Se. The highest content of Fe was recorded in Se1 treatment while the trend was recorded as: Se1 > Se2 > Se3 > CK. Similar trend was also observed in Zn content.

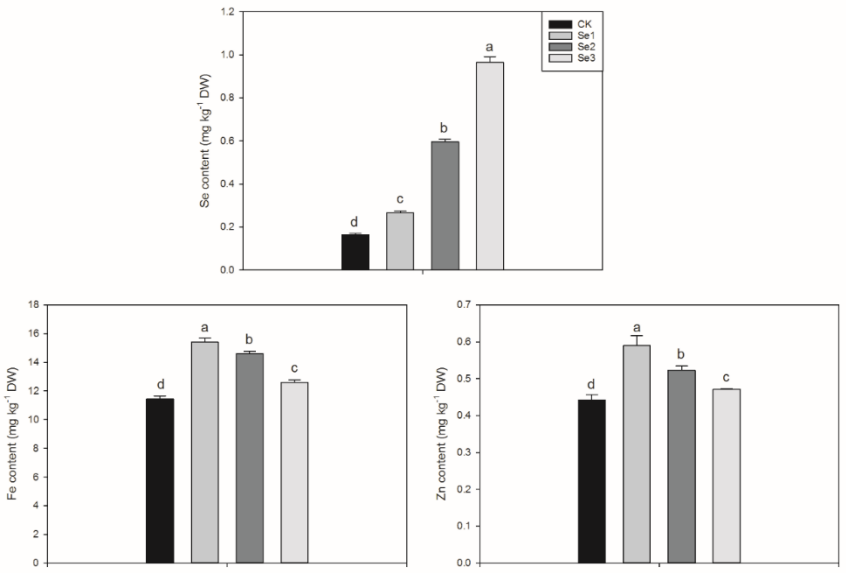

Figure 5. *Effects of exogenous sodium selenite on Se, Fe and Zn content in Chinese cabbage. Capped bars represent S.E. of three replicates. Means sharing a common letter do not differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

Analysis of variance

As shown in *Table 1*, foliar application of Se had significant effects on activities of POD, SOD, CAT, APX, GR as well as MDA content. The contents of chlorophyll a, chlorophyll b and carotenoid were significantly affected by Se application. In addition, proline, Vc, protein and sugar contents and contents of Se, Fe and Zn were significantly affected by Se application. However, foliar application of Se had no significant effect on fresh weight and dry weight of Chinese cabbage.

Table 1. *Variance analysis about the effects of Se on Chinese cabbage performances*

Parameter	*F* values	Parameter	*F* values
Fresh weight	2.61ns	Chlorophyll b	58.45**
Dry weight	3.04ns	Carotenoid	306.39**
POD	817.81**	Proline	66.92**
SOD	144.74**	Vc	120.95**
CAT	802.02**	Protein	41.59**
APX	35.53**	Sugar	199.71**
GR	465.57**	Se content	455.59**
MDA	37.32**	Fe content	76.01**
Chlorophyll a	78.15**	Zn content	20.63**

F value and significance level (**P < 0.01, *P < 0.05 and ns P ≥ 0.05)

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Discussion

Present study made the first attempt about the foliar application of Se in Chinese cabbage production and revealed the beneficial effects of exogenous Se on biomass, photosynthetic pigments, quality and antioxidant system of Chinese cabbage. The results supported both hypothesizes that foliar application of sodium selenite improves growth and development as well as quality of Chinese cabbage. At the most fundamental level, foliar application of sodium selenite increased the Se content in Chinese cabbage and the Se content increased with the increment in the applied concentrations. On the other hand, the foliar application of Se significantly increased both Zn and Fe contents and the highest Zn content and Fe content were both recorded in Se1 treatment. This result indicated that exogenous Se would enhance the absorption of Chinese cabbage to Zn and Fe which was consistent with the studies of Dai et al. (2019) and Fang et al. (2008).

As expected, foliar application of sodium selenite (Se1 treatment) produced the Chinese cabbage with higher fresh weight and dry weight. The biomass was enhanced probably due to the increments in photosynthetic pigment contents including chlorophyll a, chlorophyll b and carotenoids. Our results agreed with the study of Lai et al. (2019) who demonstrated that foliar application of Se promoted the yield of fragrant rice by increasing the chlorophyll content and enhancing photosynthesis. The investigation of Wu et al. (2000) also showed that exogenous Se applied with low concentration would promote the electron transfer rate of chloroplast in paddy rice.

Besides yield, the quality of Chinese cabbage is a determinant factor in economic returns for farmers. The quality of Chinese cabbage is influenced by parameters including protein, sugar, Vc and amino acid such as proline. Present study revealed that foliar application of sodium selenite would affect the contents of those nutritional components. There were increases in the contents of proline, protein, Vc and sugar. From the results, it can be concluded that foliar application of sodium selenite clearly increased Chinese cabbage yields and improved quality.

Interestingly, we observed that exogenous selenite application showed some antioxidant properties in Chinese cabbage according to the activities of antioxidant enzymes such as POD, SOD, CAT, APX and GR. Our study coincides with the research studies of Golubkina et al. (2019) and Zimmermann and Kohrle (2002) who indicated that element Se plays an important role in immune-modulating and antioxidant properties in both mammals and plants, by defending the organism from different kinds of oxidative stress. In our study, the activities of POD, SOD, CAT, APX and GR increased due to the foliar application of sodium selenite. This result was consistent with the study of Dai et al. (2019) who also found antioxidative enzyme activities and lipid peroxidation were positive or beneficial with the application of Se.

Present study not only improved the scientific database about the effects of Se on crops, but also enhanced the application of Se in Chinese cabbage production. However, more studies are required at physiological and molecular level to reveal the mechanism about how Se affects Chinese cabbage.

Conclusion

Foliar application of sodium selenite not only significantly improved growth (Fresh weight, dry weight) by increasing the contents of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid), but also promoted Chinese cabbage nutrient quality including Vc, proline, protein, sugar and microelements such as Se, Fe and Zn.

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Furthermore, the foliar application of Se remarkably enhanced the antioxidant system of Chinese cabbage in terms of POD, SOD, CAT, APX and GR activities. In order to reveal the mechanism about how Se affects Chinese cabbage, more studies should be conducted at physiological and molecular level

Acknowledgements. This study was supported by Shandong province key research and development project (2019GNC106121).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_74737481

IRON TOXICITY, TOLERANCE AND QUANTITATIVE TRAIT LOCI MAPPING IN RICE; A REVIEW

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(Received 14th Feb 2020; accepted 29th Jul 2020)

Abstract. Rice being a big staple food, faces many abiotic stresses, resulting in a yield reduction. Iron (Fe) has many key roles for plants in sustaining growth and production, however, Fe toxicity is a big threat to rice production worldwide. There are many regulation mechanisms in rice to ensure an adequate supply of Fe to plant and to prevent deleterious effects. Rice is adopted many physiological and molecular mechanisms to cope with Fe toxicity in flooded soils, however, rice tolerance to Fe toxicity varies greatly and this mechanism has not fully understood. Efforts are being made to unfold the genetic basis of Fe toxicity tolerance in rice. There are many regulators discussed here, which are responsible for Fe uptake and transport from rhizosphere to the plants. Toxic effects of Fe on rice, the genes advocating the regulation of Fe, and many quantitative traits loci governing Fe toxicity tolerance in rice are discussed here. Many putative QTL involved in rice tolerance to Fe toxicity are presented in this review. The identification of QTL and regulators for Fe toxicity tolerance would be more helpful in regulating Fe toxicity and developing Fe tolerant lines in rice. More efficient breeding techniques are required to screen Fe tolerant rice genotypes. This review focused on some possible ways to improve Fe toxicity tolerance in rice and provide a strong theoretical base for future research.
Keywords: *iron; toxicity, genes, QTL, regulation, rice, mapping population*

Abbreviations: AB: Abscisic Acid, NSP: Number of spikelet's/plant, BIL: Backcross inbred lines, OA: Organic Acid, BRILs: Backcross recombinant inbred lines, QTL: Quantitative trait loci, CC: Chlorophyll content, RIL: Recombinant inbred lines, Chr: Chromosomes, RL: Root length (cm), Cm: Centimeter, ROS: Reactive oxygen species, CSSL: Chromosomal segment substitution liens, DH: Double haploid, RDW: Root dry weight (mg), Fe: Iron, SWC: Shoot water content, GW: Grain weight, SL: Shoot length (cm), IL: Introgression line, LBI: Leaf bronzing index, MG: Magic population.

Introduction

Rice is the main cereal and staple crop for almost 50% of the world's population (Mahender et al., 2019; Rasheed et al., 2020a). Rice yield is expected to increase by 100 tons to feed the 9.1 billion population of the world by 2050 (Jaggard et al., 2010). Rice crop is facing many abiotic and biotic stresses including drought, heat, salinity, cold and nutrient deficiencies and most importantly the Fe toxicity (Mahender et al., 2019; Rasheed et al., 2020b). Fe toxicity is one of the leading restrictions for rice growth in many soil and a lot of studies have been conducted on understanding the genetic base of this stress (Bashir et al., 2014; Mahender et al., 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_74837498

Fe has many significant functions in rice-like, photosynthesis, homeostasis, and mitochondrial respiration (Nakanishi et al., 2006; Kim and Guerinot, 2007; Li et al., 2017). About 18% of soils globally are suffering from Fe toxicity and Fe deficiency (Saikia and Baruah, 2012; Das and Roychoudhury, 2014; Dufey et al., 2015), which results in a change in soil pH, soil fertility status and many other alterations in soil properties (Audebert and Sahrawat, 2000; Audebert, 2006). In many crops 50% reduction in grain yield has been reported owing to Fe toxicity, however, complete crop failure has also been observed during the early growth stage. Fe toxicity usually affects rice shoot length (SL), root length (RL) and also results in leaf bronzing which is a primary symptom of Fe toxicity in rice (Dufey et al., 2009).

Fe toxicity often occurs in alkaline soils, which leads to rise in pH, an increase in the amount of calcium carbonate and nitrate, a change in temperature and poor aeration (Kobayashi et al., 2014; Mongon et al., 2017). Several factors such as poor drainage, soil organic matter content, more hydrogen sulfides, low soil fertility and genotypes lead to an increase in available forms of Fe in soil (Chandel et al., 2010; Mahender et al., 2019). Rice plants adopted several mechanisms to cope with Fe toxicity in soil, like reducing Fe uptake and chelation through chelating agents, trafficking and storing in less responsive type, but most important is the expression of resistance genes (Dufey et al., 2009; Zhang et al., 2017).

QTL mapping is one of the powerful approaches to identify the genes of interest on the chromosome. In order to locate the gene of interest on chromosome, we need to screen genotypes of rice against Fe toxicity. A lot of QTL have been reported in rice for Fe toxicity tolerance using several mapping populations (Jain and Connolly, 2013; Zhang et al., 2017; Meng et al., 2017). Breeding Fe resistant varieties is an economically important approach to enhance rice production under Fe toxicity stress and Fe toxicity is controlled by many genes in rice (Dufey et al., 2012; Wainaina et al., 2018). A lot of genes have been reported in rice which is responsible for rice tolerance to Fe toxicity and these genes belong to five major protein families (OsFROs and OsFERS) (Chandel et al., 2010). Most of QTL reported in rice against Fe toxicity tolerance belongs to easily measurable traits, like seedling length, root length, seedling fresh and dry weight (Zhao et al., 2013; Dufey et al., 2015; Liu et al., 2016; Meng et al., 2017). The understanding of the Fe tolerance mechanism in rice is important to develop rice varieties tolerant to Fe toxicity. In this review we discussed the recent advancements on rice tolerance to Fe toxicity and ways to improve rice production in Fe affected soils and moreover, a view on identified QTL for Fe toxicity tolerance in rice is also discussed here.

Role of Fe in plants and rice

Fe has many roles in plants such as nutrients transport, mitochondrial respiration, photosynthesis, regulation of several enzymes and nitrogen assimilation (Bashir et al., 2010; Wu et al., 2014; Brumbarova et al., 2015). The regulation of protein stability is also performed by Fe and it also takes part in many chemical reactions such as, hydration, dehydration, redox-dependent catalysis, photo redox catalysis which detoxify excessive Fe, thus, Fe is one of the essential mineral element for plants (Zhang et al., 2013; Jain and Connolly, 2013; Dufey et al., 2015). Fe accelerates many antioxidant defense mechanisms such as, superoxide dismutase, catalase, polyphenol oxidase which protects rice plants from oxidative damage, which helps to screen Fe tolerant rice

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cultivars (Saikia and Baruah, 2012; Pennock et al., 2015). The photosynthesis process is facilitated by Fe; owing to the fact 90% of Fe is present in plastids to maintain the structural integrity of the thylakoid membrane (Rout and Sahoo, 2015; Mahender et al., 2019).

Iron toxicity in rice

In the case of Fe toxicity, cell division occurs and leaves turned into white and results in stunted growth (Vejchasarn et al., 2016; Banakrt et al., 2017). An excessive amount of Fe results in cellular oxidative damage and leads to several changes in morpho-physiological and yield traits of rice (Hell and Stephan, 2003; Sikirou et al., 2015). Iron toxicity lead to blockage of important nutrients essential for rice growth (Audebert and Sahrawat, 2000; Nughara et al., 2016) and complete crop failure can occur if Fe toxicity becomes more severe (Audebert, 2006; Li et al., 2016). A surplus quantity of Fe is firstly responsible for Fenton reaction and it produces hydroxyl radicals (−OH) and reactive oxygen species (ROS), induce permanent injury to the membrane lipid, protein and genetic material. The ROS resulting in oxidized chlorophyll and successively decrease the photosynthesis, and leads to chlorosis (a major yield-reducing factor) (Mengel, 1995; Onaga et al., 2016).

How Fe toxicity arises is soil

Fe in soil under anaerobic condition converted from Fe^{3+} to Fe^{2+} due to low pH and becomes toxic for plants. Fe prevalently occurs in the soluble and reduced ferrous form (Fe^{2+}) due to low soil redox potential arising from anaerobic conditions, which are developed when soil microorganisms and plant roots deplete oxygen by respiration. The excessive Fe molecules are transported via xylem flow to the shoot leading to Fe toxicity which is one of the main nutrient disorder in rice (Frei et al., 2016; Van Ort, 2018).

Iron uptake, transport and assimilation in rice grains

Fe uptake, transport from root to shoot and grain are essential for normal plant growth. Fe can be transported in various forms through xylem and phloem including Fecitrate, DMA–Fe (III), and NA–Fe (II). *OsFRDL1* is involved in Fe homeostasis in rice via xylem. There are 18 putative *YSL* family genes in rice, out of them Os*YSL5-7, -14* and *-17* are mainly expressed at epidermis, cortex and stele of the Fe sufficient and Fe deficient rice roots. The expression of *OsYSL1-4, 9-11* and *-18* was not studied in roots. *OsYSL12* is expressed in cortex and stele in both Fe deficient and sufficient condition whereas, *OsYSL12* is expressed in the cortex and stele under Fe sufficient *OsYSL6* was expressed in the epidermis in under Fe-sufficient condition only (Inoue et al., 2008). Among these mutant genes, O*sYSL2*, *OsYSL-15* and *OsYSL18* have been studied in detail (Aoyama et al., 2009; Ishimaru et al., 2010). *OsYSL15* transports Fe^{2+}-DMA from the rhizosphere to the roots and is involved in internal Fe homeostasis. *OsYSL15* promoter driven GUS expression was only observed in leaf tissue but also at the flowering stage (Inoue et al., 2009). These all studies indicated that *OsYSL15* is involved in Fe transport and assimilation in rice grains. *OsYSL2* is also involved in Fe assimilation in rice seeds (Koike et al., 2004). *OsYSL15* and *OsYSL12* contributed

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DOI: http://dx.doi.org/10.15666/aeer/1806_74837498

widely for Fe translocation during germination. Some of the studies suggested that *OsYSL2* is important for Fe translocation in seeds (Nozoye et al., 2007). Fe distribution and localization in grains is shown in *Figure 1*.

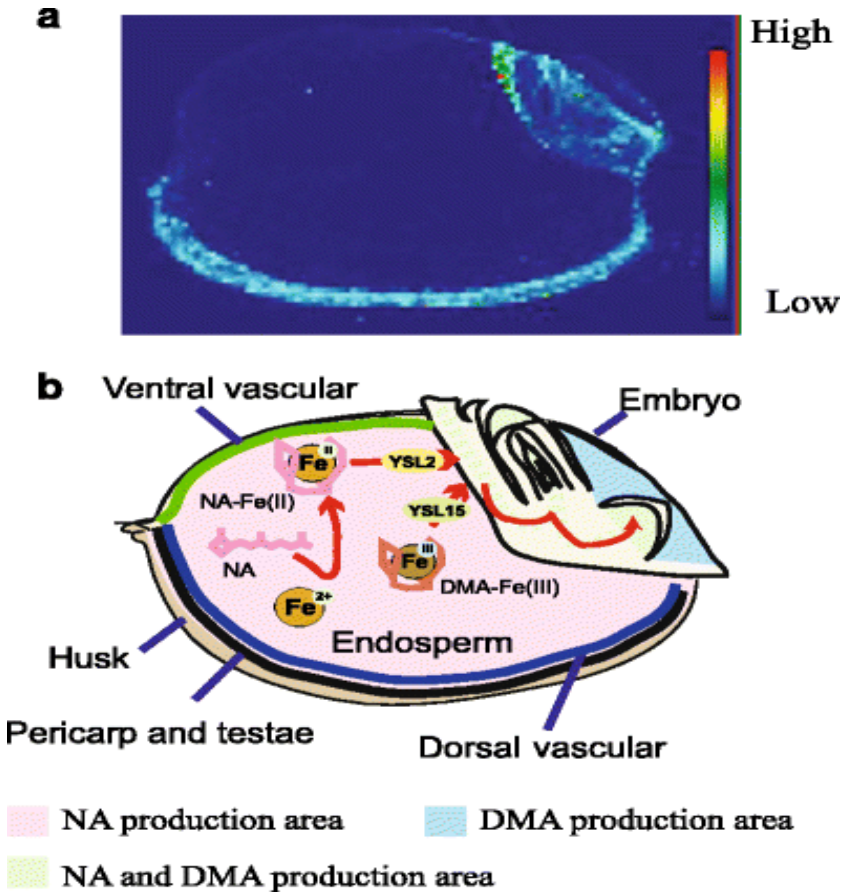

Figure 1. *Fe distribution, localization in seeds, and production of DMA, and NA, and iron distribution during germination. Taken from (Bashir et al., 2010)*

Iron toxicity tolerance mechanism in rice

Some studies give a shred of evidence; rice can be grown under Fe toxicity conditions without any loss in yield (Onaga et al., 2013; Mahender et al., 2019). There are three possible mechanisms of rice tolerance to Fe toxicity are documented; exclusion of Fe from root, storage in plant parts and tissues (Wan et al., 2003; Müller et al., 2015). The genotypes adopted three types of procedures to exclude Fe from roots, oxidation of Fe from root which result in low concentration of Fe in nutrient solution, elimination of Fe at root surface, and most remarkable strategy adopted by rice genotypes is formation of aerenchyma and lateral roots which provided low resistant pathway for oxygen transport into rhizosphere where iron is oxidized into less toxic form (Wu et al., 2019; Mahender et al., 2019). Many studies evidenced the tissue tolerance mechanism for rice tolerance against Fe toxicity (Hosseni et al., 2012; Muller et al., 2015; Frei et al., 2016), which is regulated by nitrous oxide signaling pathway, signaling storage proteins and enzymes, reactive oxygen species (ROS) and hormones etc. ROS are neutralized by nitrous oxide by its function (Darbani et al., 2013; Onaga et

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al., 2016). Nitrous oxide also regulates, kinase protein, calcium, cycling GMP, cyclic ADP-Rib, via S-nitrosylation of Cys deposits (Besson-Bard et al., 2009). N_2O also mediates Fe storage protein (ferritin) at both messenger RNA and protein level and an ARFAT an auxin-responsive element described which represented in 400 up-regulated genetic factors below Fe harmfulness (White and Brown, 2010). This mechanism is still unclear that either Fe regulates this auxin signaling protein or it is independent of Fe modulation and further studies are required on this aspect.

Iron storage in sub-cellular compartments

Once Fe is entered into the cell, there are certain steps inside the cell to stop Fe or mediate its toxicity. Many scavenging elements like nitrous oxide, DMA act as chelators of Fe and started scavenging of Fe at a sub-cellular level by forming a complex (Onaga et al., 2016). In rice the transporters of metals at inter and intracellular levels are categorized as in natural resistance-related protein (macrophage *NRAMP*) for cation carrier (Takahashi et al., 2011, Tan et al., 2019). *OsNRAMP1* is up-regulated by deficiency of Fe and *OsNRAMP7&8* showed a negative association with Fe concentration in shoots. *OsNRAMP1* most likely work as metal efflux carrier contributing in the transfer of metals from the section of vacuole to cystol and, *OsNRAMP7*, *OsNRAMP8* play role as metals influx protein which worked as sequestration of metals in the vacuole (Ogo et al., 2014). *OsNRAMP6* is regulated with *OsVIT1* in rice grown under surplus iron (Vivitha et al., 2017; Mahender et al., 2019). This showed that *NRAMP6* is responsible for transfer of Fe from cell to vacuoles and chloroplast, thereby increasing Fe toxicity tolerance in rice. *OsVIT2* is up-regulated in rice shoot and root in reaction to excessive Fe (Bashir et al., 2014), proposing that *OsVITI* & *OSVIT2* had a function in the compartmentalization of Fe (Wu et al., 2019). The transporter *VIT1* has been studied to be functional by organizing with *AtNRAMP3* and *AtNRAMP4*; the two isologs of Arabidopsis on the surface of the vacuolar membrane of roots and shoots under Fe deficiency (Onaga et al., 2016). *OsVIT1* and *OsVIT2* are more functional in rice when rice is grown under Fe toxicity stress. *OsVIT1* and *OsVIT2* are congested in rice under Fe shortage (Zhang et al., 2013). The unpredictable function of transporter (*VIT1*) in Arabidopsis and rice showed that *VIT1* also interacts with other regulators carry Fe in the vacuole, and function as diverting the release of Fe into cells of vacuole in absence of Fe through modifying the action of *AtNRAMP3* and *AtNRAMP4* (Zhao et al., 2013; Onaga et al., 2016). The transport *OsNRAMP5;* is existing in the plasma membrane and it is involved in reducing Fe concentration in roots and shoots through xylem (Ishimaru et al., 2012). Many plants carrying *OsNTAMP5i* store less Fe in shoot and xylem sap, indicating that this transporter cooperates with different other Fe transporters to regulate iron in shoots of rice (Morrissey and Guerinot, 2009; Wu et al., 2019; Mahender et al., 2019).

Chloroplast

Fe toxicity is also regulated in the chloroplast, where *FRO7* a member of *FRO* localized in plastids showed that Fe is reduced by *AtFR07* in rice and Arabidopsis and then taken by Fe^{2+} carrier. Inner membrane-localized permease is considered to be the main site for Fe distribution into chloroplast (Zhang et al., 2017). *OsFRO1* is connected to *AtFRO7*, and may show the same role converting Fe^{3+} to Fe^{2+}, in preparations for transport into the chloroplasts (Sperotto et al., 2010). The genes regulating ferritin have

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been recognized in various species of plants mainly in rice (Matthus et al., 2015). The up-regulation of genes in reaction to surplus Fe (Bashir et al., 2014; Onaga et al., 2016; Onyango et al., 2019) reveals that ferritin is effective paths of intracellular Fe in the rice (Onaga et al., 2016).

Mitochondrial chelation of Fe

The Fe can also be deposited in mitochondrial section where protein ferritin exists. (Lin et al., 2011) exhibited that over expression of a mitochondrial Fe carrier, mitochondrial RNA splicing (*MRS3*), in yeast over-whelms the expansion of Fe toxicity by declining cytosolic Fe via mitochondrial Fe increase. Orthologue of (*MSR3*) is a Fe carrier in mitochondria which was recognized as accountable for carrying Fe into mitochondria (Bashir et al., 2011; Mahender et al., 2019). The suppression of *MIT* lead to decrease in aconitase activity, specifying that mitochondrial Fe transporter is important for Fe s-cluster biogenesis in cytoplasm and mitochondria. Gross et al. (2003) and Bashir et al. (2014) have discovered the contradictory track for these genetic factors. The regulation of (*OsNAS1*, *OsNAS2*, *OsNAAT1*, and *OsNRAMP1*) was witnessed, possibly to avoid unnecessary uptake and toxicity of Fe. Five major protein families of Fe tolerance genes are *OsNRAMPs, OsFROs, OsZIPs, OsFERs* and *OsYSLs*. Fe toxicity and deficiency up and down regulated genes in rice are shown in *Figure 2*.

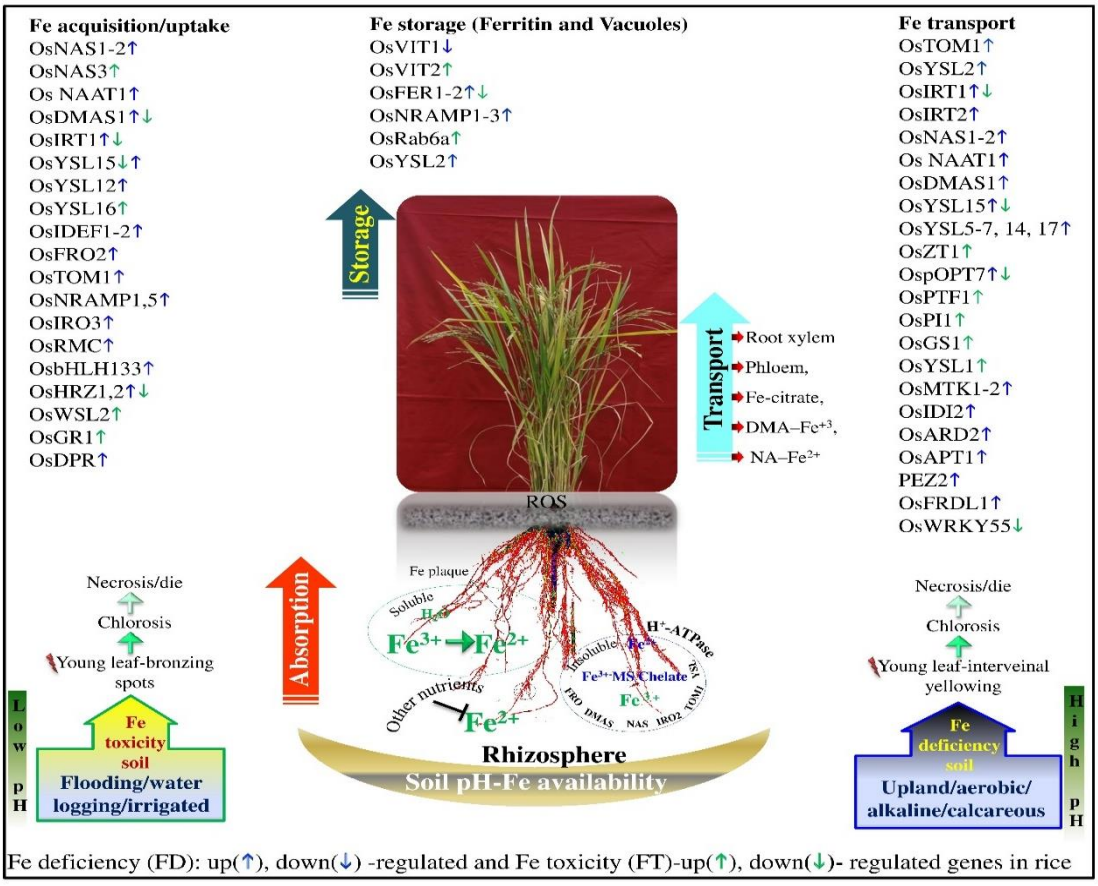

Figure 2. *Fe toxicity and deficiency up and down regulated genes showing alterations in levels of transcription through microarray and transcriptomics studies (Adopted from Mahender et al., 2019)*

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Regulation of Fe uptake

The regulation of Fe uptake is done by many *TFs* transcription factors and regulators including, signal transducers. Plants adopt strategy II in response to Fe deficiency, like enhance biosynthesis along with secretion of Fe^{3+} chelator named Mas (mugenic acid). Some enzymes, which are main target of transcription factors contributed to mugenic acids (Inoue et al., 2008). A gene (*OsFRO2*), cool with *OsNAAT1* and *OsVIT1* and protein *OsRMC* work as receptor, involved in Fe regulation in rice (Finatto et al., 2015; Onaga et al., 2016; Wu et al., 2019). The transcription factors prompted by Fe deficiency, (*OsIRO3 & OsbHL133*) were characterized in rice (Wang et al., 2012). Some other genes like (*IDEF1 & OsHRZ1-2*) were studied to catch Fe signals through binding of Fe and zinc and avoid surplus Fe uptake under sufficient Fe availability (Liu et al., 2016). Most meaningful is to conclude that there are some genes which can interact in vivo with *OsHRZ* and to find that this post-transcriptional modification can be helpful to enhance Fe toxicity tolerance in rice (Onaga et al., 2016; Mahender et al., 2019). Post-transcriptional regulation of Fe in rice has not fully understood, therefore, it will be significant to unfold the natural variation for *OsRT1* with stable disparity deposit replacement in its loop that may enhance tolerance to Fe toxicity in rice. The studies on link between amid the residue replacements in *OsIRT1* with action of *OsHRZ* and *IDEF1* can add significant benefit in studying post-translational alteration of Fe uptake in rice (Onaga et al., 2016). The earlier studies described the role of ABA (abscisic acids) and brassinosterioids, in regulation of IDE1 modulation of downstream target in Fe homeostasis single transductions mechanisms need to be unfold (Gallie, 2012; Pereira et al., 2014). Rice has a number of strategies to switch unnecessary Fe uptake.

Fe storage genes which are encoding *VOT*, *FPN2* transporter are well regulated and thus Fe may engage in old leaves. The similar genetic factors could be controlled in shoot of includer that positively stock Fe in plant aerial parts. Additionally, genes encoding *YSL4* and *YSL6* like Fe efflux carriers, could be controlled to release spare Fe from chloroplast, to stop oxidative damage. It would be valuable to detect important genes, with constant influence in reaction to the dissimilar kinds of Fe harmfulness, to use for breeding's schemes. The only one author reported the confirmed effects of Fe nutrition gene (*OsFR01*) in tolerance of Fe toxicity (Mahender et al., 2019), despite lot of research work has been accomplished. Transporters identified regarding Fe regulated genes and their function are shown in *Table 1*.

Table 1. *Transporters identified regarding Fe regulated genes and their function*

Genes	Position	Role	Reference
OsZIP4	Root	Zinc transporting proteins, Fe transport and homeostasis	(Quinet et al., 2012)
OsYSL2	Root	Fe-NA transport, Fe accumulation in seeds and translocation in grains	(Koike et al., 2004)
OSPIC1	Chloroplast	Transport Fe from root to chloroplast	(Zhang et al., 2012)
OsFRO2	Root	Alter Fe oxidation state	(Stein et al., 2009)
OsVIT1,2	Leaves/Seeds	Vacuolar Fe transporter	(Zhang et al., 2012)
OsMIR	Shoot	Fe homeostasis	(Ishimaru et al., 2010)
OsFER1	Aleurone layer	Vacuolar Fe transport and homeostasis	(Bashir et al., 2013)

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QTL identified for rice tolerance to Fe toxicity

The identification and isolation of QTL linked to Fe tolerance is a powerful way to improve the rice tolerance to Fe toxicity (Dramé et al., 2011; Mahender et al., 2019). A lot of mapping populations are used in many studies, which have identified many QTL in rice. Here we discussed some QTL identified previously and their role in iron toxicity tolerance. Wu et al. (2014) used a mapping population of RIL (recombinant inbred liens) derived from IR29/Pokkali and identified two putative QTL for leaf bronzing score *qFETOX-1-1* and *qFETOX-1-2* between the markers with 10.6% and 12% phenotypic variation. The LBI indicated the large genetic variation among the parental genotypes for tolerance to Fe toxicity. A variety of traits like, SL, RL, shoot dry weight (SDW) and root dry weight (RDW) are also used for estimation of Fe toxicity tolerance in rice. Two QTL for SL and RL were reported by Meng et al. (2017) using 873 RIL (recombinant inbred lines) derived from MAGIC populations. QTL *qSL-1* and *qRL-8* were identified with 18% phenotypic variation.

This trait indicated large genetic variability for Fe tolerance. Many genes were reported behind these QTL controlling Fe toxicity tolerance in rice. Dufey et al. (2010) used 164 RIL (recombinant inbred liens) population evaluated in multiple environment, and identified one putative QTL *qNSP-3* for number of spikelet's in rice which showed large variation for iron toxicity tolerance. Likewise, Dufey et al. (2012) identified two more QTL using same population for 1000 grain *qGW-1* weight and chlorophyll content *qCC1-7* with varying ratio of variance. Dufey et al. (2015) conducted a hydroponic experiment and evaluated 220 BC3DH (double haploid) and identified QTL, for shoot water content (SWC) *qSWC-3*, one for shoot dry weight (SDW) *qSDW-3* and one for leaf bronzing index (LBI) *qLBI-1* which strongly demonstrated that all of these QTL had significant role in rice response to Fe toxicity tolerance. Liu et al. (2016) identified two QTL using IL (Introgression lines) population derived from japonica (02428) and indica (Minghui63) and reported two QTL, *qRSDW-11*, *qRRDW-2* independent of genetic background. A QTL *qSDW-5* was identified recently which expressed under both experimental conditions with similar additive effects suggesting the genetic overlap between Fe toxicity tolerance and zinc toxicity tolerance in rice (Zhang et al., 2013). Zhang et al. (2017) identified a putative QTL *qSFW-2* with a positive additive effect which showed that the genes behind this QTL were contributed from the donor parent. These all are reported QTL for Fe toxicity tolerance and more breeding strategies and efficient screening techniques are required for the identification of tolerant genotypes of rice. Some of the putative QTL identified under Fe stress in rice are shown below in *Table 2*. *Figure 3* showed the way of novel phenotypic screening techniques to enhance Fe toxicity tolerance in rice genotypes.

Novel breeding and screening techniques for Fe toxicity tolerance in rice

Several molecular breeding techniques are used by researchers to improve Fe tolerance in rice. Molecular breeding aims to identify and transfer genes for improving heavy metals tolerance is more reliable technique. Marker assisted selection (MAS) includes using of markers for construction of linkage map to identify the putative genes and to clone the genes for speed up molecular breeding to enhance Fe tolerance in rice. This breeding approach is becoming more and more beneficial which more efficient and time saving approach is as compare to conventional breeding approaches. Use of backcross recombinant inbred lines (BRILs) populations is ideal population for targeted

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DOI: http://dx.doi.org/10.15666/aeer/1806_74837498

gene cloning and to speed up molecular breeding for improving Fe tolerance in rice (Rasheed et al., 2020a). An effective screening technique is required to characterize the resistance of genotypes at seedling stage. Seedling stage is ideal stage to identify the genotypes against different metals stress. One of the best way is to use preliminary screening technique to evaluate the genotypes against different levels of Fe toxicity. Parents and population should be grown in hydroponic condition and expose to different level of stress at seedling stage. In this way most effective dose of stress would be determined and that can be further used to screen the genotypes. Resistant genotypes could be selected for different seedling traits which are indicators of metals tolerance (Rasheed et al., 2020a). Resistant genotypes could be used for QTL mapping which lead to MAS selection. Therefore, this screening technique is needed for effective QTL mapping.

Table 2. *Putative QTL identified in rice during exposure to Fe stress*

Parents	Population	Traits	Marker	Chr	QTL	PVE%	Reference
glaberrim/ Caiapo	220 BC3DH	SWC	RM-251-RM238	3	*qSWC-3*	5.1	(Dufey et al., 2015)
glaberrim/ Caiapo	220 BC3DH	SDW	RM-60-RM22	3	*qSDW-3*	5.3	(Dufey et al., 2015)
glaberrim/ Caiapo	220 BC3DH	LBI	RM208-RM266	1	*qLBI-1*	4.7	(Dufey et al., 2015)
Azucena/IR64	164RIL	1000GW	RM034-RM246	1	*qGW-1*		(Dufey et al., 2012)
Azucena/IR64	164RIL	CCI	RM324-RM118	7	*qCCI-7*	33.9	(Dufey et al., 2012)
Azucena/IR64	164RIL	NSP	RM132-RM231	3	*qNSP-3*		(Dufey et al., 2010)
MP	873RIL	SL	SNP	1	*qSL-1*	18	(Meng et al., 2017)
MP	873RIL	RL	SNP	8	*qRL-8*		(Meng et al., 2017)
IR29/Pokkali	RIL/CSSL	LBI	173SNP/83SSR	1	*qFETOX-1-1*	10.6	(Wu et al., 2014)
IR29/Pokkali	RIL/CSSL	LBI	173SNP/83SSR	1	*qFETOX-1-2*	12	(Wu et al., 2014)
02428/Minghui63	IL	RSDW	384 SNP	11	*qFRSDW-11*	10.95	(Liu et al., 2016)
02428/Minghui63	IL	RRDW	384 SNP	2	*qFRRDW-2*	8.68	(Liu et al., 2016)
Lemont/Teqing	BIL	SDW	308SNP	5	*qSDW-5*		(Zhang et al., 2013)
Indica	222 Indica-accessions	SFW	395,553 SNP	2	*qSFW-2*	10.2	(Zhang et al., 2017)

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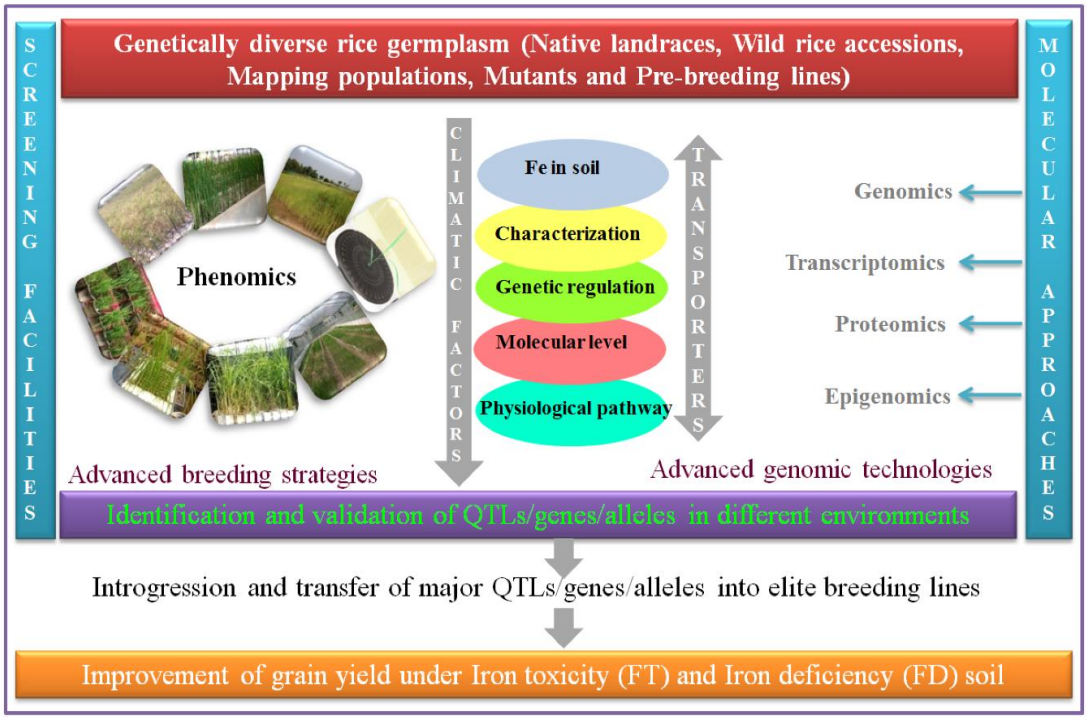

Figure 3. *A schematic way of presentation of innumerable phenotypic screening techniques and omics-based approaches to increase iron toxicity tolerance in rice cultivars (Taken from (Mahender et al., 2019)*

Wild relatives as potential source for Fe tolerance in rice

Wild relative constitutes a valuable gene pool that can be used to breed novel rice genotypes which can be tolerated to abiotic stresses like Fe toxicity. By transferring genes from these wild parents we can efficiently improve Fe toxicity tolerance in rice to sustain rice production. In a study conducted by Bierschenk et al. (2020) screened 75 rice genotypes, including 16 local genotypes, one glaberrima, and 58 wild genotypes which representing 21 species to study Fe toxicity tolerance. Plants were evaluated in green house and were treated with control and Fe stress during vegetative growth stage. Foliar Fe treatment were indicators of Fe toxicity during both stress treatments. Plants with chronic stress reduced yield due to spike fertility. Both wild and local genotypes showed variation in their response to Fe toxicity. Some of the wild relatives performed higher in individual traits towards Fe toxicity. These results showed that Fe toxicity can be improved by domestication of wild parents and to transfer their genes into domestic cultivars.

Conclusion

Fe toxicity is a polygenetic trait in rice, and there are many genetic and physiological basis of Fe toxicity tolerance. Moreover, many regulators are involved in Fe regulation at various levels. Here we provided a strong theoretical base of rice response to excessive Fe and genetic basis of Fe regulation in rice, genes and QTLs involving in regulation and tolerance of Fe toxicity. This review concluded that the use of molecular markers, high-resolution population, rice gene pool and wild relatives is a potential

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strategy to improve Fe toxicity tolerance in rice crop. Secondly many identified QTL discussed here are needed to transfer into susceptible lines through QTL pyramiding. Many Fe transporter is needed to identify to unfold their role in Fe toxicity and its regulation and more efficient breeding techniques are required to screen Fe tolerant rice genotypes.

Future perspectives

Fe is an essential element for rice and regulates rice growth when supplied in optimum concentration, but higher concentrations of Fe lead to induction of many deleterious changes in the crop which significantly reduces growth and production. There are certain levels in the cell, where Fe deficiency is regulated by using metabolic pathways. The physiological and biochemical basis of Fe regulation are clearly described in many plants but the molecular basis of Fe toxicity tolerance has not fully characterized. Here we provided a strong theoretical base of rice response to excessive Fe and genetic basis of Fe regulation in rice and genes involved in Fe toxicity tolerance. Many genes described here are associated with previously identified QTL and some of them are regulators that are controlled by *VIT*, *FNP2* like carriers, *MIT P1C1*, *FPN2* like carriers, *MIT*, *PIC1* transporters, ferritin, *OsFRO1, OsIRO3 & OsbHL133*, and *OsNRAMP7 & OsNRAMP8.* Many molecules responsible for Fe intra and inter cellular movement are still unidentified, linked to the huge number of unidentified genes in rice. Recently 3000 rice genomes were studied which highlighted the possibilities of validating these unannotated genes and identify their expression. The genome sequence for candidate gene can be accessed, moreover, the use of molecular markers that help in screening the Fe tolerant genotypes from susceptible one would be more helpful to accelerate genes pyramiding. The morphological, physiological and molecular basis of Fe toxicity tolerance would facilitate successful breeding if they are targeted to a large extent. The wild relatives of rice should be focused to screen out new sources of Fe toxicity tolerance and to transfer the novel genes in rice that could be exploited in rice breeding.

Acknowledgements. Authors are thankful to the Prof WU Ziming for his financial support during entire study. Authors are also thankful to the lab fellows for their support during manuscript preparation.

Funding. The research was supported by the National Natural Science Foundation of China (71963020 and 31760350), the National Key Research and Development Program of China (2018YFD0301102), the Jiangxi natural Science foundation（20181BAA208055 and 20202BABL205020), the Key Research and Development Program of Jiangxi Province (20171ACF60018 and 20192ACB60003), the Jiangxi Agriculture Research System (JXARS-18) and Projects of Water Science and Technology of Jiangxi Province (KT201628).

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DOI: http://dx.doi.org/10.15666/aeer/1806_74837498

USING SENTINEL IMAGE DATA AND PLOT SURVEY FOR THE ASSESSMENT OF BIOMASS AND CARBON STOCK IN COASTAL FORESTS OF THAI BINH PROVINCE, VIETNAM

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(Received 4th Mar 2020; accepted 10th Jul 2020)

Abstract. Quantitative evaluation of biomass and carbon stored in coastal forests of Thai Binh province (Vietnam) does not only contribute to coast protection, but is also required for emphasizing the forest's importance in local socio-economic development when participating in the carbon market. This estimation is primarily based on field data (diameter at breast height, height and quantity of tree in 14 surveyed plots), anatomical data (above ground biomass, total biomass and carbon content of 31 sampled trees) and an NDVI map calculated from Sentinel imagery. Mathematical models are used for expressing the relationships: above ground biomass vs. diameter at breast height, height; biomass vs. above-ground biomass; biomass vs. carbon; and above-ground biomass vs. NDVI. The results show the same behavior of above ground biomass, total biomass and carbon stock, they increase with the growth of tree. The exponential model is acceptable for correlating above ground biomass and NDVI, showing higher values in long planted areas and the lower ones in newly formed lands with recently planted mangroves. Biomass and carbon stock in the study area have average productivities of 38.8 t/ha and 19.4 t/ha, respectively. Overall estimations are ca. 292 kilotons of biomass and ca. 146 kilotons of carbon, roughly valuated of US$ 5.9 million.
Keywords: *AGB, NDVI, mangrove, remote sensing data, CO_2 valuation, Vietnam*

Introduction

Forests play a critical role in the Earth's climate system, in a number of different ways. Most importantly concerning global climate change, they capture CO_2 from the atmosphere, convert it into living biomass (stems, roots, branches and leaves) through photosynthesis and store large amounts of carbon in forest soils, absorbed through leaf litter, wood debris and roots (Brack, 2019). The estimates of carbon stored in forest ecosystems in the world vary significantly. In 2000, the Intergovernmental Panel on Climate Change estimated the total amount to be 1,100 gigatons (Gt) (Watson et al., 2000), which is 1.3 times greater than the carbon stored in fossil fuels (estimated at 800 Gt) and much larger than carbon released into the atmosphere from human activity since 1870 (about 600 Gt) (Federici et al., 2018). FAO (2010) yielded lower value of about 652 Gt, including 44%, 5%, 6% and 45% in live biomass, dry biomass, garbage and forest soils respectively. Therefore, a large amount of CO_2 will be released into the atmosphere from global deforestation. At the United Nations Climate Change Conference in Indonesia in December 2007, 187 member countries signed the Bali Agreement which outlined the program "Reducing Emissions from Deforestation and Forest Degradation" (REDD), with the aim to limit the destruction of tropical forests. Many countries will meet some of their emission reduction targets through the purchase

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

of carbon credits of developing countries made available by absorption of CO_2 by forests (United Nations Framework Convention on Climate Change - UNFCCC, 2007). So far, the carbon trading market has been divided into two types: the regulatory compliance and the voluntary markets. The regulatory compliance is the market in which carbon trading is based on the commitment of states in the UNFCCC to achieve the goal of reducing greenhouse gases. It is regulated by mandatory, mainly for Clean Development Mechanism (CDM) or Joint Implementation (JI) projects. The voluntary carbon market, outside the Kyoto Protocol framework, is based on bilateral or multilateral cooperation agreements between organizations, companies or countries. Thus, the problem posed here is to quantify the amount of CO_2 stored by forest ecosystems in developing countries, including Vietnam. This work, first of all, requires identifying the biomass and carbon accumulated in these forests.

Forest biomass could be estimated by allometric functions relating biomass and dimensions of tree or its components such as Diameter at Breast Height (DBH), tree height (H), trunk circumference, branches, roots, leaves, etc. (Clough and Scott, 1989; Comley and McGuinness, 2005; Basuki et al., 2009; Chave et al., 2014; Bao et al., 2016; Kebede and Soromessa, 2018). The CO_2 absorption of forest ecosystems is generally determined by direct measurement of physiological processes controlling carbon balance (Botkin et al., 1970; Woodwell, 1970), analyzing eddy correlation for quantifying net ecosystem exchanges of CO_2 (Wofsy et al., 1993), mathematical functions presenting the relationship between biomass, carbon stock and tree dimensions (Grier et al., 1989; Hunter et al., 2013; Ostadhashemi et al., 2014). Remote sensing data, including optical imagery (aerial photography, multispectral, and hyperspectral), Synthetic Aperture Radar (SAR) and Light Detection And Ranging (LiDAR) combined with field measurements, allometric equations, supported by GIS techniques are also used for estimating spatiotemporal variation of biomass and carbon stock in vegetation (Patenaude et al., 2004; Myeong et al., 2006; Jeyanny et al., 2011; Santoro and Cartus, 2018; Hirata et al., 2018; Pham et al., 2019). For the optical imagery, these estimations are usually based on regression models correlating Above Ground Biomass (AGB) and vegetation indices such as Normalized Difference Vegetation Index (NDVI), Green NDVI, Soil Adjusted Vegetation Index, Simple ratio, Red-edge simple ratio (Baloloy et al., 2018; Punalekar et al., 2018).

In Vietnam, biomass and forest productivity have also been studied for specific populations such as Rhizophora (Tri, 1986; Tan, 2001), *Pinus kesiya* (Phuc, 1996), *Acacia auriculiformis* (Thong, 1998), increased and diversified since the adoption of CDM (Lung and Van, 2004; Phuong, 2006; Hai et al., 2009; Trieu, 2010). Carbon stored in forest is also estimated and valuated (Ty, 2004; Nam, 2009; Tin and Loi, 2015). Recently remote sensing is applied for estimating biomass and carbon stock in mangrove (Vu et al., 2014; Pham and Yoshino, 2017; Quang and Hoa, 2018; Luong et al., 2019). For carbon trade within framework of Kyoto Protocol, there are numerous limitations impeding investments in Vietnam such as: inadequacies in approval process, lack of openness and transparency in mechanisms of financial allocation, etc. Vietnam currently has several projects towards carbon market (Lung and Van, 2004; Ty, 2004). In general, most of the studies focused on forest ecosystems in hilly and mountainous areas or mangroves in the coastal plain of southern Vietnam.

This study to evaluate the biomass and carbon stocks in coastal forests of Thai Binh province, belonging to the Red River Delta, not only fills up the lacking area but also contributes to the protection and development of these ecosystems, orienting to

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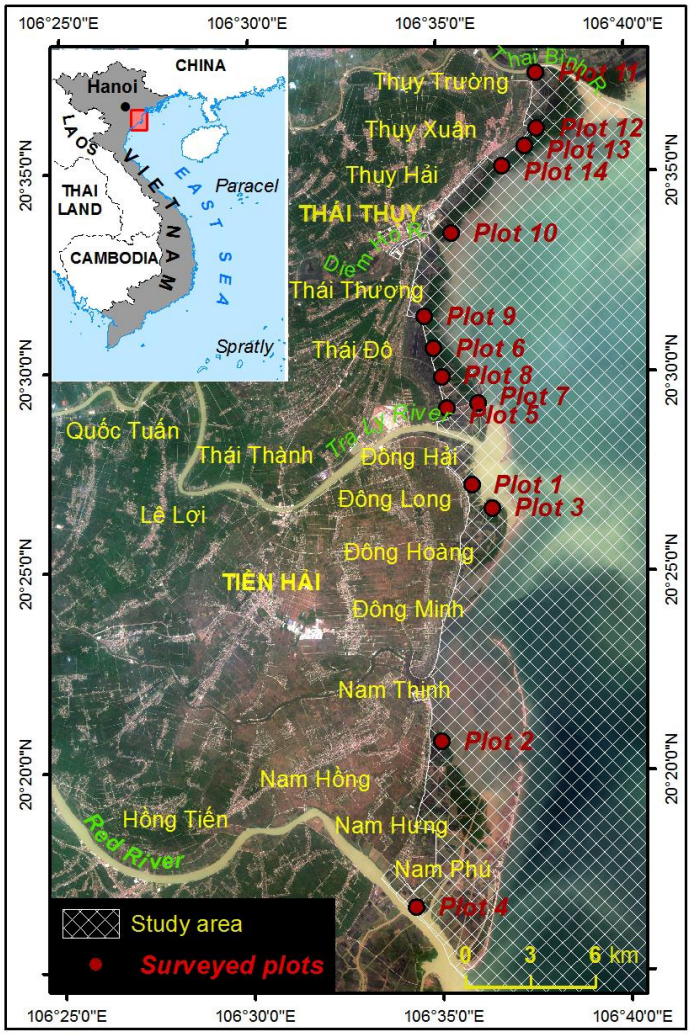
DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

participate into the carbon market, increasing the importance of coastal forest in local socio-economic development, thereby raising awareness of mangrove protection in the strategy dealing with climate change, simultaneously contributes to biodiversity conservation for the Red River Delta Biosphere Reserve.

Materials and methods

Study area

The coastal area of Thai Binh Province, located in Thai Thuy and Tien Hai districts (*Figure 1.*), is considered as one of the areas rich in biodiversity and strongly affected by climate change. Its population is estimated approximately 458,700 people with a density of 917 inhabitants/km^2 (Thai Binh Statistical Office, 2016).



ID	Coordinate
Plot 1	20°27'10.4"N 106°35'52.8"E
Plot 2	20°20'44.9"N 106°35'02.4"E
Plot 3	20°26'34.8"N 106°36'25.2"E
Plot 4	20°16'35.8"N 106°34'19.2"E
Plot 5	20°29'05.3"N 106°35'13.2"E
Plot 6	20°30'36.0"N 106°34'51.6"E
Plot 7	20°29'12.1"N 106°36'03.6"E
Plot 8	20°29'51.7"N 106°35'06.0"E
Plot 9	20°31'23.9"N 106°34'37.2"E
Plot 10	20°33'29.2"N 106°35'24.0"E
Plot 11	20°37'28.9"N 106°37'40.8"E
Plot 12	20°36'05.0"N 106°37'40.8"E
Plot 13	20°35'39.5"N 106°37'22.8"E
Plot 14	20°35'09.2"N 106°36'43.2"E

Figure 1. *Study area and surveyed plots (Image: Sentinel-2A dated 10 Aug 2015)*

This area is in tropical monsoon climate with annual average temperature about 22-24ºC, hottest in July (averagely 29.1ºC) and coldest in January (averagely 16.7ºC). The precipitation of 1,658 mm/year unevenly distributes among the months of the year.

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

The rainy season, from May to September, makes up 70 - 80% of annual total. The rest time, from October to April, the monthly rainfall is usually below 100 mm, resulting in water shortage. From May to November, especially in August, the area is hit by typhoons and tropical depressions, coming from the South China Sea with an average frequency of 2.1 times/year (Van et al., 2017).

Four major rivers, namely Thai Binh, Diem Ho, Tra Ly and Red rivers (*Figure 1.*), flowing through the study area to the sea, have complicated hydrological regime due to the influence of both Red and Thai Binh river systems. In general, there are two distinct seasons: the flood season, from June to October, accounts for 75 - 80% of the total annual flow; the dry, from November to May, makes up 20 - 25% of the total (Anh, 2016).

The wave regime, controlled by prevailing wind, has the directions: northeast in offshore area and northeast, east or southeast in coastal area for the winter; south in offshore area and southeast or south in coastal area for the summer. On the coast, winter waves can reach 0.4 - 0.9 m height in average and 3.0 m maximum; the summer waves are 0.7 to 1.2 m of average height and 6m highest. Influenced by storms and tropical cyclones, the large waves in winter are more frequent than in summer. The diurnal tide with high amplitude in relief of gentle slope creates considerably large tidal flats with a width of 4 - 5 km, even 7 - 8 km in Thai Binh estuary, favoring mangrove development for the study area (Anh, 2016).

Developed on Holocene unconsolidated sands, silts and clays, the relief of study area is relatively flat and low. The river-sea interaction produces the following landforms: river channels and floodplains, delta plains, ancient sandy dunes, high and low tidal flats, mouth bars. Among them, high and low tidal flats are the terrain suitable for mangrove development. Statistically, there are 13 soil types of sandy, saline, acid sulfate and alluvial groups in the area. Among them, the high, medium and low saline and tidal flat soils, related to mangrove forest, occupy 22,058.47 ha accounting for 37.23% of the total natural area of Tien Hai and Thai Thuy districts (Anh, 2016).

The total coastal forest of Thai Binh province occupies 3,899.1 ha, including 3,709.1 ha (95%) of mangroves, in 10 communes and towns of the two districts Thai Thuy and Tien Hai (Thai Binh Department of Agriculture and Rural Development - TBDARD, 2016). Mangroves are mainly pure or mixed planted forests with following species in majority *Sonneratia caseolaris*, *Kandelia obovata, Aegiceras corniculatum*, *Bruguiera gymnorohiza*, *Avicennia marina*. Communities of *Avicennia marina* and *Kandelia obovata*are commonly distributed in the outermost area with high salinity and deep water. *Sonneratia caseolaris*, *Kandelia obovata*, and *Aegiceras corniculatum* are situated in moderately submerged area. The community with dominance of *Sonneratia caseolaris* and underbrush *Acanthus ilicifolius*is mainly found in the estuarine area (Thuy et al., 2016). In Thai Thuy district, there are more than 2000 hectares of mangroves concentrated in 5 communes: Thuy Truong, Thuy Xuan, Thuy Hai, Thai Phuong and Thai Do. An inventory in Thuy Truong lists 38 plant families with 111 species in which 12 species are true mangrove sand 30 others are mangrove associates (Cuc and Tan, 2004). In Tien Hai district, mangroves are distributed mainly in five communes: Nam Thinh, Nam Hung, Nam Phu, Dong Long and Dong Hoang, in which the first three belong to Tien Hai Wetland Nature Reserve occupying an area of 1450 ha. This Reserve has: 11 species of true mangroves with 1 fern and 10 angiosperms; and 37 species of mangrove associates with 17 monocotyledons and 20 dicotyledons (Tuan and Anh, 2008). Outside of the reserve such as in Dong Long,

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

the flora has quite high diversity with 66 species of 33 families, whose 8 species are true mangroves and 19 others are mangrove associates (Tam, 2013). For whole coastal zone of Thai Binh, there are 14 species of true mangrove in the communities of natural forest, planted forest, aquaculture ponds and new-land pioneer as follows (Van et al., 2017):

- 1 fern: *Acrostichum aureum* L.;
- 12 dicotyledons: *Acathus ebracteatus* Vahl, *Acathus ilicifolus* L., *Sensuvium portulacastrum* L, *Avicennia marina* (Forsk) Veirh), *Lumnitzera racemosa* (Gaud.) Presl., *Derris trifoliata* (Benth) Barker, *Excoecaria agallaocha* L, *Aegiceras corniculatum* (L.) Blanco, *Bruguiera gymnorohiza* (L), *Kandelia obovata* Sheue Liu & Yong, *Rhizophora stylosa* Griff.and *Sonneratia caseolaris* (L.) Engl.;
- 1 monocotyledon: *Cyperus stoloniferus* Retz.

Methods

Biomass and carbon evaluation for the study area is firstly based on field data (DBH, H and tree quantity in surveyed plots), anatomical data (AGB, total biomass and carbon content of sampled trees) and NDVI map calculated from Sentinel imagery. From the anatomical data, the mathematical models expressing AGB vs. DBH and H, Biomass vs. AGB, Biomass vs. Carbon relationships are set up. The mean AGB in pixel (100 m^2) and mean NDVI are determined for each surveyed plot, and then expressed in a mathematical model, enabling to map AGB from NDVI for the study area. The biomass map can be produced from AGB map by Biomass vs. AGB expression and then carbon stock from biomass by Biomass vs. Carbon expression.

Field survey

In the study area, 14 key plots with sizes of 100 m^2 (10 m x 10 m), 500 m^2 (25 m x 20 m) or 2,500 m^2 (50 m x 50 m), selected on the basis of topographic features and current status of mangroves, were surveyed in November 2015 (*Figure 1.*). In each plot, survey work was conducted: statistics of the number of trees, measurement of DBH and H of all trees, species identification, abnormal characteristics of trees (banyan tree, buttress tree, diameter of buttress, buttress height, etc.) and weighting of fresh biomass of sampled trees.

A total of 31 trees, representative for key plots, are sampled for laboratory analysis. However, *in situ* identification of sampled trees is carried out and precise measurements are also done for diameter at the stump (position 0.0 m), DBH, stem length (from the stump to the highest), height below the branches (from the position 0.0 m to the main branching point of the tree), trunk length from the base (position 0.0 m) to the point of 10 cm diameter; buttress height and diameter, etc. For the below-ground parts, the coarse roots, greater than 2 mm in diameter, are dug out for biomass estimation; and the fine roots, less than 2 mm in diameter, are ignored in this estimation because of the difficulty in separating them from other organic matter in soil. After the *in situ* measurements, the sampled trees are separated into their parts such as the trunk, branches, leaves, roots and buttress (if present), then weighted for fresh biomass estimation.

Finally, each tree is sampled in stem, branch, leaf and root for laboratory analysis. Stem sample includes 2 - 3 cutting boards or radial cutting boards (if the tree is large) with a mass accounting for 0.2% of fresh stem. Branch is taken with 4 small cutting

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

boards, weighted of 0.5 - 1.0 kg in total. All samples are packed in plastic bags and tied tightly to prevent evaporation.

Carbon analysis in tree samples

Samples taken from roots, stems, branches and leaves of mangroves are dried at 70°C for estimating dry mass and moisture content. These dry samples are grinded to the size through 0.2 mm sieve for analysis. The organic carbon content is determined by Walkley Black method specified in Vietnam Standards (TCVN 9294: 2012).

Mathematical modeling

Based on the anatomical data of tree samples and imagery data, the mathematical models are used to quantify the relationship between:

- DHB, H and AGB, i.e. biomass sum of trunk, branches and leave.
- AGB and total biomass, i.e. biomass sum of trunk, branches, leave and root.
- Biomass and accumulated carbon
- AGB and NDVI

The coefficient of determination - R^2 (*Eq. 1*) and the Standard Error of the regression - SEreg (*Eq. 2*) are used for measuring the goodness of fit of the models. F value (*Eq. 3*) for F-test is also used to assess whether any of the independent variables in a multiple linear regression are significant.

$$R^2 = \frac{SSreg}{SStot} = 1 - \frac{SSres}{SStot} = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2} \quad \text{(Eq.1)}$$

$$SEreg = \sqrt{\frac{\sum_i (y_i - f_i)^2}{n - 2}} \quad \text{(Eq.2)}$$

$$F = \frac{MSreg}{MSres} = \frac{(\sum_i (f_i - \bar{y})^2)/(p-1)}{(\sum_i (y_i - f_i)^2)/(n-p)} \quad \text{(Eq.3)}$$

where, SSreg is regression sum of squares; SSres is residual sum of squares; SStot is total sum of squares; y_i is the measured value; $\bar{y}$ is the mean of y_i; f_i is mathematically calculated value; n is number of observations; p is number of regression parameters.

The coefficient of determination (R^2) ranges from 0 to 1. In general, the higher the R^2, the better the model fits your data and the model could be acceptable if $R^2 \geq 0.5$.

The standard error of the regression could be used to obtain a rough estimate of the 95% prediction interval. About 95% of the data points are within a range that extends from ±2.SEreg from the fitted line.

In F-test with a significance level α, in these cases α = 0.05, models are accepted if F-value > F-significance and P-value is less than 0.05.

The Microsoft Excel with ANOVA add-in is used for the above-mentioned analyses and mathematical modelling.

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

Remote sensing and GIS

The images should be dated around the moment of field survey for synchronizing the data. Given the weather condition and available data, the image of Sentinel 2A, taken at 10:15:36.027 (local time) on 10 August 2015, the best image close to survey time in November, is chosen for analysis (*Figure 1*). Its three bands Green (559.8 nm), Red (664.6 nm) and Near InfraRed - NIR (832.8 nm) with 10 m resolutions are used for calculating NDVI and Normalized Difference Water Index (NDWI):

$$NDVI = (NIR-Red)/ (NIR+Red) \quad \text{(Rouse } et\ al.\text{, 1974)} \quad \text{(Eq.4)}$$

$$NDWI= (Green-NIR)/ (Green+NIR) \quad \text{(McFeeters, 1996)} \quad \text{(Eq.5)}$$

The NDVI value, ranging from -1 to +1, increases with increased green biomass as a result of increased red reflectance due to greater absorption of incident red light by plant chlorophylls and decreased near-infrared reflectance associated with radiation scattering by the hydrated wall of leaf cells.

NDWI is used to distinguish land and sea by identifying water body with a value greater or equal to threshold of 0.3 for Landsat (McFeeter, 1996, 2013) or 0.1 for Sentinel (Kaplan and Avdan, 2017).

The GIS with ARCGIS software is applied for integration and production of maps presenting spatial variation of data and for extraction of information.

Results and discussion

Results of plot survey

Detailed surveys are conducted for all 14 plots with total area of 10600 m^2 (*Table 1.*). 700 trees counted in these plots have mean values of 10.69 cm and 4.66 m for DBH and H respectively. Plot 11, forest of *Sonneratia caseolaris* in Thuy Truong commune (Thai Thuy district), is the largest mean DBH (19.19 cm). The smallest mean DBH with value of 5.88 cm is Plot 7, a recently planted forest of *Sonneratia caseolaris - Kandelia obovata - Bruguiera gymnorohiza* in Con Den (Tien Hai district). In terms of average height for each plot, Plot 6 is the highest (9.11 m) and Plot 10 is the lowest (3.11 m). The highest density is Plot 3 (7600 trees/ha) and the lowest is Plot 12 (100 trees/ha). In general, the older forests have mean DHB and height higher than the overall average because they have long time for growing their dimensions. The high density is statistically related to forests having mean DHB and height lower than the overall average. These forests may be planted on a large scale recently.

Biomass and carbon stored in sampled trees

Biomass and carbon storage are determined for 31 sample trees with DBH from 5.41 cm to 14.01 cm and height from 1.5 m to 5.5 m (*Table 2*). The analytical results show that the AGB, total biomass and accumulated carbon range 4.11 - 41.04 kg, 4.34 - 43.65 kg and 2.18 - 21.83 kg, respectively. The same changing behavior with minimum in Tree 8 and maximum of Tree 31 (*Table 2.*) shows a close relationship among AGB, total biomass and accumulated carbon. In general, they increase with the growth of tree, i.e. the increase of DHB and height.

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Table 1. *Plot survey*

Plot number	**Dominant species**	**Length x Width** (m)	**Area** (m^2)	**Tree Quantity**	**Mean of DHB** (cm)	**Mean of Height** (m)	**Density** (trees/ha)
1	*Son.*	25 x 20	500	92	10.45	5.65	1840
2	*Son.*, *Kan.,Bru.*	25 x 20	500	40	11.24	4.79	800
3	*Son.*, *Kan.,Bru.*	10 x 10	100	76	7.92	3.74	7600
4	*Son.*	25 x 20	500	82	15.62	5.88	1640
5	*Son.*	25 x 20	500	103	8.27	3.83	2060
6	*Son.*, *Kan.,Bru.*	10 x 10	100	9	14.85	9.11	900
7	*Son.*, *Kan.,Bru.*	10 x 10	100	67	5.88	3.90	6700
8	*Kan.*	10 x 10	100	21	9.02	4.71	2100
9	*Son.*, *Kan.,Bru.*	50 x 50	2500	32	11.31	3.96	128
10	*Son.*, *Kan.,Bru.*	10 x 10	100	54	7.96	3.11	5400
11	*Son.*	10 x 10	100	14	19.19	8.64	1400
12	*Son.*, *Kan.,Bru.*	50 x 50	2500	25	16.42	5.72	100
13	*Son.*, *Kan.,Bru.*	50 x 50	2500	36	14.66	5.00	144
14	*Son.*, *Kan.,Bru.*	25 x 20	500	49	12.65	4.29	980
Overall			**10600**	**700**	**10.69**	**4.66**	**660**

Note: Son. - Sonneratia caseolaris; Kan. - Kandelia obovata; Bru. - Bruguiera gymnorohiza

Table 2. *Dry biomass and carbon storage in sampled trees*

Tree	**Parameters**		**Dry biomass** (kg)						**Total carbon storage**(kg)
	DBH (cm)	H (m)	**Trunk**	**Branch**	**Leaf**	**Root**	**AGB**	**Total**	
1	10.98	3.9	9.5	11.03	3.74	1.89	24.27	26.16	13.09
2	11.14	4.2	13.82	10.8	4.5	1.85	29.12	30.97	15.49
3	5.57	1.5	2.93	1.89	1.08	0.95	5.9	6.85	3.44
4	10.98	4.45	15.26	4.32	3.15	1.4	22.73	24.13	12.07
5	8.59	3	4.55	7.34	2.93	1.53	14.82	16.35	8.19
6	6.68	1.8	3.56	4.19	3.02	1.44	10.77	12.21	6.11
7	5.57	1.6	3.24	1.04	0.59	0.5	4.87	5.37	2.69
8	5.41	1.5	2.93	0.68	0.5	0.23	4.11	4.34	2.18
9	10.03	3.5	9	10.35	3.6	1.67	22.95	24.62	12.32
10	11.94	4.1	13.95	11.25	4.95	2.03	30.15	32.18	16.11
11	7.96	2.5	5.85	3.6	1.8	0.77	11.25	12.02	6.02
12	11.62	4.5	15.3	4.5	3.6	1.58	23.4	24.98	12.49
13	8.91	2.9	4.95	7.65	3.15	1.53	15.75	17.28	8.66
14	7	2	4.05	4.5	3.15	1.49	11.7	13.19	6.61
15	6.05	1.9	3.24	1.04	1.04	0.59	5.32	5.91	2.96
16	11.94	4.5	15.98	5.18	3.38	1.62	24.54	26.16	13.08
17	9.87	4	9.9	10.8	3.15	1.53	23.85	25.38	12.7
18	12.73	4.2	15.3	11.25	4.05	2.12	30.6	32.72	16.37
19	6.37	2	3.15	2.25	1.35	0.54	6.75	7.29	3.66
20	10.19	3.7	13.95	3.15	2.25	1.08	19.35	20.43	10.23
21	10.5	3.4	9	7.2	4.95	2.16	21.15	23.31	11.66
22	6.37	1.7	3.56	4.19	3.02	1.49	10.77	12.26	6.14
23	6.68	2.1	4.05	2.25	1.35	0.77	7.65	8.42	4.23
24	10.66	4.6	14.63	11.7	4.5	2.12	30.83	32.95	16.48
25	11.94	4.4	11.25	12.6	4.5	2.07	28.35	30.42	15.22
26	12.57	4.1	13.05	10.35	4.05	1.89	27.45	29.34	14.69
27	8.28	2.6	5.85	3.83	2.25	1.04	11.93	12.97	6.5
28	11.46	4.8	16.16	5.22	4.05	2.03	25.43	27.46	13.74
29	14.01	5.1	9.9	14.85	5.85	2.12	30.6	32.72	16.37
30	9.87	3.8	7.11	8.37	6.03	2.16	21.51	23.67	11.85
31	13.69	5.5	13.5	4.14	23.4	2.61	41.04	43.65	21.83

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Analyzing the correlation of DBH and H with AGB, ABG with total biomass, biomass with carbon storage (*Table 3.*), the following suitable expressions could be chosen:

$$AGB = 2.110606*DBH + 3.302734*H - 11.8674 \quad \text{(Eq.6)}$$

$$Biomass = 1.0548*AGB + 0.4502 \quad \text{(Eq.7)}$$

$$Carbon = 0.4999*Biomass + 0.0127 \quad \text{(Eq.8)}$$

Table 3. *Analysis of DHB, H, AGB, biomass and carbon correlations from anatomical data*

	Coefficients	***SE***	***P-value***	**R^2**	***SEreg***	***F***	***Significance F***
ABG ~ DBH, H							
Intercept	-11.8674	2.4435	4.1041E-05	0.9265	2.7342	176.3669	1.35E-16
DBH	2.1106	0.7472	8.6232E-03				
H	3.3027	1.5973	4.8028E-02				
Total biomass ~ ABG							
Intercept	0.4502	0.1111	3.48346E-4	0.9993	0.2749	41898.9073	2.22E-47
ABG	1.0548	0.0052	2.21622E-47				
Carbon storage ~ Biomass							
Intercept	0.0127	0.0023	7.1E-06	0.9999	0.0057	24664592	1.54E-87
Biomass	0.4999	0.0001	1.54E-87				

Correlation between AGB and NDVI

NDVI is produced from Red and NIR bands of Sentinel image (*Eq. 4*). This product is integrated with study area (*Figure 2.a*) and land – sea distribution (*Figure 2.b*), mapped from NDWI (*Eq. 5*) with the threshold of 0.1 (Kaplan and Avdan, 2017), to obtain NDVI map for the study area (*Figure 2.c*). For the study area, NDVI varies from -0.30 to 0.66, its high value is found in Thuy Truong – Thuy Hai, Thai Thuong and south of Nam Thinh, the low value is commonly around the river mouth. The mean values of surveyed plots (*Table 4*) extracted from NDVI map are in range of 0.18 (Plot 12) – 0.50 (Plot 3).

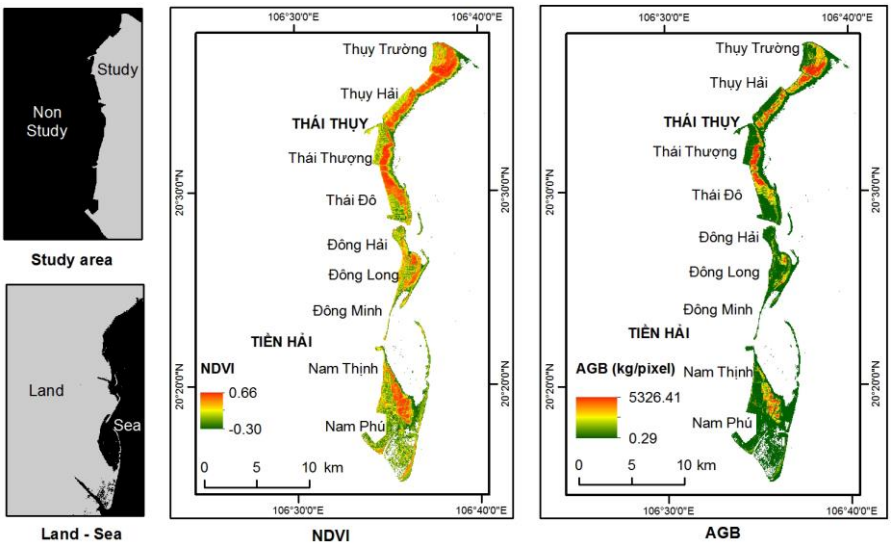

Figure 2. *Study area (a), Land – sea distribution (b), NDVI (c) and AGB (d)*

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

Table 4. *Estimation of AGB and IDVI for surveyed plots*

Plot number	**AGB** (kg)	**Area** (m^2)	**AGB productivity** (kg/100m^2)	**Mean NDVI**
1	2666.99	500	533.40	0.42
2	1112.42	500	222.48	0.29
3	1315.14	100	1315.14	0.50
4	3334.79	500	666.96	0.36
5	1888.67	500	377.73	0.31
6	448.20	100	448.20	0.48
7	904.37	100	904.37	0.48
8	479.93	100	479.93	0.46
9	806.21	2500	32.25	0.31
10	826.17	100	826.17	0.43
11	803.49	100	803.49	0.46
12	1045.78	2500	41.83	0.18
13	1285.58	2500	51.42	0.29
14	1426.26	500	285.25	0.34

Note: 100 m^2 = 1 pixel (10 m x 10 m)

The AGB of each plot (*Table 4.*) is estimated from mean values of DBH and H (*Table 1*) by the mathematical model expressing their relationship (*Eq. 6*). AGB values vary from 448 kg (Plot 6) to 3335 kg (Plot 4). However, in term of productivity, AGB in an area unit, 100 m^2 or 1 pixel for Sentinel image with used bands in this case, the minimum and maximum are 32.25 kg/ 100 m^2 (Plot 9) and 1315.14 kg/ 100 m^2 (Plot 3).

The AGB productivity and NDVI of plots (*Table 4.*) could be correlated as follow (*Figure 3.*):

$$AGB = 6.5135\ e^{10.234NDVI} \quad \text{or} \quad \ln(AGB) = 10.234*NDVI + 1.8739 \qquad \text{(Eq.9)}$$

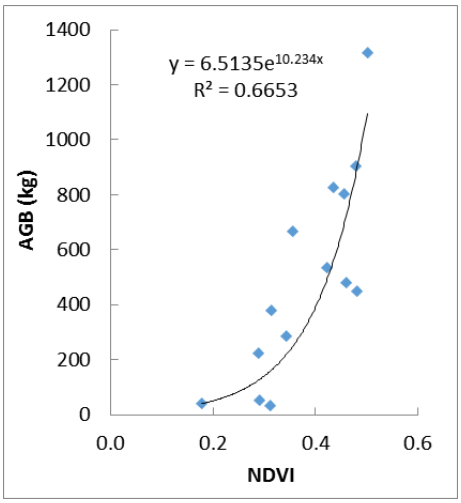

Figure 3. *Graph expressing AGB vs. NDVI*

Using the coefficient of determination R^2 and F-test (*Table 5.*) show that this expression of AGB - NDVI relation (*Eq. 9*) is acceptable.

An AGB map is produced from NDVI by the model expressing their relationship (*Eq. 6*). The AGB in the study area (*Figure 2.d*) ranges from 0.29 kg/pixel to 5326.41 kg/pixel with high value in Thuy Truong – Thuy Hai, Thai Thuong and south of Nam Thinh, where also the NDVI is high.

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

Table 5. *Analysis of ln(AGB)~NDVI correlation*

	Coefficients	*SE*	*P-value*	R^2	*SEreg*	*F*	*Significance F*
Intercept	1.8739	0.8183	0.0409	0.6653	0.7246	23.8493	0.0004
NDVI	10.234	2.0955	0.0004				

Biomass and carbon stock in coastal forests

Biomass as well as carbon stored in forest depend heavily on the species, age and density of trees. In Giao Lac commune (Nam Dinh province), next to the study area, the amount of carbon accumulated in *Kandelia obovate* forests of 1, 5, 6, 8 and 9 years increases with the ages (Hanh, 2009). In mixed forests of *Kandelia obovate* and *Sonneratia caseolaris* with 10, 11 and 13 years of Nam Phu commune (Thai Binh province), accumulated carbon in also increases with the age and it in *Kandelia obovate* (1.60 - 2.39 kg/tree) is much lower than in *Sonneratia caseolaris* (23.22 - 37.23 kg/tree); however, due to the tree density, carbon productivity in *Kandelia obovate* (49.31-124.56 t/ha) is higher than in *Sonneratia caseolaris* (12.51 - 32.74 t/ha) (Hanh, 2015).

For the study area, biomass is estimated from AGB (*Eq. 7*) and then carbon storage is from biomass (*Eq. 8*). Biomass and carbon storage (*Figure 4.*) have high value in the region of Thuy Truong – Thu Hai, Thai Thuong – Thai Do, Nam Hung, where mangrove is planted for long time, and low value around the river mouth, the newly formed land with recently planted mangrove. Biomass extracted from its map ranges from 76 kg/ha to 561875 kg/ha with an average of 38796 kg/ha. Carbon storage varies from 39 kg/ha to 280882 kg/ha with an average value of 19395 kg/ha. Overall estimations for whole study area, covering approximately 7527 ha (752690 pixel), are 292,014,265 kg biomass and 145,987,493 kg carbon.

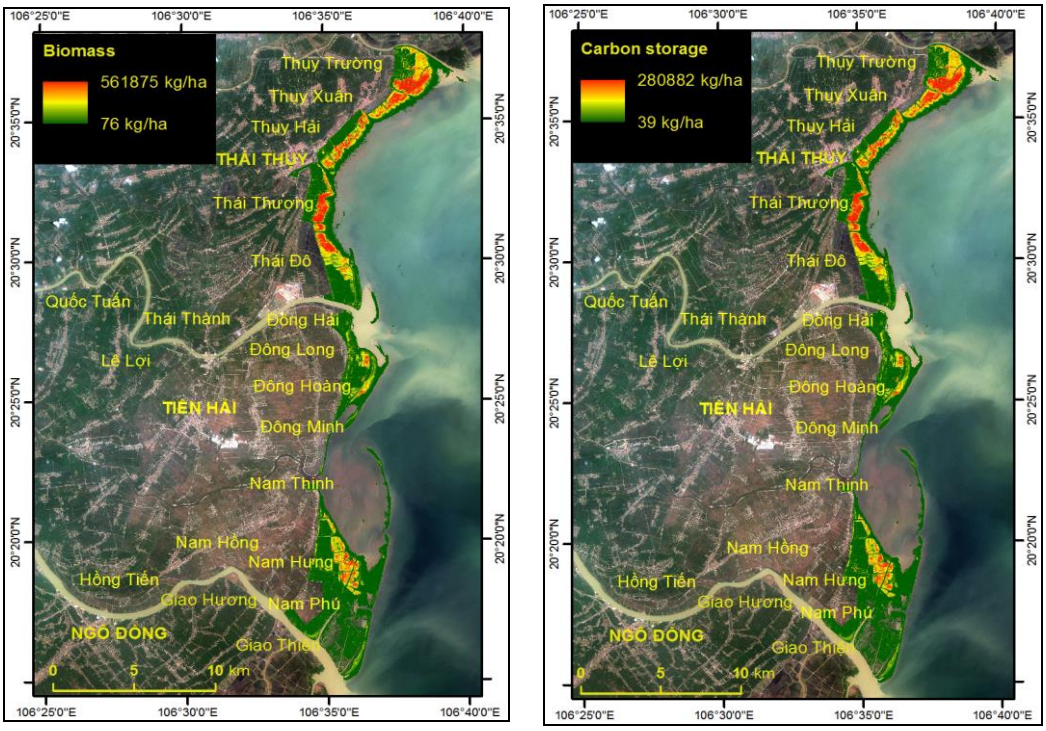

Figure 4. *Total biomass (left) and carbon storage (right)*

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

Forest CO_2 valuated through carbon market participation

The carbon market is seen as the main tool to reduce CO_2 emissions, one of the four greenhouse gases. The carbon market activity is supported by four main mechanisms outlined in the Kyoto Protocol, including emissions trading mechanism, CDM, JI and REDD (Höhne et al., 2015). In Vietnam, given the CO_2 price from US$5/t to US$10/t, the carbon stored in production forests is valuated from VND 61 million/ha (restored forest) to VND 119 million/ha (rich forest) for Southern Vietnam, VND 50 - 121 million/ha for the Central Vietnam and VND 46 - 100 million/ha for the Northern Vietnam (Vietnamese Academy of Forest Sciences, 2013).

For the study area, as mentioned above, the total carbon in coastal forest is estimated of 145987.49 tons, so the converted CO_2, as much as 3.6667 times of carbon, is 535287.47 tons. Based on the lowest CO_2 price of US$11/t in carbon market price, the total CO_2 absorbed in coastal forest of Thai Binh province is valuated of US$ 5,888,162. This is a significant value for forest managers, especially for local habitants managing the community forests in Tien Hai and Thai Thuy coastal districts of Thai Binh province.

General remarks

According to TBDARD (2016), coastal forests of Thai Binh province is landward limited by the sea dikes. These dikes are also used as inner limit of study area; the outer is the land - sea boundary, defined by McFeeter (1996) as NDWI = 0.3 (*Figure 2.b*). The study area covers 7527 ha, but the statistics of TBDARD (2016) gave a smaller number, 3,899.1 ha of coastal forest for Thai Binh, in which 3,709.1 ha (95%) is mangroves. This difference is probably due to the study area defined by NDWI takes into account of forests and non-forested area, i.e. lands for residential, garden, crops, public work or other purposes and newly accreted land while the data of TBDARD is only administratively managed forests. Furthermore, the superficies of study area is influenced by imaging time related to tide and weather condition, spatial resolution of image and delineation of outer limit by NDWI. In the case of Fresno city (California, USA), using NDWI to detect swimming pools for Mosquito Abatement has overall classification accuracy of 91.2% and an overall Kappa coefficient of 0.806 (McFeeters, 2013).

All the mathematical models are assessed by coefficient of determination R^2 (*Eq. 1*), Standard error of the regression (*Eq. 2*) and F-test with significance level $\alpha = 0.05$ from F value (*Eq.3*). The F-test and high R^2 show all the chosen mathematical models expressing relationships among DBH, H, AGB, NDVI, biomass and carbon storage, (*Eq. 6*), (*Eq. 7*), (*Eq. 8*) and (*Eq. 9)*, are acceptable. There are close correlations between AGB and DBH and H (*Eq. 6*), biomass and AGB (*Eq. 7*) and carbon and biomass (*Eq. 8*), expressed by $R^2 > 0.9$. Lower correlation between NDVI and AGB, $R^2 = 0.67$, may be due to different time between Sentinel data (Aug 2015) and plot survey (Nov 2015) and also due to few number of sample plots (14 plots).

The study area includes not only mangroves, but also other vegetation in small percentage such as forests of *Casuarina equiselifolia* and trees planted in gardens, residential and public areas.

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

Conclusion

The coastal forests of Thai Binh province are largely made up of mangroves with dominant species of *Sonneratia caseolaris*, *Kandelia obovate*, *Bruguiera gymnorohiza* and *Aegiceras corniculatum,* planted for a long time up to now. On an area of 10,600 m^2 of 14 plots, there are 700 trees with a mean DBH of 10.69 cm mean height of 4.66 m. Forests with DBH larger than overall average often have the height above overall average due to longtime plantation. Dense forests are often associated with small trees, recently planted on a large scale.

There are close relationships among DHB, H, AGB, total biomass and accumulated carbon. The AGB, total biomass and accumulated carbon change in the same behavior and they increase with the growth of tree, i.e. the increase of DHB and height.

The relationship between AGB and NDVI could be presented in acceptably exponential model. They have higher value Thuy Truong – Thuy Hai, Thai Thuong and south of Nam Thinh and lower value around the river mouths. Biomass and carbon stock do the same with the average productivities of 38.8 t/ha and 19.4 t/ha respectively. Overall estimations for whole study area covering approximately 7527 ha are 292 kilotons biomass and 146 kilotons carbon, worth about US$ 5.9 million. Most of this value is contributed by mangrove, showing its role not only in coastal protection from erosion but also in economic interest of local community.

The forest biomass and carbon stock could be evaluated from satellite data in combination with plot surveys. In future study, the more accurate assessment requires the synchronous data of both imageries and field survey as well as the adequate plots covering all vegetation's types.

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DOI: http://dx.doi.org/10.15666/aeer/1806_74997514

TEMPORAL VARIATION GOVERNS THE PROLIFERATION OF DIFFERENT TAXA OR GUILDS OF ULTRAPLANKTONIC FUNGI IN A LARGE RESERVOIR

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(Received 9th Mar 2020; accepted 29th Jul 2020)

Abstract. The fungus play an important role within the aquatic ecosystem. However, the ultraplanktonic fungus diversity in reservoir ecosystems is still greatly unexplored. In this study, we used Illumina sequencing technology to analyze the diversity of ultraplanktonic fungi (size class 0.7–5 μm in this study) from two sampling sites along the Three Gorges Reservoir of the Yangtze River in China at four seasons in 2015. *Basidiomycota* was the most abundant phylum (36.69% of the total fungal reads), followed by *Ascomycota* (27.05%), *Cryptomycota* (18.06%), *Chytridiomycota*, (15.81%) and *Blastocladiomycota* (1.51%). Our study showed that ultraplanktonic fungi were changing with time, and a shift in dominant taxonomic groups was observed between time points. A marked temporal variation in functional guilds of fungi was also observed, suggesting that temporal variation governs the proliferation of different taxa or guilds of fungi in the reservoir. Our results suggest that inputs from fungal species of terrestrial origin may be responsible for the observed changes in pelagic community composition. This result also implies that terrestrial inputs may be an important source of fungal species in reservoir ecosystems. Additionally, it is possible that the chytrids were limited by hyperparasitic *Cryptomycota*.
Keywords: *reservoir ecosystem, aquatic fungi, high-throughput sequencing, diversity and distribution, functional guilds*

Introduction

Fungi represent one of the last frontiers of the unexplored biodiversity that challenges microbial ecology today (Lepère et al., 2018). The kingdom of fungi is highly diverse. Recent estimates indicate that the number of aquatic fungal species lies between 3000 and 4000 (Grossart and Rojas-Jimenez, 2016). Aquatic fungi have been detected from a wide range of habitats, including the ocean (Pachiadaki et al., 2016; Hassett et al., 2019), coastal regions (Gao et al., 2010; Duan et al., 2018), lakes (Lefèvre et al., 2012; Comeau et al., 2016; Wahl et al., 2018), and rivers (Duarte et al., 2015; Seena et al., 2019). However, relatively few studies have investigated the biodiversity and ecological role of mycoplankton in reservoir ecosystems.

Fungi play an important role in aquatic ecosystems. They influence and shape them by cycling organic matter and channeling nutrients across trophic levels (Nilsson et al.,

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DOI: http://dx.doi.org/10.15666/aeer/1806_75157533

2019). Some fungi decompose leaves and wood (Canhoto et al., 2017), and others are important sources of food and nutrients for filter-feeding zooplankton and copepods (Rasconi et al., 2011; Sime-Ngando et al., 2011; Frenken et al., 2018). Moreover, rather than just serving as prey and a source of nutrients, fungi have been found to potentially establish alternative trophic links between large inedible phytoplankton and zooplankton through the "mycoloop" pathway (Kagami et al., 2014; Agha et al., 2016; Gerphagnon et al., 2019).

The majority of aquatic fungi occur in freshwaters (Shearer et al., 2007). As threats to freshwater biodiversity escalate, there is an urgent need to survey, collect and isolate freshwater fungal species. Most aquatic fungi are microscopic (Wurzbacher et al., 2011), hence ultraplanktonic fungi have a major role in aquatic environments. Despite their ecological importance, ultraplanktonic fungi have remained poorly described because of their small size and lack of morphological characteristics. Therefore, it is important to understand ultraplanktonic fungi diversity in freshwater ecosystems. In some studies, culture-independent molecular techniques have been exclusively used to determine fungal diversity in freshwater habitats. Several studies have surveyed the composition, distribution, and function of planktonic fungi in reservoir ecosystems (Chen et al., 2018). Unfortunately, most studies of large freshwater systems are conducted at one time points. For diversity studies, the inclusion of time surveys allows for the detection of a wider diversity than time-point studies (Simon et al., 2015). Nevertheless, knowledge is scarce regarding temporal series diversity of ultraplanktonic fungi in large reservoir ecosystems. Lack of information on ultraplanktonic fungi in large reservoir ecosystems limits the comprehensive understanding of aquatic ecosystems.

The Three Gorges Reservoir is located in the upstream reaches of Yangtze River in China. Presently, it is one of the largest reservoirs in the world (Han et al., 2015). In this study, a high-throughput Illumina sequencing technology was used to analyze the diversity of ultraplanktonic fungi (size class 0.7–5 μm in this study). Two sites were considered along the Three Gorges Reservoir of the Yangtze River and sampled over four seasons in 2015. The objective of our study was to provide a taxonomic and functional characterization of the ultraplanktonic fungal community of a large reservoir at two diametrically opposed sites on 4 occasions during the year.

Materials and methods

Sampling and measurement of physico-chemical parameters

In total, eight water samples were collected at two sites (CJ01 and CJ05) from the Three Gorges Reservoir of the Yangtze River of China in the following months of 2015: April (spring), August (summer), October (autumn), and December (winter) (*Fig. 1*). The Three Gorges Reservoir is a canyon-shaped, typical river-type reservoir. The water body type of the two sample sites were the same. These sites are in a transition-type region (Zheng et al., 2006). The sampling sites were located in the middle of the river; CJ01 (E 110.99990, N 30.82111) is approximately 1.6 km and CJ05 (E 110.84682, N 30.88436) is approximately 200 m away from the riverbank. The sampling site CJ01 was located approximately 1 km upstream of the Three Gorges Dam.

Water samples were collected from a depth of about 0.5 m beneath the water surface by using a 5-L sampler. These samples were pre-filtered through 5-μm pore-size polycarbonate filters (Millipore, USA). Then, these samples were filtered through GF/F

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DOI: http://dx.doi.org/10.15666/aeer/1806_75157533

filters (0.7 μm, Whatman). The filters were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis.

Physico-chemical factors, such as water temperature (WT), dissolved oxygen (DO), pH, and turbidity, were measured *in situ* with an YSI model Professional Plus multiparameter probe (YSI, USA). According to standard methods described in previous studies, we determined the following parameters: chemical oxygen demand (COD), oxidation-reduction potential (ORP), the concentrations of total nitrogen (TN), orthophosphate (PO_4^{3-}), nitrate (NO_3^-), total phosphorus (TP), and ammonium (NH_4^+) (Huang et al., 1999). Trophic states of different sampling sites and times were determined from comprehensive nutritional status index (trophic level index, TLI) by using the values of following parameters: TN, TP, and COD (Tian et al., 2017). Trophic states were set as follows: $TLI \leq 30$, oligotrophic; $30 < TLI \leq 50$, mesotrophic; $50 < TLI \leq 60$, slightly eutrophic; $60 < TLI \leq 70$, moderately eutrophic; $TLI > 70$, highly eutrophic (Meng and Zhao, 2007).

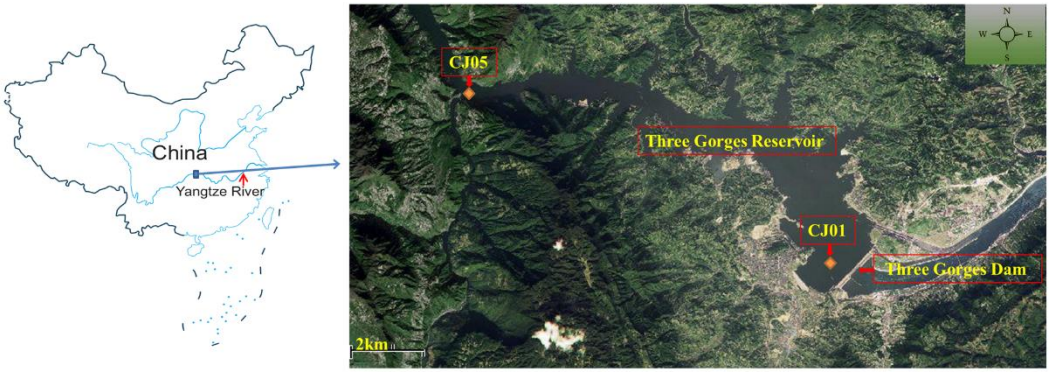

Figure 1. *Map of the study area and sampling site*

DNA extraction, amplification, and sequencing of 18S rDNA fragments

A previously described method was used to extract DNA from filters (Countway et al., 2005). To amplify the 18S small subunit rRNA gene fragments from samples, we used the following fungal-specific primers: 817F (5′-TTAGCATGGAATAATRRAATAGGA -3′) and 1196R (5′-CCTTTGAGTGGTCCAGGTCT -3′) (Borneman and Hartin, 2000). To modify primer sequences, Illumina adaptor was added to the ends of the forward primer and another adaptor was added to the ends of the reverse primer. After amplification, the amplicons were purified directly by using UNIQ-10 PCR purification kit (Sangon Biotech Ltd, Shanghai, China); the purified amplicons were quantified with TBS-380 (Turner BioSystems Inc, USA). Paired-end sequencing of purified amplicons was carried out on an Illumina MiSeq PE250 platform (Majorbio Bio-Pharm Technology Ltd, Shanghai, China).

Sequence processing

The raw demultiplexed reads were first quality trimmed Mothur software version 1.28.0 (Schloss et al., 2009). Following these filtering parameters, some reads were removed due to quality considerations: the length of nucleotides was less than 300 bp; it contained ambiguous bases, which were imperfectly matched with a sample-specific

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DOI: http://dx.doi.org/10.15666/aeer/1806_75157533

barcode; and the average sequencing quality scores were less than 25 for the whole sequence. Potentially chimeric sequences were identified and removed using UCHIME (Edgar, et al., 2011). By using the furthest neighbour method (http://www.mothur.org/wiki/Cluster), we clustered unique sequences into operational taxonomic units (OTUs) at 97% sequence identity. Subsequently, a representative sequence from each OTU was aligned against the SILVA 123 reference alignment using the RDP classifier (Wang et al., 2007). The functional group (guild) of fungal OTUs was determined by using FUNGuild v1.0 database (Nguyen et al., 2016).

Statistical analyses

Diversity indices (Shannon-Wiener and Pielou's evenness index) of each sample were calculated by using the R (Ihaka and Gentleman, 1996) package vegan (Oksanen et al., 2016). Bray-Curtis distances were used to compare community structures with non-metric multidimensional scaling (NMDS). The significance of observed differences in community structure was determined by permutational multivariate analysis of variance (PERMANOVA), which is based on Bray-Curtis distance using 9999 permutations. Similarity percentage analysis (SIMPER) was used to identify which OTUs contribute the most for observable difference between groups. Moreover, NMDS, PERMANOVA and SIMPER were carried out using R package vegan. The non-parametric Mann-Whitney (two groups) and Kruskal-Wallis tests (more groups) were employed to evaluate differences. These tests were performed by using SPSS software version 24 (IBM, USA). R pheatmap package (Kolde, 2019) was used to construct a heatmap of the top 10 most abundant OTUs. We identified the OTUs that differed significantly ($q < 0.05$) for two time points with White's non-parametric t-test and Storey's multiple testing correction; the Statistical Analyses of Metagenomic Profiles (STAMP) software version 2.1 was used to perform these tests (Parks et al., 2014).

To determine the correlation between ultraplanktonic fungi communities and environmental factors, canonical-correlation analysis (CCA), was performed using R package vegan. The CCA was performed using the relative abundance of reads data for the fungal phyla and all the physico-chemical factors. To identify environmental factors that significantly correlate with ultraplanktonic fungi communities, an alternative CCA model was constructed using the step function in R and Akaike's information criterion (AIC).

Nucleic acid sequence accession numbers

In this study, the raw sequencing data were submitted to the Sequence Read Archive (SRA) of NCBI (National Center for Biotechnology Information) (Accession number SAMN06107068).

Results

Characterization of environmental variables

In this study, we measured 11 physico-chemical factors at the two sampling sites: CJ01 and CJ05 (*Table A1*). No significant differences were found in the physicophysical-chemical factors between the two sampling sites ($p > 0.3$, Mann-Whitney U-test). Moreover, these factors did not significantly differ across the four time

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points (all $p > 0.08$, Kruskal-Wallis test). The whole trophic state of Three Gorges Reservoir is mesotrophic, except CJ05 site, which was slightly eutrophic in summer (TLI = 51.4). At the CJ01 and CJ05 sampling sites, the trophic state did not differ significantly (Mann-Whitney U-test, $p = 1.0$); however, the TLI indexes of CJ05 site were greater than that of CJ01 site (except in summer) (*Table A1*).

Overall community composition of ultraplanktonic fungi

In this study, 283,191 reads (most between 390 and 400 bp) were obtained after removing denoising and chimera. After plotting rarefaction curves of OTU richness for each sample, we inferred that total diversity could be recovered from all the samples (*Fig. A1*). We obtained a total of 35,393 high-quality fungal reads, ranging from 1923 to 7444 per sample. All reads were grouped into 192 OTUs by using a cut-off value of 97% sequence identity. Of the 192 OTUs, 61 OTUs belong to fungi. These fungi OTUs ranging from 28 to 46 OTUs per sample (*Table 1*). The other 131 OTUs mainly affiliated to algae and protozoan. Only three and seven fungal OTUs were unique for CJ01 and CJ05 sites, respectively (*Fig. A2*).

Table 1. *Summary of the diversity index of each sample*

Season	Sample sites	Reads	OTUs	Shannon-Wiener	Pielou evenness
Spring	CJ01	1923	32	1.454	0.419
	CJ05	3140	28	1.442	0.428
Summer	CJ01	3826	43	3.165	0.831
	CJ05	6679	46	2.941	0.764
Autumn	CJ01	6529	44	2.397	0.63
	CJ05	7444	41	2.522	0.671
Winter	CJ01	3526	28	2.517	0.755
	CJ05	2326	30	2.281	0.671

OTU: operational taxonomic unit

Fungal reads were assigned to the following five fungal phyla: *Basidiomycota*, *Ascomycota*, *Chytridiomycota*, *Cryptomycota*, and *Blastocladiomycota* (*Fig. 2A*). Furthermore, there was the group fungi_LKM15, which named after the first clone encountered in an environmental DNA survey in Lake Ketelmeer and referred to some undescribed fungi (van Hannen et al., 1999). The classification of 61 fungal OTUs is as follows: nineteen fungal OTUs belonged to *Ascomycota* (27.05% of total fungi reads); eighteen fungal OTUs belonged to *Basidiomycota* (36.69% of the reads); ten fungal OTUs belonged to *Chytridiomycota* (15.81% of the reads); six fungal OTUs belonged to *Cryptomycota* (18.06% of the reads); one fungal OTU belonged to *Blastocladiomycota* (1.51% of the reads); two fungal OTUs belong to LKM15 group (0.35% of the reads); and five fungal OTUs were considered as unknown fungi (0.53% of the reads). The 10 most frequently observed OTUs constituted 74.07% of total fungal reads, these OTUs belong to *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Cryptomycota* (*Fig. 2B*).

By matching sequences with *Ascomycota*, we observed that there was high affinity with four known classes. Most *Ascomycota* reads were affiliated with *Dothideomycetes* (15.69% of the total fungal reads). Meanwhile, *Eurotiomycetes* and *Sordariomycetes* constituted 3.59% and 4.23% of the total fungal reads, respectively. The most common

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Basidiomycota classes were as follows: the *Microbotryomycetes* (15.51% of the total fungal reads), *Exobasidiomycetes* (9.08%), and *Agaricomycetes* (8.03%). *Cryptomycota* was mostly composed of incertae sedis taxa. The diversity of most *Chytridiomycota* was represented by *Zygorhizidium* (74.84% of the total *Chytridiomycota* reads).

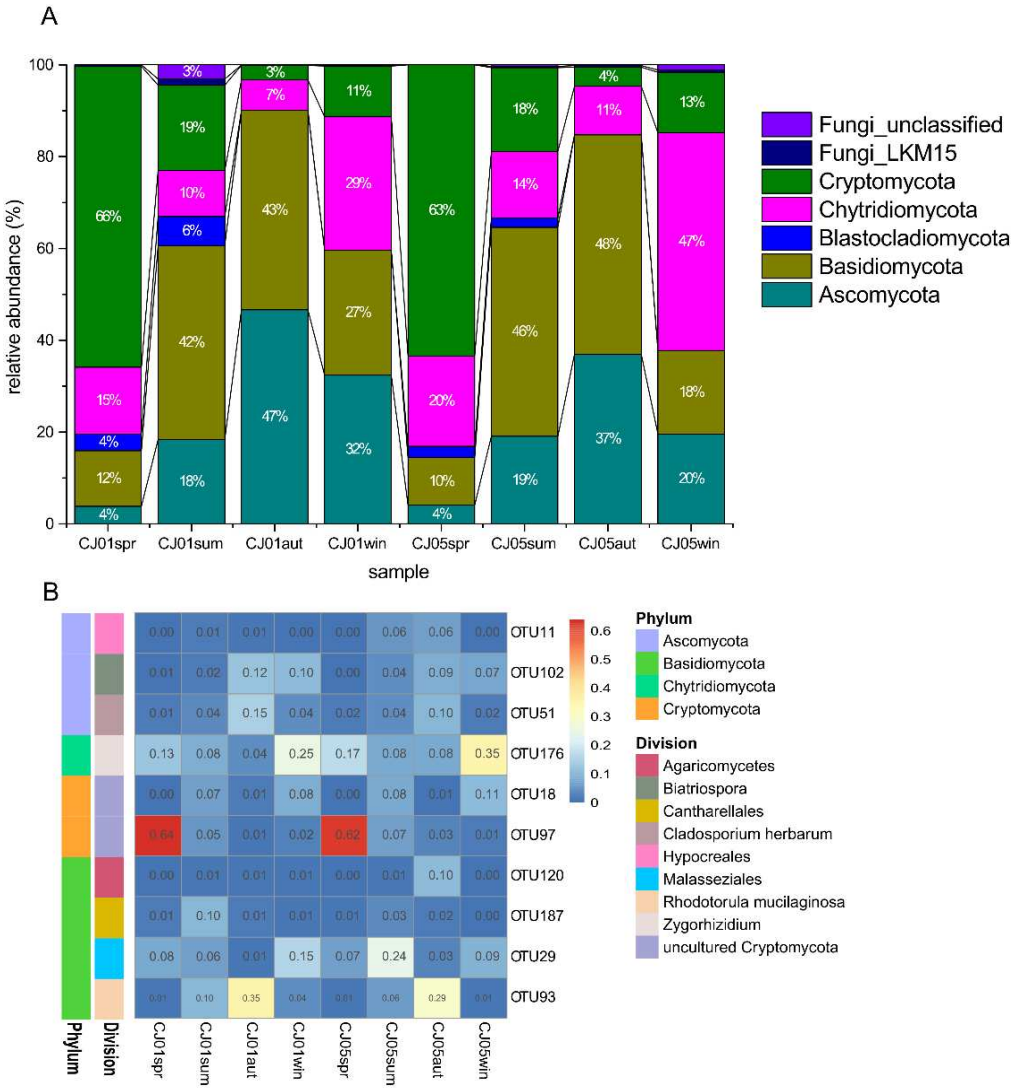

Figure 2. *Variation in fungal community compositions at each sampling site within one year. (A) Taxonomic composition at the phylum level. (B) Heat map showing the relative abundance of the top 10 most abundant operational taxonomic units (OTUs) among the 61 fungal OUTs. Spr: spring, sum: summer, aut: autumn, win: winter*

Only 30 fungal OTUs were assigned to a functional group by FUNGuild database. In this study, six trophic modes and eleven functional guilds were found (*Fig. 3*). Saprotrophs occupied most of the OTUs (14 OTUs); pathotroph-saprotroph possessed most reads (15.52% of total fungal reads) despite containing only one OTU. Saprotroph-symbiotroph occupied one OTU and least reads (2.6% of the total fungal reads). Among the 11 functional guilds, undefined saprotroph had the largest percentage of assigned OTUs (12 OTUs). Plant pathogen (six OTUs) and fungal parasite-undefined

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saprotroph (four OTUs) were the other moderately abundant guilds. Animal Endosymbiont-Undefined Saprotrophs comprised of one OTU but most percentages of fungal reads (15.52% of total fungal reads).

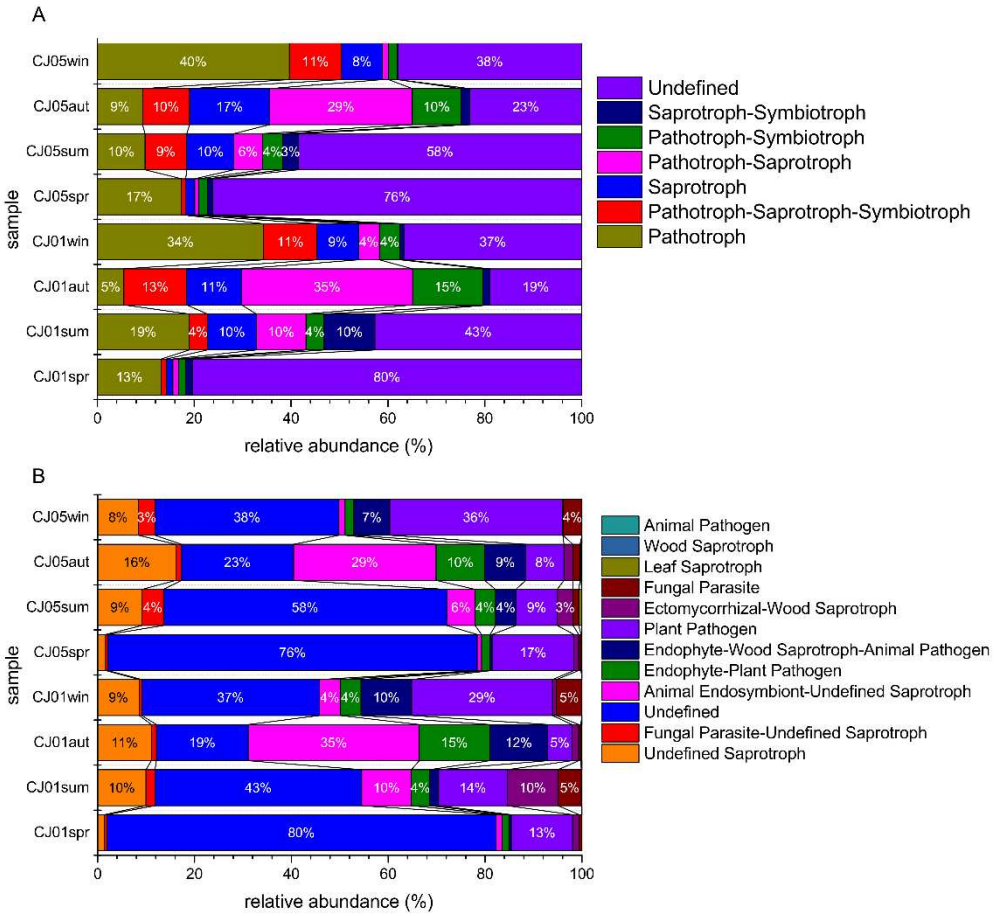

Figure 3. *Compositions of fungal functional guild and trophic mode inferred by FUNGuild. (A) Functional guild. (B) Trophic mode*

Basidiomycota, *Ascomycota*, and *Chytridiomycota* were assigned to nine, four, and two functional guilds, respectively. *Basidiomycota* was assigned to five trophic modes, while *Ascomycota* was assigned to four trophic modes. The trophic modes of *Chytridiomycota* were pathotroph (two OTUs) and saprotroph (one OTU).

Variations of community structure and composition

The diversity indices (Shannon-Wiener and Pielou's evenness) did not differ significantly between the two sampling sites: CJ01 and CJ05 sites (all $p > 0.8$, Mann-Whitney U-test). Moreover, the diversity indices did not change significantly among the four time points (Kruskal-Wallis test, $p > 0.09$). Fungal communities were primarily differentiated by time (*Fig. 4*) and differences in community structure of ultraplanktonic fungi were significant (PERMANOVA, $p = 0.0101$); no significant difference were found between sampling sites (PERMANOVA, $p = 0.8278$). Based on the overall fungal community composition (at the OTU resolution), SIMPER analysis indicate that

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temporal differences were mainly associated with the following OTUs: OTU97 (*Cryptomycota*, 23.94%), OTU93 (*Basidiomycota*, *Rhodotorula*, 15.94%), OTU29 (*Basidiomycota*, *Malasseziales*, 7.196%), OTU51 (*Ascomycota*, *Cladosporium*, 5.94%), OTU102 (*Ascomycota, Biatriospora,* 5.52%) and OTU176 (*Chytridiomycota*, *Zygorhizidium*, 5.28%).

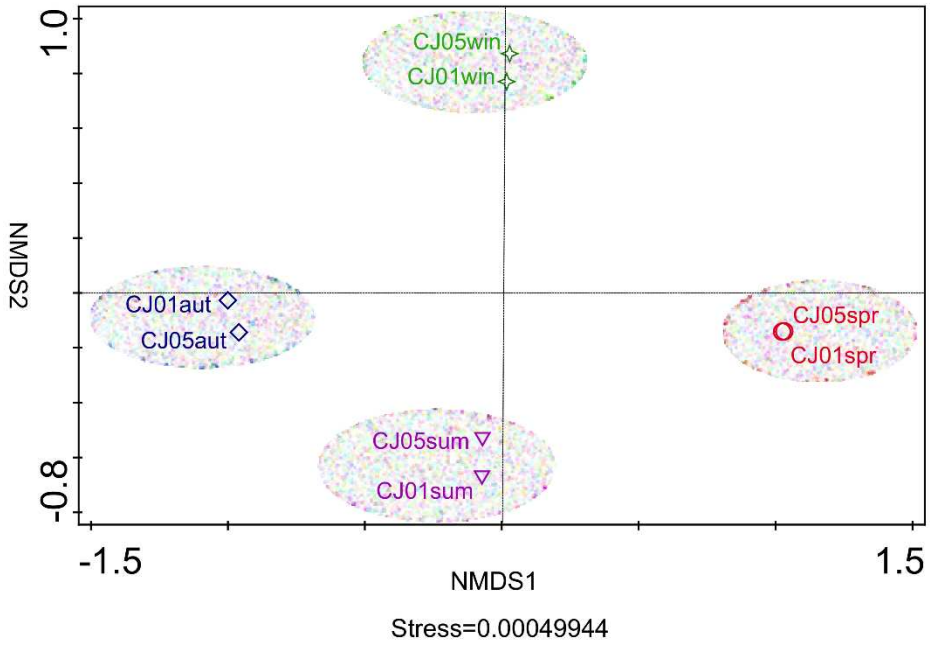

Figure 4. *Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis distances*

In this study, dominant phyla varied with time (*Fig. 2A*). *Cryptomycota* and *Chytridiomycota* were dominant in spring and winter seasons, respectively. In warm seasons (summer and autumn), *Basidiomycota* and *Ascomycota* dominated total fungal reads. Most of the top 10 abundant OTUs showed temporal variations (*Fig. 2B*). Seven of the top 10 abundant OTUs achieved maximum abundance in the warmer season, while the other OTUs showed maximum abundance in winter or spring season. For example, OTU176 (*Chytridiomycota*, *Zygorhizidium*) was present in all seasons; however, the abundance of OTU176 was maximum in winter. Moreover, OTU93 (*Basidiomycota*, *Rhodotorula*) was predominant in autumn. Furthermore, OTU97 (*Cryptomycota*) accounted for over 60% of the fungal reads in spring. We found that the relative abundance of three of the 61 OTUs varied significantly between two time points (*Fig. 5*). Compared to summer, the relative abundance of OTU97 (*Cryptomycota*) was significantly higher in spring (White's non-parametric t-test, $q < 0.01$). Moreover, OTU86 (*Ascomycota*, *Trematosphaeria*) was more abundant in autumn than in summer (White's non-parametric t-test, $q < 0.01$). Furthermore, OTU145 (*Basidiomycota*, *Phallus*) was far more abundant in autumn than in winter (White's non-parametric t-test, $q < 0.01$).

Individual OTUs showed variable patterns of temporal change on a finer scale (*Fig. 6*). The read numbers of most OTUs were more abundant in a particular season. Temporal patterns were observed in the relative abundance of these OTUs. Few OTUs showed consistent abundance, regardless of season (for example, OTU 176,

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Chytridiomycota). Some OTUs had a relatively low abundance in most seasons; however, they showed a relatively high relative abundance in a certain season (OTU 93, *Basidiomycota*, showed relatively higher abundance in autumn; OTU 97 and OTU 189, *Cryptomycota*, showed relatively higher abundance in winter) or in some seasons (OTU 18, *Basidiomycota* were relatively abundant in following seasons: summer and winter). Furthermore, several OTUs were found consistently in a certain season (OTU 86, *Ascomycota* was found consistently in autumn).

By performing a comparative analysis of the defined functional guild structures in the four time points, we observed a distinct distribution. In the CJ01 site, endophyte-plant pathogen accounted for 15% and 1.46% of fungal reads in autumn and spring, respectively (*Fig. 3*). The trophic mode of pathotroph-saprotroph peaked to 35% in autumn, but it was very low in spring. The percentage of some defined functional guilds, such as undefined saprotroph, was relatively higher in autumn. (11% and 16% in CJ01 and CJ05 sampling sites, respectively) (*Fig. 3A*).

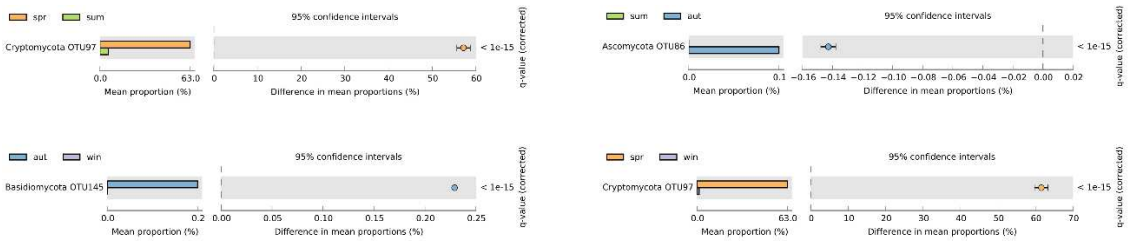

Figure 5. *OTUs that were significantly different between two seasons*

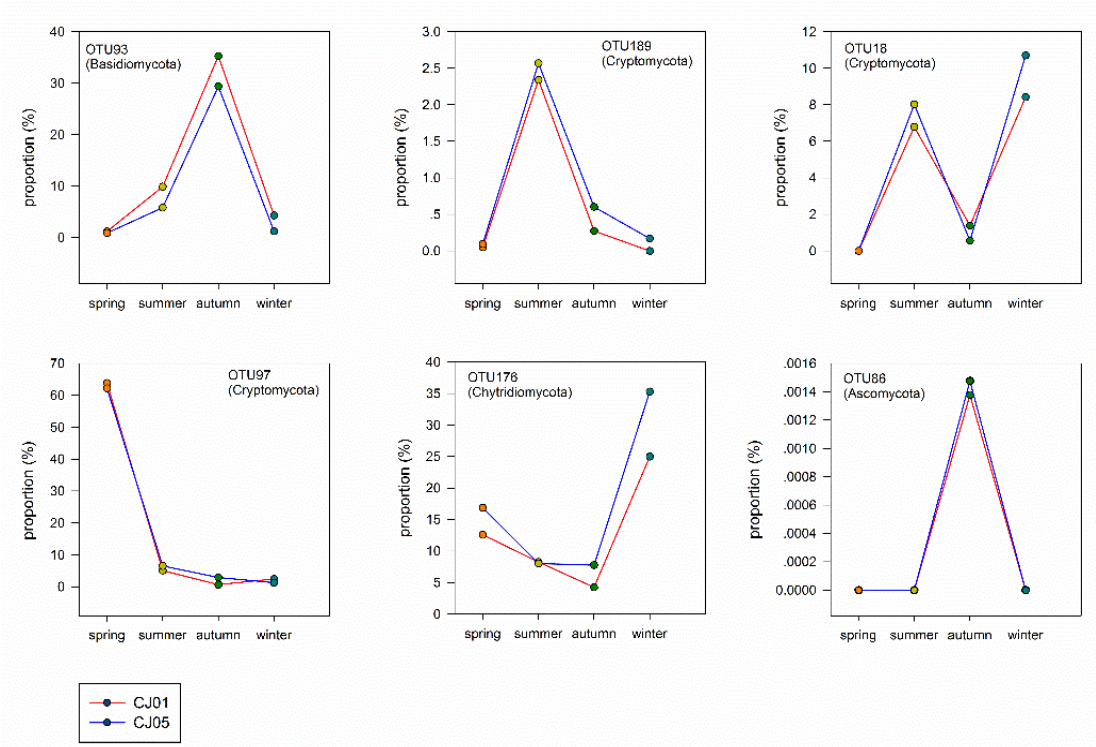

Figure 6. *Relative abundances of six fungal OTUs showing different types of dynamics*

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Correlation of ultraplanktonic fungi and physico-chemical parameters

CCA analysis was conducted for five phyla and three (from 11) forward selected environmental variables (WT, pH, and PO_4^{3-}) (*Fig. 7*). Environmental variables accounted for 91.42% of the variance associated with ultraplanktonic fungi community structure. The first canonical axis accounted for 77.67% of the total variance explained in fungal community structure and were mostly correlated with WT and PO_4^{3-}, while the second canonical axis accounted for 15.35% of the variance and was mostly correlated with pH. The CCA biplot showed that *Basidiomycota* and *Ascomycota* were more related with high temperature. While *Chytridiomycota* and *Cryptomycota* with higher levels of PO_4^{3-}.

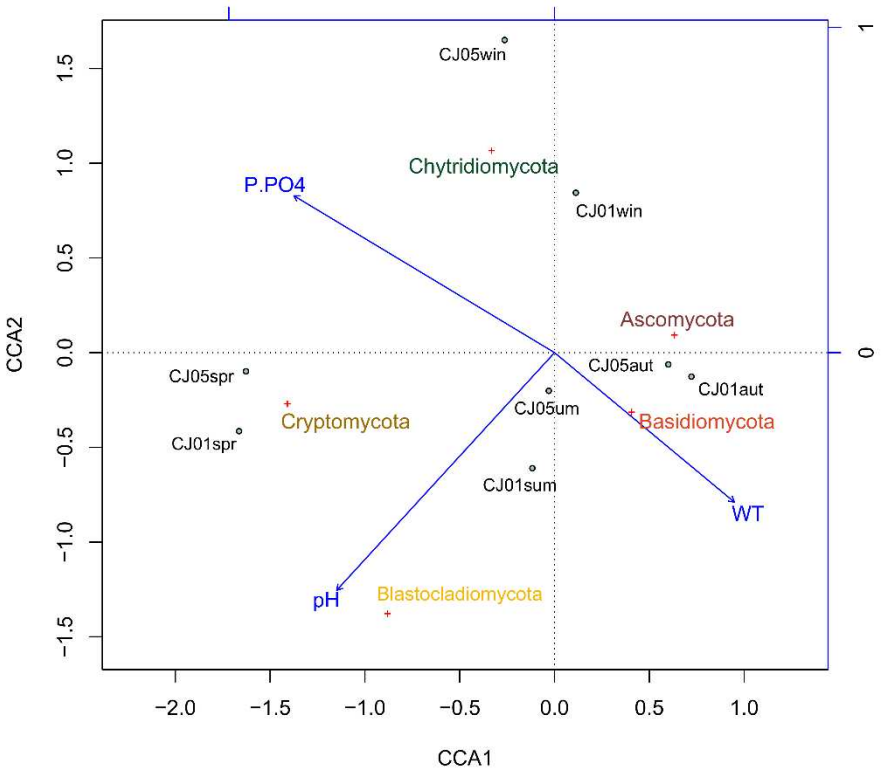

Figure 7. *Canonical correspondence analysis (CCA) ordination plot of fungal phylum and environmental parameters. WT: water temperature; P-PO4: orthophosphate*

Discussion

It is well known that various commonly used primers used to study microbial diversity of environmental samples may introduce biases during amplification (Tedersoo et al., 2015). Some researchers prefer the SSU rDNA sequence and others prefer ITS regions to survey fungi from environmental samples (Richards et al., 2012). The ITS approach is useful for determining species diversity, but it is of limited use for extrapolating early diverging fungal lineages and investigating novel groups (Horton and Bnms, 2001), therefore ruling out its use as the only marker for phylogenetic studies (Frenken et al., 2017). For early diverging fungal lineages, sequences from the small subunit (SSU) rRNA gene (18S) can provide the affiliation of higher taxonomic ranks (Cole et al., 2014). Some researchers have focused on sampling the SSU rRNA

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gene to identify novel fungal diversity among higher taxonomic groups (Jebaraj and Raghulnimar, 2009; Preston, et al., 2012; Kivlin and Treseder, 2015), despite that this gene cannot discriminate between closely related fungal species. In some studies, the small subunit has been particularly useful for phylogenetic placing of *Chytridiomycota* and *Cryptomycota* and other aquatic fungi (Ishii et al., 2015; Panzer et al., 2015). We pay more attention to the new fungal diversity among higher taxonomic groups, so we prefer the SSU rDNA sequence primers.

The significance of planktonic fungi in the large reservoir is unclear, mainly because data on fungal taxonomy and function in this habitat have been limited. Taxonomically, aquatic hyphomycetes that typically decompose leaf litter and wood are mainly associated with Ascomycota, and only a small percentage are affiliated with *Basidiomycota*. In this study, *Ascomycota* and *Basidiomycota* were the dominant phyla in the warm season, but the relative abundance of these phyla was low in the spring (*Fig. 2A*). This change may reflect changes in the function of the whole fungal community. For instance, *Biatriospora* (*Ascomycota*), *Cladosporium* (*Ascomycota*) and *Rhodotorula* (*Basidiomycota*) reach their maximum relative abundance in autumn. *Biatriospora* is associated with plant material found in aquatic habitats (Kolařík et al., 2017). *Cladosporium* species are agents of decay, deterioration, or a cause of allergies or even plant or animal disease, and often have a high environmental impact (Schubert et al., 2007). *Rhodotorula* is a saprophytic fungus that can decompose many substances (Wang et al., 2018).

In this study, the relative abundance of *Chytridiomycota* was the lowest in spring (*Fig. 2A*). In general, the number of parasitic chytrids increases with the growth of phytoplankton (Ibelings et al., 2004). Compared to winter, the relative abundance of *Chytridiomycota* was still much lower in spring (*Fig. 2A*). This phenomenon may be attributed to the dominance of *Cryptomycota* in spring (more than 60%). The dominance of *Cryptomycota* was mainly due to OTU97 (LKM11). Environmental clades LKM11 and *Rozella* formed the deepest-branching clade of fungi (Lara et al., 2010). *Cryptomycota* are parasitic fungi, and some *Cryptomycota* are parasitic in chytrids (Gleason et al., 2014). We hypothesize that OTU97 may be a hyperparasite. Furthermore, the abundance of chytrids may be limited by a large number of *Cryptomycota*. This conforms to the theoretical assumption that both a decline in phytoplankton populations and an increase in hyperparasite populations as the growing season progresses would result in a decrease in chytrid populations (Gleason et al., 2014). Another possible explanation for the dominance of *Cryptomycota* in the spring is that some *Cryptomycota* can parasitize green algae (Held, 1981). In the Three Gorges Reservoir, green algae dominate in spring and reach maximum biomass (Wang et al., 2015). A large number of green algae provide hosts for *Cryptomycota* so that *Cryptomycota* can thrive.

In winter, diatom is the dominant group in the Three Gorges Reservoir, its biomass accounts for the highest proportion (Wang et al., 2015). In the Three Gorges Reservoir, the average water temperature is higher than that of air temperature by 10 ℃ in winter (Zhou et al., 2019). This higher temperature is conducive to algae growth. In this survey, the relative abundance of *Chytridiomycota* was the highest in winter. Generally, the dominance of parasitic fungi in winter is unexpected, given the usual lack of suitable algal hosts during this period. Diatoms reach their maximum biomass in winter could explain the dominance of *Chytridiomycota* with parasitic lifestyles, diatoms provides suitable algal hosts for chytrids. In this study, most taxa of *Chytridiomycota* belong to

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the parasitic genus *Zygorhizidium* (80.01% of total *Chytridiomycota* reads). *Zygorhizidium* genus is conducive to the growth of cladoceran zooplankton (Kagami et al., 2007). Thus, chytrid acts as a direct link between inedible or poorly nutritious phytoplankton and filter-feeding zooplankton (Wurzbacher et al., 2010; Haraldsson et al., 2018). This indicates the ecological importance of mycoloop in freshwater ecosystems.

Terrestrial filamentous fungi can be introduced into lakes through spores and pieces of mycelia during inflowing stream, rainwater and wind events (Voronin, 2014). The vegetation on both sides of the Three Gorges Reservoir is dense, and a large number of wood and leaf litter enter the reservoir via flowing streams in the warm season. This litter carries a large number of fungi. In our survey, we found that 18 OTUs only occur in warm seasons. In the *Basidiomycota* and *Ascomycota* phyla, terrestrial taxa are the most dominant taxa. For example, OTU187 (*Cantharellales*) and OTU51 (*Cladosporium*) are considered terrestrial taxa (El-Nagdy and Nasser, 2000). The relative abundance of these terrestrial fungi varies according to time. This variance may be one of the reasons for the temporal variations in the fungal community. Based on our observations, it can be implied that allochthonous inputs are an important source of fungal species in aquatic ecosystems.

In this survey, we also observed an obvious temporal variation in functional guilds of fungi (*Fig. 3*). In cold seasons (spring and winter), fungal parasites dominated functional guilds. With an increase in temperature, the proportion of saprotrophic functional guilds also increases. The highest proportion of saprotrophic functional guilds occurs in autumn. There is tremendous leaf fall in autumn, and a lot of organic matter is input into the reservoir. Saprotrophic fungi depend strongly on the organic carbon content across biomes (Fierer et al., 2009). The input of allochthonous organic matter can impact the fungal community in the reservoir. Therefore, saprotrophic fungi increase as a result of increased organic matter inputs. Seasonal allochthonous inputs are likely important drivers of the fungal community in the reservoir. Interestingly, phytoplankton biomass was highest in summer and lower in autumn in the Three Gorges Reservoir (Wang et al., 2015). The increase in pathotroph-saprotroph OTUs in summer and autumn may be related to the presence and senescence of algal taxa during phytoplankton succession along the season (Sun et al., 2017). However, in this study, only 30 OTUs (30/61, 49.2%) were annotated by the FUNGuild annotated database. The FUNGuild database has been cited by a large number of studies (Lu et al., 2018; Toju et al., 2018; Philpott et al., 2018; Jacobsen, et al., 2018). A previous study revealed that fungal guild characteristics were well represented and that guild assignment relies heavily on accurate OTU taxonomic identification (Nguyen et al., 2016). There are a large number of unassigned groups of fungi in our results, and more accurate results need to be further studied.

Chytridiomycota and *Cryptomycota* were mostly related with higher PO_4^{3-} levels. Fungi react indirectly to physico-chemical factors; this implies that PO_4^{3-} ions may be indirectly affecting these fungi. Furthermore, PO_4^{3-} ion is a limiting nutrient in aquatic systems, and it is especially critical for autotrophs (McMahon and Read, 2013). The presence of phosphorus in lakes is positively correlated with phytoplankton (Lv et al., 2011). In this study, some of the recovered *Cryptomycota* and *Chytridiomycota* OTUs (e.g. OTU125, *Rhizophydiales*) are parasites of phytoplankton. These parasites extract nutrients from phytoplankton (Arce Funck et al., 2015). So, plankton can be beneficial for the growth of fungi. Therefore, PO_4^{3-} levels can influence the abundance of fungi. In

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our investigation, we found that the diversity of fungi is reduced by eutrophication. This finding has also been reported by researchers in previous studies (Duarte et al., 2015).

Conclusion

To conclude, our study showed that the Three Gorges Reservoir region harbors high-level fungal diversity with an obvious temporal variation of community composition and functional guilds. Parasites thrive in the spring and winter and saprotrophs prevail in the autumn. This highlights a temporal change in the dominant functional role of fungi in the reservoir. Our results show that terrestrial inputs may be an important source of fungal species in reservoir ecosystems. A high species richness and many aquatic hyphomycetes were highlighted, suggesting important ecological roles for fungi in the reservoir region. Finally, to obtain insight into the activity of aquatic fungi and their associated functions, more effective approaches will be necessary in future studies.

Acknowledgments. This work was supported by the National Natural Science Foundation of China (41171045), the Research Fund for Science and Technology Innovation Team of University (T201504). We are thankful to Dr. Yonghong Bi and the Three Gorges reservoir's ecosystem field station for the assistance of sampling. We are grateful to the editor and the anonymous referees for their professional assistance and comments on this manuscript.

Competing interests. The authors declare no competing financial interests.

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DOI: http://dx.doi.org/10.15666/aeer/1806_75157533

APPENDIX

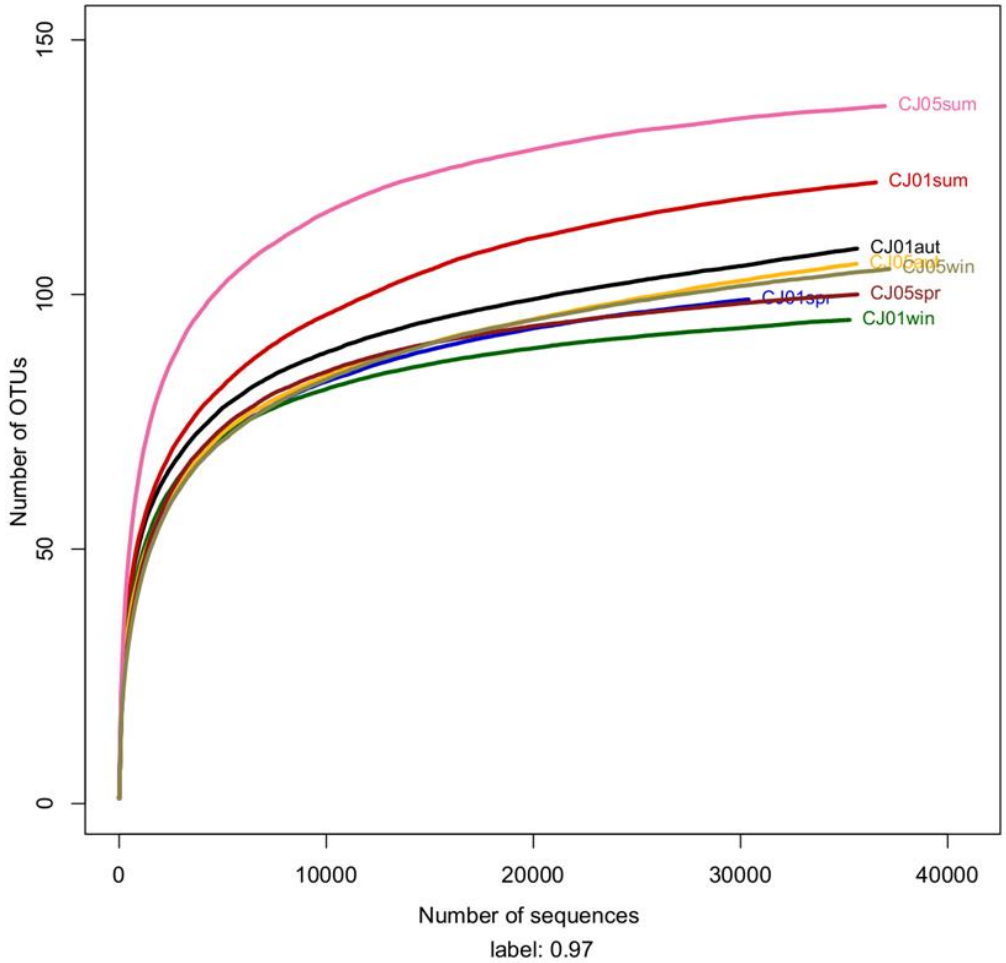

Figure A1. *Rarefaction curves. (Spr: spring, sum: summer, aut: autumn, win: winter)*

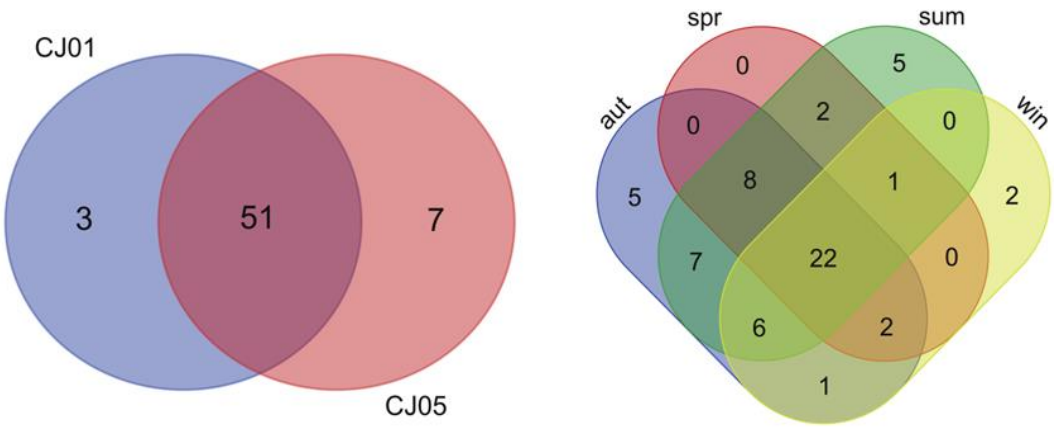

Figure A2. *Venn diagrams showing shared and unique seasons and sampling sites by operational taxonomic unit (OTU)*

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DOI: http://dx.doi.org/10.15666/aeer/1806_75157533

Table A1. *Physical and environmental characteristics of the sampling sites*

	CJ01spr	CJ01sum	CJ01aut	CJ01win	CJ05spr	CJ05sum	CJ05aut	CJ05win
TP (g/L)	0.075	0.084	0.079	0.074	0.079	0.035	0.139	0.077
TN (mg/L)	1.911	2.006	2.122	1.800	1.889	2.880	2.255	2.033
COD (mg/L)	1.143	1.451	2.222	1.569	1.219	1.725	2.343	2.000
P-PO4 (mg/L)	0.076	0.054	0.051	0.064	0.076	0.050	0.054	0.075
N-NH4 (mg/L)	0.027	0.026	0.069	0.094	0.006	0.039	0.028	0.072
N-NO3 (mg/L)	1.667	1.378	1.149	1.718	1.635	1.327	1.184	1.787
WT (℃)	14.800	26.820	22.280	18.440	15.000	26.570	22.210	18.410
DO (mg/L)	9.130	6.730	7.530	9.120	10.050	6.720	7.850	9.370
ORP (mV)	332.400	127.000	186.000	247.000	335.500	83.000	195.000	258.000
pH	8.750	8.470	8.020	8.160	8.700	8.460	8.050	7.490
Turbidity (NTU)	1.200	4.500	2.100	1.500	1.400	5.800	1.900	1.400
TLI	40.692	43.703	47.433	43.144	41.488	42.429	51.414	46.178

Spr: spring, sum: summer, aut: autumn, win: winter. WT: water temperature, DO: dissolved oxygen, TN: total nitrogen, P-PO4: orthophosphate, N-NO3: nitrate, TP: total phosphorus, N-NH4: ammonium, COD: chemical oxygen demand, ORP: oxidation-reduction potential, TLI: trophic level index

ELECTRONIC APPENDIX

This manuscript has five electronic appendices with basic data:

Appendix 1. Information on the classes and orders identified from the 8 samples
Appendix 2. Compositions of fungal functional guild inferred by FUNGuild
Appendix 3. The distribution of fungal functional guild in the phylum level
Appendix 4. The distribution of fungal trophic mode in the phylum level
Appendix 5. Similarity Percentage analysis (SIMPER) of the 61 fungal OTUs

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75157533

EFFECTS OF FERTILIZATION AND STRAW INCORPORATION ON BACTERIAL COMMUNITIES IN BLACK SOIL, NORTHEASTERN CHINA

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(Received 20th Apr 2020; accepted 13th Aug 2020)

Abstract. The effects of three treatments including no fertilizer or straw returned to the field (CK), chemical fertilizer with straw not returned to the field (F) and no fertilizer with straw returned to the field (W) on the composition, diversity and structure of the soil bacterial community were examined. The results showed that a total of 1,833,232 reads and 25,843 operational taxonomic units (OTUs) were obtained through sequencing. The dominant bacteria of the black soil in the northeast (>10%) were Proteobacteria, Acidobacteria, Bacteroidetes, Chloroflexi and actinomycetes. The soil bacterial diversity was greatly improved, but fertilization had little effect on the soil bacterial diversity. A stratified clustering map showed that the soil bacterial community structures after W and F treatments were similar to one another at genus level, and the soil bacterial community structures after treatment with W and F were different from those after treatment with CK. The effect of fertilizer application on the composition, relative abundance and community structure of dominant groups of soil bacteria was greater than that of straw mulching, which significantly reduced the soil bacterial richness, whereas straw mulching significantly increased the soil bacterial diversity.
Keywords: *chemical fertilization, straw residue, high-throughput sequencing, Mollisol, bacterial composition*

Introduction

Soil microorganisms are not only an important part of the soil, but are also the primary promoters of soil nutrient cycles. Changes in the soil microorganism community can reflect changing trends in soil quality to a certain extent (Zhang et al., 2005). Bacteria are the most abundant and widely distributed microbial community in plural, participating in nearly all biochemical cycles of terrestrial ecosystems, and playing an important role in maintaining the health of farmland ecosystems and soil productivity (Han et al., 2014). However, the research on soil bacteria is limited because most of them are uncultured due to the limitations of traditional molecular biological methods, such as denaturing gradient electrophoresis (DGGE) (Cao et al., 2013). In recent years, high-throughput sequencing technology has provided a powerful tool for the study of microbial molecular ecology, which has been widely applied in the study of environmental and soil microorganisms (Bartram et al., 2011). Chen et al. (2015) used Miseq high-throughput sequencing technology based on the Illumina platform to compare the soil bacterial community structure in different iron mining areas, which

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75357550

resulted in a deepened understanding of the mechanism by which heavy metal pollution influences the soil bacterial community structure and diversity. Fields such as those in one study (Tian et al., 2013) that were based on the Illumina platform of Miseq high-throughput sequencing technology, the leakage of CO_2 sequestration scenario was studied to evaluate changes in the richness of the diversity and community structure of the farmland soil bacterial community; the results showed that changes occurred in the richness of the diversity and community structure of the cornfield soil bacterial community structure due to leakage of CO_2 sequestration. The soil acid bacillus (Acidobacteria) bacteria showed a relative increase in May as a biological monitoring indicator of the effect of leakage of geologic storage of CO_2 on the soil ecological system. High-throughput sequencing is simple, the results are stable, the repeatability is strong, and the consistency of the sequencing results is as high as 99.99%; thus, this method allows the possibility for in-depth study of soil bacteria (Liu et al., 2014; Zhang et al., 2014).

Black soils (Mollisols) are a type of soil with characters of inherently fertile and productive. Around the world, there are four dominant Mollisol distribution areas, which are located in North America, in Russia and Ukraine, in South America and in north-eastern of China (Liu et al., 2019). Black soil is one of the important soils in the northeast plain of China and is primarily distributed in this area, covering an area of approximately 1.02 million square kilometres (Xing et al., 2005). The soil type is classed as a phaeozem (IUSS Working Group WRB, 2006). The black soils in Heilongjiang province are fertile, with relatively high cation exchange capacity, epipedon macroaggregate stability and high organic matter content (Hu et al., 2017). Crop stalks produced in the process of crop production are one of the important organic fertilizer sources. Simultaneously, crop stalks that are returned to the field as an important link in global organic agriculture have positive effects on maintaining farmland fertility, reducing fertilizer use and improving the land-soil carbon sink capacity (Zhang et al., 2013).

In recent years, returning straw to the field has been widely applied in agricultural production practice, and good results have been achieved with regard to fertilizing black soil and increasing crop yield (Wei et al., 2013; Zhu et al., 2014). Fertilization is one of the most profound agricultural measures affecting soil quality and its sustainable use. Different fertilization systems, soil microbial populations, quantities and activities lead to differences in soil biological fertility, and these differences affect the soil structure. Fertility and productivity have a profound effect (Wang et al., 2014).

Application of chemical and organic fertilizers has a certain effect on microorganism community structure and diversity in farmland soil. Wang et al. (2015) showed that the application of organic fertilizers could improve the diversity of soil fungi. Luo (2014) showed that long-term fertilization of brown soil significantly changed the soil microbial community structure and activity and significantly increased the diversity of bacteria and fungi in the brown soil. In a greenhouse simulation, Sapp et al. (2015) showed that fertilization had a certain effect on soil bacterial communities. Therefore, changes in the bacterial community structure, dominant populations and diversity in farmland soil after fertilization are important for maintaining soil fertility and the farmland ecological environment (Bao et al., 2000). Currently, the effects of fertilization and returning of straw to the field on microorganism groups in the uplands of black soil are rarely reported. Therefore, this study primarily adopted field experiments using the Miseq Illumina platform for high-throughput sequencing of soil

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DOI: http://dx.doi.org/10.15666/aeer/1806_75357550

bacterial 16S rDNA to study the effect of different fertilization systems on the composition, structure and diversity of bacteria in black soil of an arid farmland ecosystem. The results will deepen the understanding of soil microbial groups and changes in soil microbial community structure, providing a theoretical basis for improving soil biological fertility of chernozems.

Materials and Methods

Overview of the test site and test materials

The trial began in 2012 at the Heilongjiang Academy of Agricultural Sciences in the Heilongjiang Province modern agriculture demonstration area (45°49′N, 126°48′E, and altitude 117 m) (*Figure 1*). The area has a mid-temperate continental monsoon climate with a short winter and summer, an annual average temperature of 4.5 ℃, an annual ≥10 ℃ effective accumulated temperature of over 2700 ℃, an annual crop growth period of approximately 155 d, an annual average precipitation of 569.1 mm, and precipitation primarily concentrated in June-September. The soil type is black soil, and soil nutrient contents in the 0-20 cm soil layer are as follows: organic matter of 29.56 $g \cdot kg^{-1}$, total nitrogen of 2.81 $mg \cdot kg^{-1}$, available nitrogen of 79.56 $mg \cdot kg^{-1}$, available phosphorus of 55.84 $mg \cdot kg^{-1}$ and available potassium of 168.42 $mg \cdot kg^{-1}$. The rice variety tested was long rice 21.

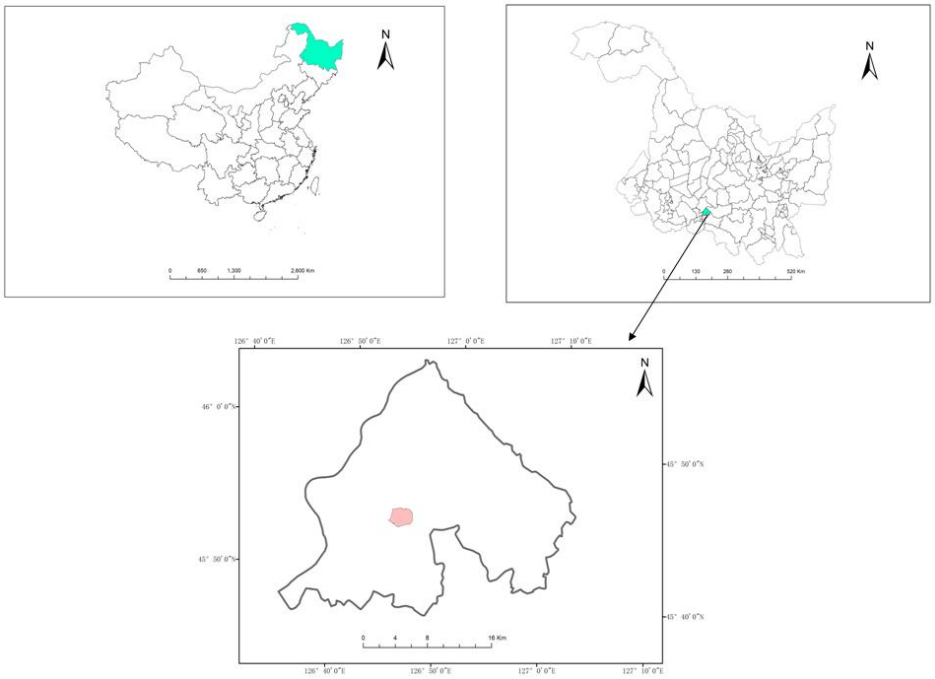

Figure 1. *The research location of this experiment*

Experiment design

The stubble crop before the experimental field was rice, and the planting mode was single-season rice, leaving the field free of crops in the winter. For the plots, the area was 24 m^2. The straw used in the experiment was the rice straw harvested in the

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previous season, which was dried and cut into approximately 5 cm pieces and returned to the field evenly. To ensure the uniformity of the test, all the stubble straw was removed before the site was cleared and then returned to the field accurately according to the treatment. The crushed straw was spread evenly on the surface of the plot, mixed in the soil layer by rotary tiller, and the stubble field was transplanted to rice after a week.

Three treatments for the amount of straw returned to the field were used in the experiment, namely, no fertilizer or straw returned (the amount of straw was 0 kg/hm^2) to the field (CK), fertilizer with no straw returned (the amount of straw was 0 kg/hm^2) to field (F) and no fertilizer with straw returned (the amount of straw was 7500 kg/hm^2) to field (W). Each treatment was replicated 3 times in the field. All treatments were applied with the same amount of fertilizer (N3). Approximately 133 kg·hm^{-2} of nitrogen fertilizer (pure nitrogen) was applied, 46 kg·hm^{-2} of pure phosphorus was applied, and 75 kg·hm^{-2} of pure potassium was applied. Phosphate fertilizer was used as a base fertilizer (100%) once. The fertilizer was applied to a base fertilizer (50%), a turning green fertilizer (30%) and a tillering fertilizer (20%), and the amount of straw returned was 7500 kg/hm^2. Random permutations were used with 3 replicates. For the plots, the size was 24 m^2. Seeding was performed on April 15; seedling planting was performed on May 15; and the seedling specifications were 30 cm (row spacing) x 13 cm (plant spacing) x 3 plants/hole. Other management measures were implemented uniformly according to the local conventional cultivation requirements. From 2012, we always continued these two treatments (N and straw) every year in order to investigate the effects of long-term treatment on soil bacterial diversity and structure.

The arable layer soil (0-20 cm) was sampled in October 2017, with each processing district thoroughly incorporated with multipoint sampling. Each soil sample was divided into two parts. One hundred grams was returned to the lab in sterilized bags and stored at -80 °C for the next step of molecular biology research. Approximately 800 g that remained was used to analyse the basic physical and chemical properties of the soil (*Table 1*); the basic physical and chemical properties of soil were determined with reference to the methods described by Bao et al. (2000).

Table 1. *Chemical properties of the soil after different treatments*

Treatment	pH	Organic matter (OC) (g/kg)	Total N (g/kg)	Available P (AP) (mg/kg)	Available K (AK) (mg/kg)
CK	7.09±0.02a	16.01±0.15b	1.01±0.02b	8.02±1.02b	117.56±5.45b
F	5.47±0.03c	17.05±0.20a	1.22±0.03a	26.08±1.56a	145.64±6.52a
W	6.25±0.01b	16.56±0.78ab	1.08±0.01b	15.81±3.20b	137.65±6.52ab

Note: Different small letters in the same column indicate significant differences among treatments at a significance level of 0.05. The same applies to the results described below. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned

Extraction of total DNA from soil microorganisms and high-throughput sequencing of bacteria

A strong soil DNA extraction kit from the MOBIO Power Soil ® DNA Isolation Kit (USA) was adopted, and soil microbial genomic DNA was extracted according to the operation manual. A nucleic acid quantifier (NanoDrop ND-1000) was used detect the concentration and purity of the total DNA extracted from the soil. After the samples

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were purified, the DNA was sent to the MiSeq Illumina platform of Beijing Biomaker Company for sequencing.

Synthesizing specific primers with bar codes according to the designated sequencing area was the primary step of the high-throughput sequencing. PCR used TransGen AP221-02 and TransStart Fastpfu DNA Polymerase, with PCR conducted on an ABI GeneAmp ® 9700. All samples were conducted in accordance with the formal experimental conditions, and each sample was replicated 3 times. The PCR products of the same sample were mixed and detected by 2% agarose gel electrophoresis. PCR amplification was performed using primers 338F (5 '-actcctacgggaggcagca-3') and 806R (5 '-ggactachvgggtwtctaat-3'), which amplified the V3-V4 region of 16S rDNA of soil bacteria (Zhang et al., 2015). The 20-μLPCR reaction system was as follows: 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mmol·L^{-1} dNTPs, 0.4 μL of forward primer (5 μmol·L^{-1}), 0.4 μL of reverse primer (5 μmol·L^{-1}), 2 μL of template (10 ng DNA), 0.4 μL of FastPfu Polymerase (Trans Start Fastpfu DNA Polymerase, TransGen, China), and 10.8 μL of ddH2O. PCR amplification procedure was performed as follows: 95 °C pre degeneration for 2 min, modified at 55 °C and 95 °C for 30 s and 30 s, annealing at 72 °C, and extending for 30 s, for a total of 30 cycles; completed at 72 °C and eventually extended to 10 min. According to the results of the electrophoresis, preliminary quantitative PCR products were detected with the QuantiFluor™- ST blue fluorescence quantitative system (Promega). Then, according to the volume of sequencing, each sample was quantified according to the corresponding percentage of hybrid.

High-throughput sequencing data analysis

The experiment used pair-end sequencing. First, quality control of raw data was conducted, and the sequences were connected with the Flash software (Magoč and Salzberg, 2011) to discard sequences that could not be connected. According to the test requirements, the bases below the mass value of the tail of the read were filtered with the window set to 50 bp. When the average mass value in the window was lower than 20 bp, the base at the back end from the window was cut off, and the reads below 50 bp were filtered after quality control. According to the overlapping relationship between PE reads, the paired reads were spliced (merged) into a sequence, and the minimum overlap length was 10 bp. The maximum error ratio allowed in the overlapping region of the mosaic sequence was 0.2, and the non-conformance sequence was screened. The box sequence at the end of the sequence was detected, and the minimum mismatch number was 0. The sequence containing box at the beginning was inversely complemented, and the box was removed. The barcode on the test sequence was detected, and the samples were distinguished. The number of the barcode mismatches was 0, the maximum primer mismatches was 2, and the final sequence for analysis was obtained (Edgar et al., 2011).

With the application of QIIME (Quantitative Insights into Microbial Ecology), the sequences were classified into multiple OTUs (operational taxonomic units) according to similarities between the sequences. After OTU production, the OTUs contained in each sample and the number of sequences contained in each OTU was calculated. The uparse OTU (version 7.1; http://drive5.com/uparse/) method was used for clustering, with the OTU sequence similarity set at 97%, to obtain representative OTU sequences (Edgar et al., 2011). Using uchime (version 4.2.40; http://drive5.com/usearch/manual/uchime_algo.html), PCR amplification of chimeric

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sequences was detected and removed from the OTUs (Quast et al., 2012). Using the usearch_global method (Edgar, 2010), the map of the optimized sequence was compared back to the OTU representative sequence, and the abundance statistics table of each sample sequence of an OTU was obtained. To obtain each OTU corresponding species classification information, the RDP classifier Bayesian algorithm at a 97% similarity level was used for analyses of the taxonomic OTU on behalf of the sequences, and at all levels (phylum, class, genus) for the statistical community composition of each sample, we specified the dominant population at the phylum, and class level relative abundance was greater than 10%, with the relative abundance of the genus level greater than 1%. The comparison database was as follows: Silva (Release115 http://www.arb-silva.de) (Chao et al., 1984). The OTUs with a similarity at 97% were selected to generate the expected dilution curve, and mothur was used to calculate the abundance indexes, Chao1 (1984) and ACE (1993), and the diversity indexes, Shannon (Shannon, 1948a,b) and Simpson (Simpson, 1949). The bacterial community richness was represented by the Chao1 and ACE indexes, and relatively high values indicated high community species richness. The Shannon index reflected the degree of diversity of a sample, and relatively high values indicated high diversity of community species. The Simpson index reflected the dominance of species.

Statistical analysis

Difference in soil physicochemical characteristics, Reads, bacterial alpha diversity indices of different treatments were compared using one-way analysis of variance, which was followed by the Duncan's difference test performed in IBM SPSS (version 17.0; Chicago, IL, USA) (Banerjee et al., 2016).

The shared and unique OTUs among different treatments were used to generate Venn diagrams using R software (version 3.3.2; R Development Core Team, 2017). Rarefaction curves were conducted by R software with "vegan" package based on OTU level (version 3.3.2; R Development Core Team, 2017). The heatmap representation of the top 50 classified genera in per treatment was built using the R software with "ggplot" and "pheatmap" packages (version 3.3.2; R Development Core Team, 2017). In order to find the relationships of soil physicochemical properties and soil bacterial community, Redundancy analysis (RDA) was used to analyze the soil physicochemical properties and soil bacterial community at OTU level. The RDA was performed by R software with "vegan" package.

Results

Soil physicochemical properties

As seen in *Table 1*, soil pH value ranged from 5.47 to 7.09. Soil pH value under F treatment was the most acidic with 5.47, followed by W treatment, while CK contained the highest soil pH value. There was significant difference among different treatment regarding soil pH, OC, TN, AP and AK ($P<0.05$, *Table 1*). Interestingly, OC, TN, AP and AK exhibited highest value in the F treatment, with the values of 17.05 g/kg, 1.22 g/kg, 26.08 mg/kg and 145.64 g/kg, respectively.

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Sequencing results of soil samples and validation of sampling depth

Using the bacterial 16S rDNA V3-V4 sequencing area, with low quality sequences filtered from the original sample, a total of 210,383 valid sequences and 183,232 readings (reads), clustering under 97% similarity for OTUs for species classification, and the statistics of each sample for different OTU abundances were obtained; the nine samples produced 25,843 OTUs. As shown in *Table 2*, the soil treated with W contained the maximum number of OTUs, followed by the CK treatment, and the minimum number of OTUs occurred after treatment with F. Compared with the CK treatment, the number of soil OTUs decreased by 4.70% after treatment with F, whereas the number of soil OTUs increased by 11.9% after treatment with W.

Table 2. *Reads and OTU numbers of soil bacterial sequences after the different treatments*

Treatment	Reads	OTUs
CK	16889±5 259a	2655±172ab
F	20539±6 225a	2530±95b
W	21447±4 298a	2973±46a

Different small letters in the same column indicate significant differences among treatments at a significance level of 0.05. The same applies to the results described below. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned

The rarefaction curve reflects the sample depth and can be used to evaluate whether the sequencing volume is sufficient to cover all groups. *Figure 2* shows the dilution curve for all samples in this test under the condition of similarity of 0.97. As shown in *Figure 2*, all soil sample dilution curves tended to flatten, indicating that sampling was reasonable, and the confidence in the bacterial community structure in the actual environment was high, which could reflect the bacterial community of a soil sample in a relatively real way.

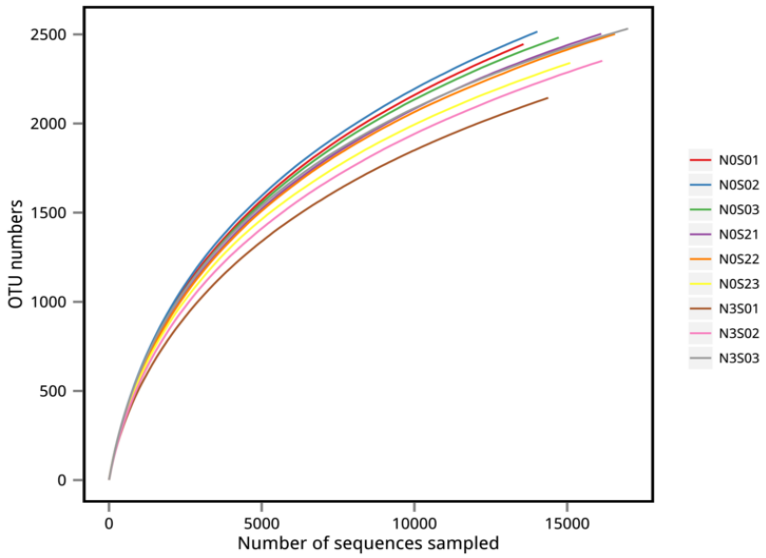

Figure 2. *Rarefaction curves of OTUs clustered at 97% sequence identity across different soil samples. Note: N0S0 represents CK; N3S0 represents F; N0S2 represents W. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned*

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Analysis of soil bacterial community richness and diversity

From *Table 3*, the soil richness indexes (Chao1 and ACE indexes) of the F treatment were significantly lower than those of the CK and W treatments. No significant difference was detected between the bacterial richness indexes of the W and CK treatments. The ACE index of the F treatment was 27.4% lower than that of the CK treatment. The results showed that fertilization significantly reduced the richness of soil bacterial species, but straw returned to the field had little effect on the richness of soil bacterial populations. The Shannon index for soil bacteria treated with F was significantly different from that for the bacteria treated with CK (*Table 3*), whereas the Shannon index for soil bacteria treated with W was significantly lower than that of those treated with CK (6.94), and the Shannon index for soil bacteria treated with W was 2.4% higher than that of those treated with CK. The results showed that the soil bacterial diversity was greatly improved, but fertilization had little effect on the soil bacterial diversity. The Simpson index of the F treatment was significantly higher than that of the CK and W treatments, and the Simpson index of the F treatment was 25.0% higher than that of the CK treatment, whereas the smallest Simpson index was obtained with the W treatment, which was significantly lower than that of the CK treatment by 25.0%. The above results indicated that fertilization significantly reduced the soil bacterial richness and increased the soil bacterial dominance. Straw returned to the field improved the soil bacterial diversity and reduced the soil bacterial dominance.

Table 3. *Richness and diversity indexes of soil bacteria after different treatments*

Treatment	ACE index	Chao1 index	Shannon index	Simpson index
CK	4468.75±72a	4421.57±67a	6.94±0.03b	0.004±0.0001b
F	3241.91±106b	4218.49±60b	6.92±0.04b	0.005±0.0001a
W	4479.60±63a	4330.81±52a	7.11±0.12a	0.003±0.0001c

Different small letters in the same column indicate significant differences among treatments at a significance level of 0.05. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned

Analysis of soil bacterial groups

At the phylum level, bacteria were distributed in 12 known phylum groups, in addition to 11 candidate phyla and an unclassified group. As shown in *Figure 3*, Proteobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Actinobacteria, Gemmatimonadetes, Nitrospirae, Verrucomicrobia, and Ingavibacteriae were detected. The relative abundance of a total of 11 bacterial phyla was greater, and the sum of the relative abundances accounted for more than 95% of the total amount of bacteria in the three treated soil samples.

The dominant bacterial groups in the CK and F treatments were Proteobacteria, Acidobacteria, Bacteroidetes, Chloroflexi and Actinobacteria. The return of straw reduced the relative abundances of Bacteroides and Actinobacteria, which decreased by 10.5 and 9.7%, respectively. The relative abundances of Proteus and Acidophilus increased by 4.7 and 18.5%, respectively. The composition and relative abundance of the dominant bacteria in soil treated with W were different from those in soil treated with CK and F. The dominant bacteria in soil treated with W were deformed bacteria, Acidobacteria and Chloroflexi. Compared with treatment with CK, treatment with W

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increased the relative abundances of Proteobacteria, Acidobacteria and Chloroflexi by 7.5, 15.5 and 32.4%, respectively. The relative abundance of Bacteroidetes decreased by 23.8%.

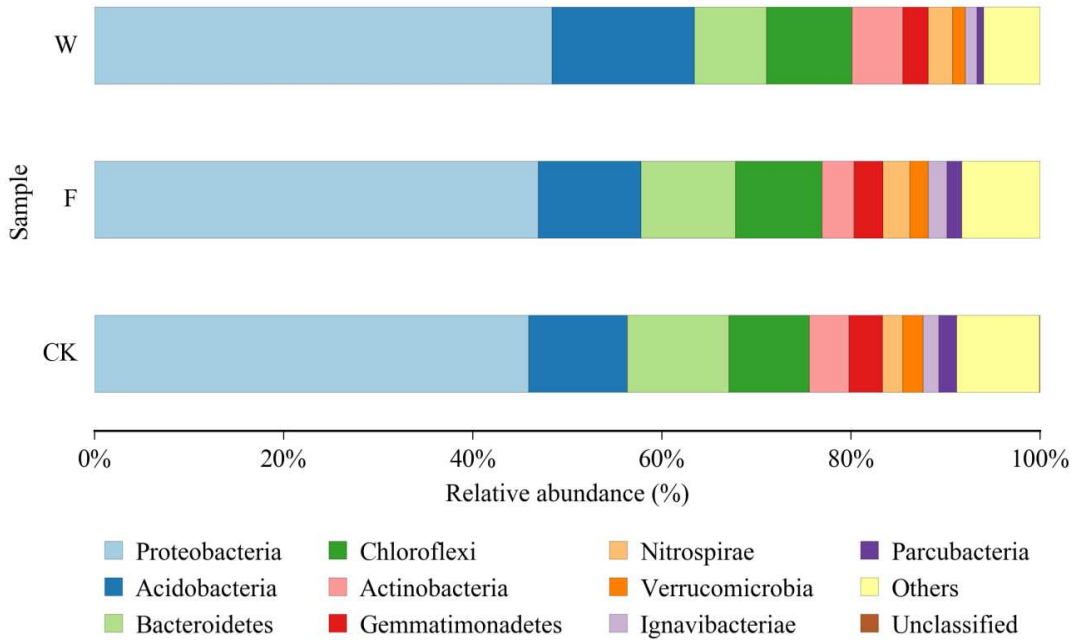

Figure 3. *Comparison of bacterial groups at the phylum level after different treatments. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned*

At the class level, a total of 21 known classes of bacteria were obtained. In addition to unclassified classes, 40 candidate classes of bacteria were obtained. As shown in *Figure 4*, Betaproteobacteria, Deltaproteobacteria, Alphaproteobacteria, Gammaproteobacteria, Sphingobacteriia, Anaerolineace, Solibacteres, Nitrospira, Holophagea, Gemmatimonadetes, Actinobacteria, Cytophagia, Acidobacteria, Ignavibacteria, Blastocatellia, and the Bacteroidetes_vadinHA17 group were relatively abundant classes of bacteria, and the sum of the relative abundances accounted for more than 80% of the total amount of soil bacteria in the three treated soil samples.

Differences in the composition and relative abundance of the three dominant soil bacterial programmes were observed (*Figure 4*). The dominant bacteria in the soil treated with CK were α-proteobacteria, β-proteobacteria and acid bacillus, and the dominant bacteria of the F treatment were α-proteobacteria, sphingolipids, actinomycetes, and γ-proteobacteria.The dominant bacteria in the soil treated with W were α-proteobacteria, β-proteobacteria, and sphingolipidae. Compared with the CK, fertilization increased the relative abundance of e. perciformis, actinomycetes, and sphingolibacilli by 19.2%, 46.4% and 45.3%, respectively. The relative abundance of wild-deformation bacteria was reduced by 36.1%. The relative abundance of bacillus costunica increased by 36.0%, and the relative abundance of candidia-proteobacterium, extension-proteobacterium and acid-bacilli decreased by 5.8%, 18.8% and 28.1%, respectively.

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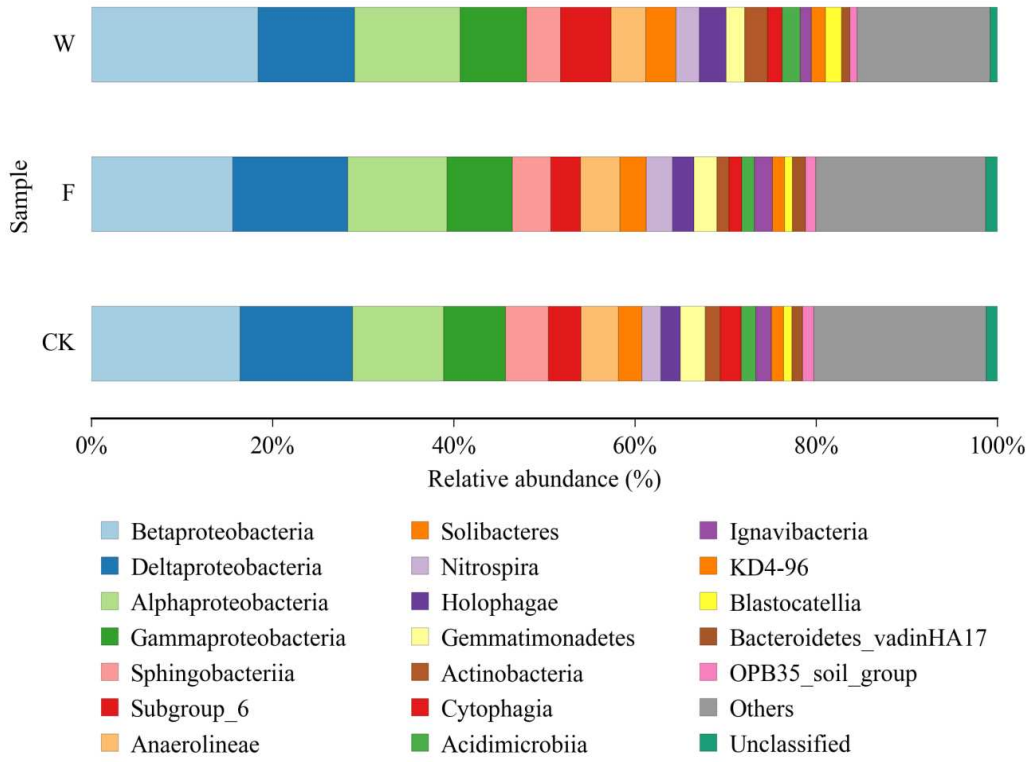

Figure 4. *Comparison of bacteria groups at the class level after different treatments. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned*

A Venn diagram (*Figure 5*) was used to analyse the number and unique dominant genera (relative abundance >1%) shared by three fertilization-treated soil bacterial communities. The dominant genera with the most species (636) were in the F treatment group. In the CK treatment, 610 dominant genera were identified. Fertilizing and returning of straw to the field both significantly increased the number of dominant species in soil of which the dominant population in soil was the largest. The three samples had high relative abundance of Nitrosomonadaceae, Anaerolineaceae, and Gemmatimonas. The unique advantage of CK treatment was that 15 of the genera of rose bend occurred, and 24 genera had a unique advantage from W treatment. The maximum number of bacterial genera in the soil sample treated with F was 35.

The relative abundance level of the top 50 bacteria was used to build a hierarchical clustering figure (hierarchical heatmap; *Figure 6*). The hierarchical clustering figure uses colour changes to visually define the size of the data values by colour depth, and the degree of the colour gradient reflects the similarities and differences in the data. As shown in *Figure 6*, the 9 soil samples were divided into two categories. The bacterial community structure of the 6 soil samples treated with F and W was similar, and the bacteria were clustered into one group. However, the bacteria from the 3 soil samples treated with CK were clustered into another group. The conclusion is that the effect of fertilization and straw return on the soil microbial community structure was significant at the genus level. The red part in the stratified cluster diagram shows the genus with the highest relative abundance, and as is clearly shown, the abundance of the primary soil bacterial communities under different treatments was different.

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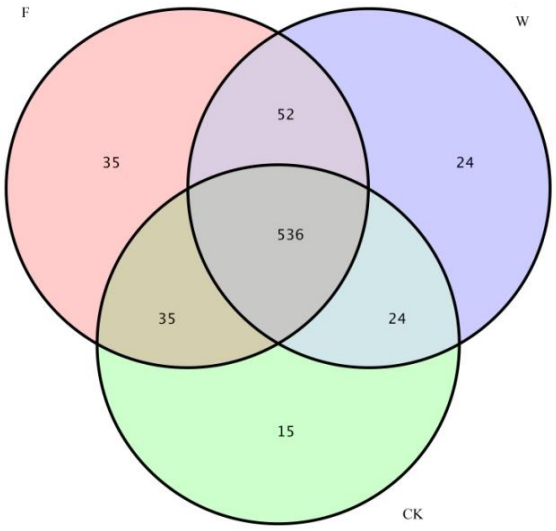

Figure 5. *Venn diagram of the dominant genera among different soil treatments. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned*

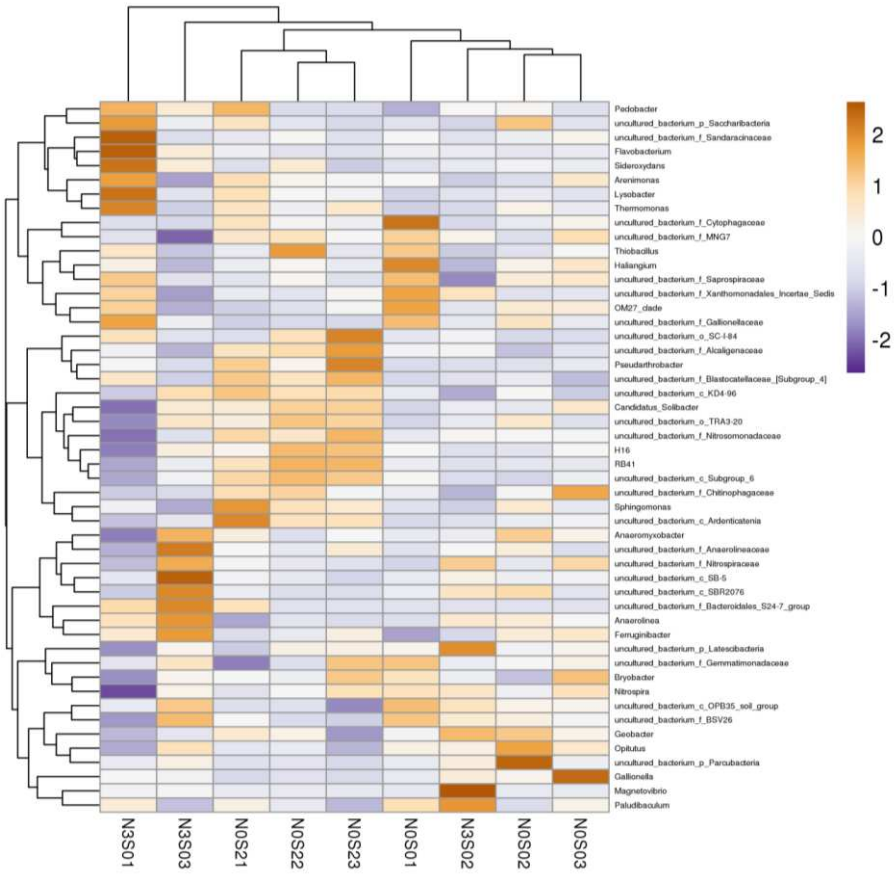

Figure 6. *Hierarchical clustering diagram of bacteria at the genus level after different soil treatments. Note: N0S0 represents CK; N3S0 represents F; N0S2 represents W. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned*

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Redundancy analysis of soil physicochemical properties and soil bacterial community

RDA was used to evaluate the relationship of soil physicochemical properties and soil bacterial community (*Figure 7*). The first tow axes of the principal components analysis accounted for 34.03% of the total variance. The biplot showed a clear spatial separation among different soil samples. In fact, the axis 1 discriminated for the W treatment, while the axis 2 discriminated F treatment. The AP was situated in the first quadrant, soil pH in the second quadrant; soil TN in the third quadrant and OC in the fourth quadrant. The soil pH and AP was significant correlation with F treatment and the OC and TN was significant correlation with W treatment.

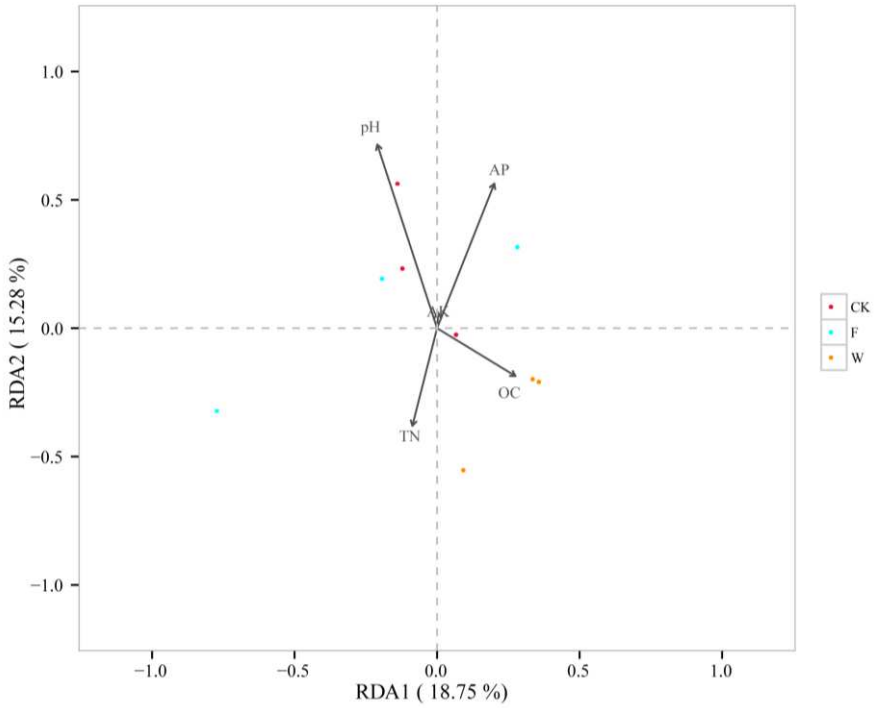

Figure 7. *Redundancy analysis of soil physicochemical properties and soil bacterial community. Note: N0S0 represents CK; N3S0 represents F; N0S2 represents W. CK: no fertilizer or straw returned to the field; F: fertilizer with no straw returned; W: no fertilizer with straw returned*

Discussion

This study used Illumina platform Miseq high-throughput sequencing technology to conduct amplification sequencing of 16S rDNA V3-V4 of soil bacteria, and the good coverage index of each sample shows that under the condition of a similarity of 0.97, OTUs covered more than 99% of the bacteria in the soil. Simultaneously, sequencing also identified many unclassified bacteria, indicating that high-throughput sequencing can obtain comprehensive biological information and also identify some unrecognized and classified bacteria. However, the relative abundance of the unclassified bacteria in the soil among the 3 treatments did not differ significantly at each taxonomic level.

Soil microbial diversity is relatively stable, and short-term fertilization has no significant effect on soil microorganisms (Shi et al., 2010), but long-term reasonable fertilization can have a certain effect on soil microbial community structure and diversity (Rousk et al., 2010; Liu et al., 2014) showed that the physical and chemical

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properties of soil, particularly the soil pH and organic matter, had significant effects on the soil bacterial community composition and diversity.

In this paper, straw returned to the field significantly improved soil bacterial diversity. The straw returned to the field not only increased the soil organic matter content in the process of decomposition but may also provide rich nutrition for microbes in the straw products produced during the process of decay, such as sugars, eggs, endoplasmic reticulum, amino acids, vitamins, organic acids, and phenols, among others (Sapp et al., 2015). Simultaneously, straw returned to the field can improve soil structure and increase soil permeability, thereby increasing microbial species and soil bacterial diversity. Fertilizer application significantly reduced the richness of soil bacteria and led to a significant decrease in soil pH and soil consolidation, which was not conducive to the growth of aerobic bacteria.

The relative abundance of dominant bacterial groups in soil has a certain correlation with the physical and chemical properties of soil (Wang et al., 2015). In this study, the relative abundance of acid-bacilli was negatively correlated with total nitrogen and available potassium in soil. The relative abundance of Bacteroides was positively correlated with total nitrogen, available potassium and available phosphorus in soil. The relative abundance of SPP was positively correlated with soil pH and negatively correlated with organic matter, total nitrogen and phosphorus. The relative abundances of ore-deformis and campus-deformis were positively correlated with pH and negatively correlated with the contents of organic matter, total nitrogen and available phosphorus. The relative abundance of probiotics was negatively correlated with pH and positively correlated with the contents of organic matter, total nitrogen and available phosphorus. The relative abundance of Nitrospira was significantly positively correlated with pH and negatively correlated with organic matter, total nitrogen and available phosphorus (Lauber et al., 2008).

In the black soil of northeast China, bacterial groups were abundant, and the dominant bacteria were Proteobacteria, Actinomycetes, and Bacteroides, and the relative abundance of Proteobacteria was the largest (36.5-45.2%). Bacterial classes at an advantage were beta bacteria, phylum Δ-deformation, alpha deformation phylum, and gamma, with beta deformation bacteria being the largest phylum based on relative abundance. These results are consistent with those of (Liu et al., 2014) regarding bacteria in black soil in northeast China and with those of Sapp et al. (2015). The application of chemical fertilizer and the return of straw to the field changed the physical and chemical properties of the soil. The relative abundance of Proteobacteria in black soil in northeast China is less than that in the three treated soil groups. As the largest group of bacteria, many groups can perform nitrogen fixation and adapt to various complex environments (Liu et al., 2014). Therefore, changes in environmental conditions have little effect on their distribution and relative abundance. Acidophilus bacteria are widely distributed in soil and sediment and are acidophilic. However, in this paper, the lowest values of soil pH and relative abundance of Acidobacteria occurred after fertilization, confirming results of (Liu et al., 2014) that the growth of Acidobacteria in soil was not only affected by soil pH but also by other factors such as soil organic matter and crop growth status. Actinomycetes can promote the decomposition of animal and plant residues in soil, play an important role in the decomposition process of crop stalks after they are returned to the field, and play a certain role in the natural nitrogen cycle (Lauber et al., 2008). Fertilization treatment and straw returned to the field significantly increased the relative abundance of

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Actinomycetes in the soil, which might be related to the increase in total nitrogen content after treatments with fertilization and straw returned to the field. Fertilization and straw return to the field also had certain effects on some functional bacteria related to elemental transformation. For example, Sphingomonas is a new type of biological resource that is primarily involved in the decomposition of aromatic compounds. Compared with the CK treatment, straw return to the field significantly reduced the relative abundance of Sphingomonas. Fertilization and straw return to the field also had a certain effect on the relative abundance of bacteria associated with biological nitrogen fixation. Fertilization and straw return to the field significantly reduced the relative abundance of Nitrospira, which led to a large increase in the relative abundance of Rhizobium. Fertilization significantly reduced the relative abundance of Gemmatimonas associated with phosphorus metabolism.

The effect of fertilization on the composition, relative abundance and community structure of dominant groups of soil bacteria was greater than that of straw returned to the field. The mechanisms by which fertilization and straw return to the field affect the community structure of black soil bacteria in northeast China require further research.

Conclusions

The long-term effects of fertilizer application on the composition, relative abundance and community structure of dominant groups of soil bacteria were greater than those of straw returned to the field. Additionally, fertilizer application significantly reduced soil bacterial richness, whereas straw returned to the field significantly increased soil bacterial diversity.

Our results found that long-term fertilizer application was the main factor that resulted the soil bacterial community changed in black soil. This provided a guidance to explore environmentally friendly agricultural fertilization measures in the future: while increasing soil fertility and increasing crop yields, as the same time measures should also be taken to maintain a suitable pH of the soil, so as to achieve equal emphasis on agricultural production and ecological benefits. Of course, this study only analyzed the changes of the soil bacterial community. For special functional groups (such as nitrogen-fixing bacteria, nitrifying bacteria, etc.) and other microbial species (such as archaea, fungi, and miniature animals) that play an important role in the soil ecosystem did not carried out. The systematic study of various types of microorganisms in the soil ecosystem will be an important aspect of our future research. This will be of great help for us to understand the function of the soil ecosystem and the relationship between the soil microbial community and soil fertility and crop yield. Furthermore, it provides a solid foundation for us to explore environmentally friendly and sustainable agriculture.

Acknowledgement. National key R&D plan (2016YFD0300204); Heilongjiang Youth Fund Project (QC2018026) as well as Heilongjiang Academy of Agricultural Sciences-Agricultural Science and Technology Innovation Project (HNK2019CX1302).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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HABITAT SELECTION OF INSECTIVOROUS BIRDS IN WESTERN BLACK SEA REGION OF TURKEY

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(Received 21st Apr 2020; accepted 29th Jul 2020)

Abstract. Understanding the habitat preferences of species is crucial to their conservation. Bird species in particular are thought to be strongly affected by habitat structure (canopy cover, tree height, etc.) and land use. Due to their importance in pest control in both natural and agricultural environments, the habitat preference of insectivorous birds in particular was assessed by this study. To this end, the abundance and the number of insectivorous birds across different habitat types and land uses were investigated in Western Black Sea Region of Turkey. A total of 78 insectivorous bird species belonging to 6 orders and 25 families were identified and 10635 individuals were counted in the study. Canopy cover, vegetation layer, elevation and habitat heterogeneity positively affected insectivorous bird diversity. Forest management plans should include precautions to ensure the maintenance of tree canopy and habitat heterogeneity. Natural vegetation should be protected and improved when lacking in farmlands and urban areas in order to ensure the conservation of bird species.
Keywords: *bird habitat, vegetation layer, canopy cover, elevation, insectivorous bird*

Introduction

There is much research related to habitat preferences of birds, providing insight into the impacts of natural and human factors that affect birds (Heikkinen et al., 2004). Habitat use is associated with mechanisms responsible for habitat selection (Morris, 1987). Rosenzweig (1981) revealed three important concepts concerning the habitat selection of bird species; generalist, specialist and opportunist. Habitat selection is a hierarchical process of behavioral responses that results in a use of resources out of proportion to their availability, influencing the fitness and survival of individuals (Jones, 2001; Broughton et al., 2012). Individuals can forage in suitable habitats where probability of survival is higher. Species that exploit all habitat types are thought of generalists. Since the density of resources among habitat types is different and provides a diversity with food resources (MacArthur and Levins, 1967; Morris, 1987).

Vegetation gives terrestrial habitats their characteristic physical structure. That spatial structuring or physiognomy has repeatedly been shown to be important in determining the distribution and abundance of birds in habitat selection and, presumably, as an ultimate factor by its association with critical resources such as food, nesting sites, or cover from predators (Hilden, 1965; Rotenbery and Wiens, 1980). Forest structure (canopy, tree height, vertical and horizontal stratification and diversification) in particular has a strong relationship with bird diversity. And high insect abundance and diversity being responsible for greater bird diversity and numbers observed in tall and dense stands (Beskardes et al., 2018a).

Bird species are evaluated as ecological indicators of habitat or ecosystems (Canterbury et al., 2000). They are among the animals that consume insects most (Perrins, 1987; Bezzel, 1996) and particularly play significant role in ecosystems (Turan, 1990; Ozkazanc, 2016). Because of prey size, energy and other nutrients

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(Kaspari and Joern, 1993), granivorous birds feed on insects during their breeding season, while insectivorous birds feed on insects as long as they can find them (Arslangundogdu, 2010). Insects and other invertebrates provide proteins and nitrogen in bird diets, which are particularly important for growing birds (Stratford and Şekercioğlu, 2015). It has been proven that birds can significantly reduce the abundance of herbivorous insects in tropical, temperate, and boreal forests. Birds in forests account for 75% of the annual prey consumption of the world's insectivorous birds (≈ 300 million tons year^{-1}) (Nyffeler et al., 2018).

The foraging strategies can be different among bird species (Yard et al., 2004). Insectivorous birds can be classified as aerial (*predominantly feeding on insects in the air*) and terrestrial/arboreal that classifying is based on foraging strategy (Kissling et al., 2012). The number of terrestrial insectivorous bird is 4903 (55% of all birds) species and aerial insectivores 225 species on earth. (Kissling et al., 2012). Insectivorous birds have diverse strategies for finding insects. Terrestrial insectivores, for instance, forage in the leaf litter but there are various methods of foraging in litter including leaf tossing, searching the surface or searching under living leaves while walking across the surface of leaf litter (Stratford and Stouffer, 2013).

Based on the importance of insectivorous birds in ecosystems, in this study, it was aimed to determine the number of insectivorous bird species, abundance and their habitat preference, and to make recommendations for habitat management of important insectivorous bird species to landowners and wildlife managers in the region.

Material and Methods

Study area

This study was conducted in Duzce province which is located at the western border of the Western Black Sea Region of Turkey (*Figure 1*). Surface area of Duzce is 264296 ha. Forest covers around 138460 ha, farmlands, meadows and wetlands 108425 ha, settlements 17411 ha. Deciduous (beech, hornbeam, oak, chestnut, linden) forests, coniferous (fir, yellow pine, black pine) forests, and a mixture of these are all under the influence of the Black Sea climate. Forest areas are found in regions where groups of high slopes rise to 1830 m (Karduz plato) (Keten, 2017). Kucuk Melen, Ugursuyu, Asarsuyu, Aksu, Buyuk Melen rivers and Efteni Lake are the important wetlands surrounding with agricultural and rural areas. The average temperature is 13.3 °C and the average rainfall is 816 mm (1950-2014). The climate (according to Thornthwaite) is classified as moist, with mesothermal water moderately deficient in the summer and a summer evaporation rate of 51% (MGM, 2018).

Samplings

Sampling was conducted from middle of the March to August of 2014 in 351 sampling points (*Figure 1*). Bird observations were done during the morning from sunrise to 11:00 hours and from 15:00 hours to sunset. Only breeding insectivorous birds were considered for this study. Counts were made by directly observing and hearing sounds of birds in transect lines with two belts in the sampling plots (Bibby et al., 2000) which were recorded on bird cards. The length of the transect lines is 100 m in forests, the width of the transect is 100 m in forest habitats (25 m for each belts),

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200 m for settlement/farmland habitats (50 m for each belts) (*Figure 2*). A total of 819 ha area (0.31%) was surveyed in study area.

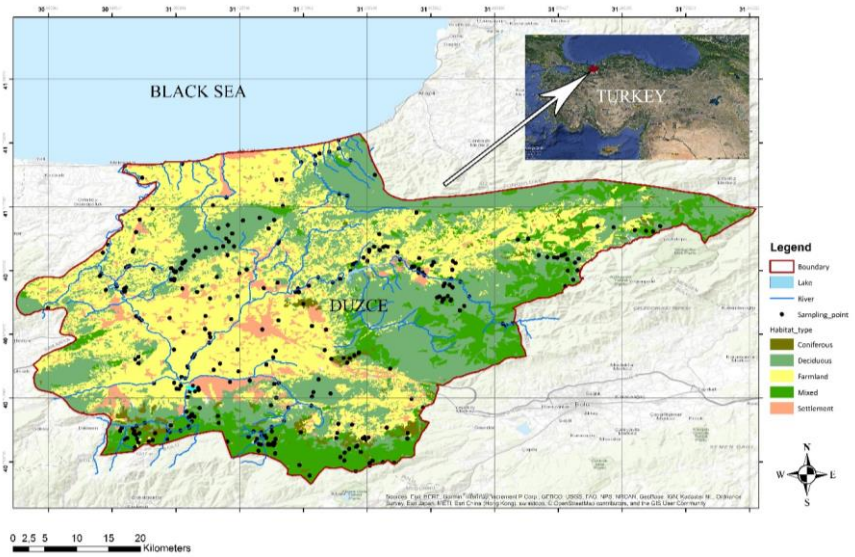

Figure 1. *Duzce province and Sampling points*

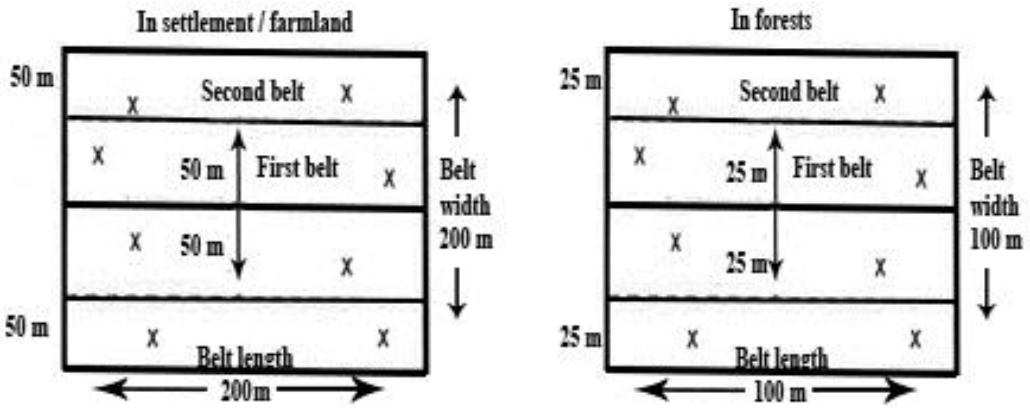

Figure 2. *In Settlement and Farmland (belt width 200 m) / In Forest (belt width 100 m)*

GPS was used to determine sampling plot coordinates and elevation. In addition, habitat types of localities were recorded. The species identification was based on several field guides (Heinzel et al., 1995; Mullarney et al., 1999; Hume, 2002) and the insectivorous bird species were categorized according to Perrins (1987). The map of Duzce province and sampling points was prepared by ARCGIS 10.2.

Data analysis

The sampling plots were classified as coniferous forests, deciduous forest, mix forest, farmland and settlement. Insectivorous bird species richness was calculated as the total number of species recorded at the plots, and species diversity using the Shannon-Wiener index (Magurran, 1988). We used descriptive statistics and one-way analysis of variance (ANOVA) to compare number of bird species with habitat types.

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Constrained Correspondence Analysis (CCA) was conducted to assess the habitat types based on all parameters measured in the study (Legendre and Legendre, 2003). CCA was conducted in habitat variables; latitude, longitude, distance to settlement (m), vegetation layer, elevation (a.s.l., m), canopy cover (%) and aspect.

In evaluating the relationship between bird species and habitat factors using CCA, only sites that were sampled at least 10 times on different days were used in order to avoid observation bias. The similarity in habitats among the five habitat types were estimated with Ward's methods that uses minimum variance values and classifies by hierarchical clustering (Murphy, 2004). Venn diagram were used to analyze in habitat selection of birds. Significance was set at the $\alpha = 0.05$ level. All analysis was conducted using Program R Gui (R Development Core Team, 2018).

Results

A total of 78 insectivorous bird species belonging to 6 orders and 25 families were identified and 10635 individuals were counted. Although, farmlands had more bird species abundance (61 species) and 6256 individuals. The conifer habitats had lower bird species abundance (15 species) and 119 individuals. However, among habitat types, the mix forest habitats had the most bird diversity (2.9), the settlements had the lowest (0.75) (*Table 1*). The number of insectivorous birds per ha was 17.02 in settlements, 13.03 in farmlands, 11.01 in mix forests, 9.90 in deciduous forest and 5.41 in conifer forests (*Table 2*). All bird species in the study and the habitats in which they were recorded are listed in the supplementary materials.

Table 1. *Biodiversity indexes for each habitat observed in the Duzce province study area*

	Conifer F.	Deciduous F.	Mix F.	Farmland	Settlement	Total
Total species	15	39	47	61	27	81
Total individuals	119	851	958	6256	2451	10635
S_W Index	2.16	2.79	2.9	1.35	0.75	1.83
Evenness	0.8	0.76	0.75	0.33	0.23	0.42

Table 2. *Estimated insectivorous bird number and density at each habitat observed in the Duzce province study area*

Habitat type	Habitat (Area - ha)	Sampling number	Sampling area (ha)	Total individual	Bird number per ha
Conifer	4341.5	22	22	119	5.41
Mix	38155.7	87	87	958	11.01
Deciduous	95963.2	86	86	851	9.90
Farmland	108425.3	120	480	6256	13.03
Settlement	17410.5	36	144	2451	17.02
Total	264296.2	351	819	10635	12.99

The results of variance analysis comparing 5 variables revealed that factors such as vegetation layer, canopy closure, elevation and distance to settlement were effective in the habitat selection of birds (<0.001) (*Table 3*). On the contrary, aspect was found to be insignificant (P=0.171).

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Table 3. *Descriptive statistics (Mean and standard error) and ANOVA*

	Conifer F	Deciduous F	Mix F	Farmland	Settlement	F	P
Number of species	2.50 ± 0.40	3.53 ± 0.20	3.68 ± 0.20	3.18 ± 0.46	3.90 ± 0.51	0.909	0.459
Number of individual	7.44 ± 1.94	7.81 ± 0.86	8.40 ± 0.69	63.84 ± 52.68	116.71±97.15	1.119	0.347
Vegetation layer	1.88 ± 0.05	1.38 ± 0.05	1.39 ± 0.05	1.92 ± 0.06	1.67 ± 0.11	17.54	<0.001
Canopy closure	2.44 ± 0.18	2.67 ± 0.05	2.40 ± 0.06	1.00	1.00	191.00	<0.001
Aspect	2.19 ± 0.23	2.10 ± 0.09	1.82 ± 0.08	2.03 ± 0.09	2.00 ± 0.20	1.61	0.171
Elevation	840 ± 127	642 ± 29	1088 ± 36	283 ± 27	257 ± 54	90.61	<0.001
Distance Settlement	1558 ± 311	2636 ± 202	3039 ± 331	721 ± 79	0	18.51	<0.001

The prepared Venn diagram shows that 11 of the bird species (*Delichon urbicum, Erihtacus rubecula, Motacilla alba, Periparus ater, Cyanistes caeruleus, Parus major, Poecile palustris, Phoenicurus ochruros, Phylloscopus collybita, Turdus merula, Turdus philomelos*) were common species, and observed in all habitat types. The highest bird species diversity was identified with 61 taxa in agricultural areas followed by 47 taxa in mixed forests. The least number of species was determined in coniferous forests with 15 taxa. Additionally, there was no insectivorous bird species that preferred only coniferous forests (*Figure 3*).

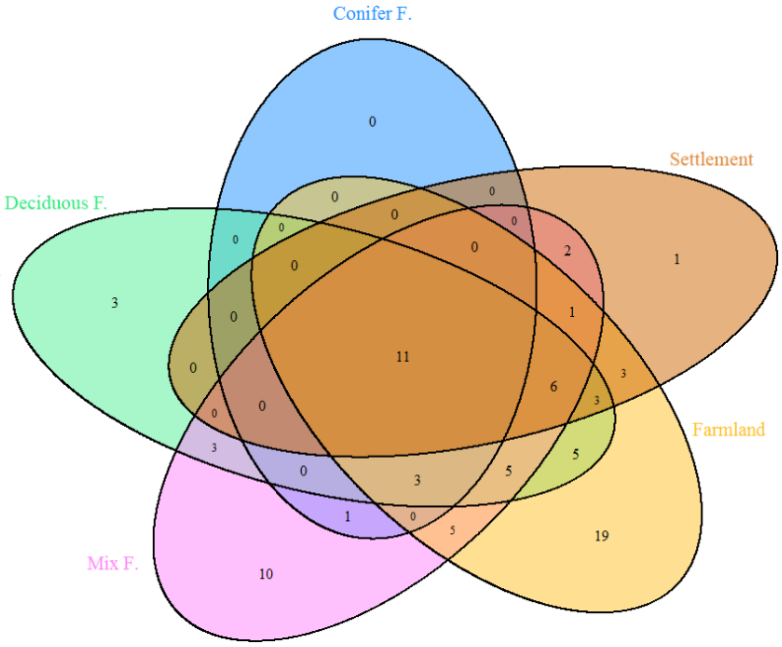

Figure 3. *Venn diagram of bird species' number with habitats*

Farmlands and settlements showed similarity in terms of bird species diversity and abundance (*Figure 4*). Overall, forested plots had similar bird community composition (*Figure 4*). However, within forest areas, deciduous and mixed forests were clustered together and slightly apart from coniferous plots in terms of bird composition (*Figure 4*).

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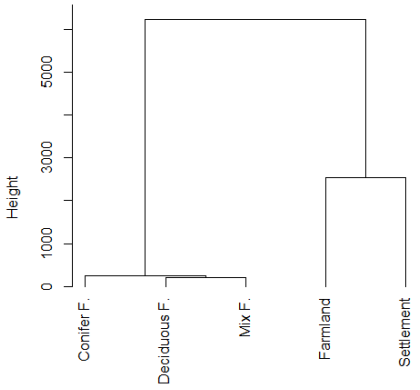

Figure 4. *Cluster analysis (Ward's Method)*

In the study, there were 23 bird species that were observed more than 10 times. The most important factors in driving bird species composition were elevation and cover followed by vegetation layer (*Figure 5*). However, several bird species including *Turdus merula, Muscicapa striata, Erithacus rubecula, Picus viridis, Parus major, Cyanistes caeruleus, Motacilla cinerea, and Dendrocopos major* being centered in the CCA graphic were not strongly affected by those factors. Farmlands and settlements included many of the same bird species such as *Sturnus vulgaris* and *Delichon urbicum. Hirundo rustica* and *Lanius collurio* were affected by vegetation layers in open areas (*Figure 5*), and farmlands and settlements had the most individual numbers. Cover and distance to settlement in deciduous forests positively affected bird species richness. *Periparus ater* and *Poecile palustris* were the species most affected by altitude and preferred mixed forests.

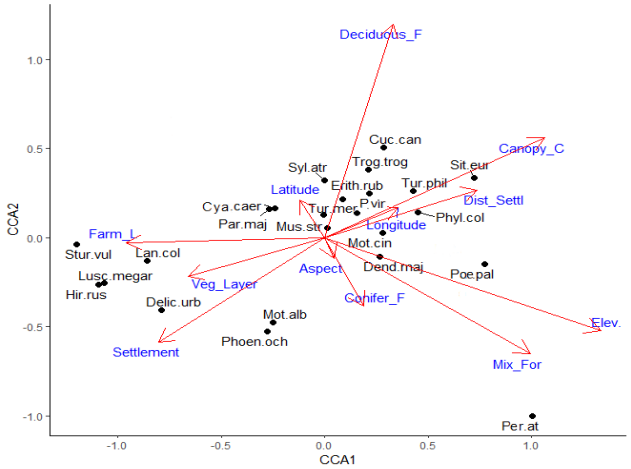

Figure 5. *Constrained Correspondence Analysis for insectivorous birds (Sturnus vulgaris – Stur.vul., Lanius collurio –Lan.col., Luscinia megarhynchos – Lusc.megar., Hirundo rustica Hir.rus., Delichon urbicum – Delic.urb., Phoenicurus ochruros – Phoen.och., Motacilla alba – Mot.alb., Cyanistes caeruleus – Cya.caer., Parus major –Par mar., Muscicapa striata – Mus.str., Turdus merula – Tur.mer., Sylvia atricapilla – Syl.atr., Erithacus rubecula – Erith.rub., Picus viridis – P.vir., Motacilla cinerea – Mot.cin., Dendrocopos major – Dend.maj., Poecile palustris – Poe.pal., Periparus ater – Per.atr., Phylloscopus collybita – Phyl.col., Turdus philomelos – Tur.phil., Troglodytes troglodytes – Trog.trog., Cuculus canorus – Cuc.can., Sitta europaea – Sit.eur.)*

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Discussion

Deciduous and mix forests in Duzce province were composed of similar bird species, and differentiated from coniferous forests. Agricultural areas and settlements also has many of the same species, which were not common in forested areas. Bird abundance per hectare was lowest in conifer forests, the highest in settlements, and moderate for deciduous, mix forests and agricultural areas. Oak forests in our region had higher bird density than that observed in Sitka spruce plantations in Ireland (Sweney et al., 2010), and had higher bird species richness than other pine stands in Turkey (Bergner et al., 2015). Species richness were found to be higher in edge habitats and open areas on forest exteriors rather than forest interior in Belgium (Paquet et al., 2006). Similar to my findings, bird species richness was significantly higher in mixed oak-pine forests than in pinewoods in Spain (Diaz, 2006). Duzce province is a developing region with much of the landscape being composed of rural areas. A major lake in the region, Efteni lake, is located within the settled areas of the city of Duzce, and most rivers in the region pass through settlement and agricultural areas. In Romania, rivers were shown to have positive effects on the bird community composition by providing of many resources in Romania (Domokos and Domokos, 2016). Thus the concentration of natural water features may explain the greater insectivorous bird density in settlements and agricultural areas in Duzce province.

In this study, 10635 individuals were counted, and 78 insectivorous bird species recorded that constitutes 42.6% of 183 bird species known for the region (Nuhungemisi, 2019). Breeding bird species density ranged between 8.9 and 21.4 birds in deciduous forests in USA (Anderson, 1970; Holmes et al., 1986). In this study, the density of breeding birds was found also within that range (*Table 2*). Birds hold down arthropods or insects' population by eating adults, larvae, pupae and eggs. While looking for these nutrients, birds find insects under tree barks, among wood litter, in the soil and from mud (Turan, 1990). Insectivorous birds consume 404.6 million tons of insects and $2.7x10^{18}$ joule $year^{-1}$ in the world (Nyffeler et al., 2018). Thus, insectivorous birds are highly important biological agents that protect agricultural areas and the other ecosystems from pest outbreaks.

Although 61 insectivorous bird taxa were detected in agricultural areas, the lowest biodiversity was found in these ecosystems. The birds forming large groups especially *Sturnus vulgaris* and *Hirundo rustica*, caused a lower Shannon and Evenness value in agricultural and settlements. Nutrition plays important role in the habitat preference of *Sturnus vulgaris* (Tinbergen, 1980). Albeit, they are feeding on different habitat types, rural and agricultural areas provide perfect feeding habitats. For this reason, those areas are included more individuals of *S. vulgaris*.

In ecosystems, the coexistence of generalists' bird species depended strongly on the species' dispersal abilities (Büchi and Vuilleumier, 2014). In this study, 11 insectivorous birds were observed across all habitat types (*Delichon urbicum, Erithacus rubecula, Motacilla alba, Periparus ater, Cyanistes caeruleus, Parus major, Poecile palustris, Phoenicurus ochruros, Phylloscopus collybita, Turdus merula, Turdus philomelos*). These birds can be described as common species. Since they can be found on any kind of habitat and are less affected by habitat fragmentation and disturbance than other species (Devictor et al., 2008). Although *Phylloscopus collybita* and *Turdus philomelos* could be found in all habitats, they were mostly observed in deciduous forests with high canopy cover, and preferred to stay away from settlements. The four tit species *Parus major, Periparus ater, Cyanistes caeruleus, Poecile palustris* also

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75517562

observed in all habitat types. *P. ater* were closely associated with coniferous forests and *P. palustris* with deciduous forests (Beskardes et al., 2018b). *P. ater* and *P. palustris* were affected by elevation. In addition, both species preferred mixed forest than conifer and deciduous forests. Mix forests has provided more food and cover than pure coniferous forests for insectivorous birds, especially in breeding season.

A total of 23 bird species was detected more than 10 times. The forested areas further away from settlements have greater insectivorous bird number and species. Especially, *Sitta europaea* were affected by canopy cover mostly. Farmland and rural area species such as *Sturnus vulgaris*, *Delichon urbicum*, *Hirundo rustica*, *Lanius collurio* has affected positively by habitat patches. Plant height diversity (Lancaster and Rees, 2011) and horizontal layer (stratification) in habitat affected birds' population positively (Arslangundogdu and Yılmaz, 2011). The fact that species richness was most strongly related to understory cover emphasizes the importance of woodland vegetation structure to birds in my study region (Sweney et al., 2010). Canopy cover, vegetation layer, elevation and heterogeneity of habitat in mix forests also positively affected insectivorous bird diversity. Complex vegetation structure and floristic composition heterogeneity increase niche diversity, which is thought to also increase avian diversity (Diaz, 2006).

In Duzce province, intensive agricultural activities are carried out and also 120 tones pesticides (insecticide, herbicide, fungicide etc.) are used every year (PDEU, 2018). It is known that farmland bird population has declined in Europe in the past three decades with agricultural intensification and the use of pesticides are known to be a major threat to farmland birds (Hallmann et al., 2014). The higher bird density in agricultural areas and settlement observed in this study should not be interpreted as a basis for a lack of threat to bird species in these areas as agricultural practices may have effects on birds on a regional scale while providing good habitat for certain species. Long term bird censuses are needed in agricultural and surrounding areas in order to understand how the application of pesticides or other agricultural practices are effecting bird diversity in Duzce Province.

Conclusion

One of the most important areas for bird species in Duzce is the Efteni lake and the Melen river. Especially in agricultural areas, these water resources are the most important factors that increase the biodiversity and bird species, and their preservation is of utmost importance to wildlife. Birds need cover to breed and to raise their fledglings successively during breeding. For this reason, maintenance of forest and shrub cover are also critical for bird conservation. Increasing vegetation cover and layers in marginal areas increases stratification and edges which effects birds positively. Thus, wildlife managers and landowners should be supported to protect vegetation layers (bushes, wooded etc.) in farmlands for birds, and landowners should be warned not to use pesticides during bird breeding seasons. In addition, the future studies are necessary to investigate the effects of pesticide using on birds in agricultural areas and how agricultural practices affect the diversity of bird species.

Acknowledgements. This study was supported by the Düzce University Scientific Research Projects (DU-BAP). I would like to thank Akif KETEN, Zeynel ARSLANGUNDOGDU and Ergun BACAK for their valuable help. I also thank Kemal Burak PEKIN for helping to evaluate and improve the article. And

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75517562

special thanks to my wife Ozlem BESKARDES who provided me with a peaceful and perfect working environment at home and helped me with everything.

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SUPPLEMENTARY MATERIAL

Species list and individual numbers of birds

Habitat	Conifer	Deciduous	Mix	Farmland	Settlement	Total
Total Species	15	39	47	61	27	81
Total individual	109	851	958	6256	2451	10635
Tachymarptis melba	0	35		300	5	340
Apus apus		5	2	2		9
Cuculus canorus	1	9	11	2		23
Upupa epops			1	4		5
Merops apiaster				180		180
Dendrocopos major	2	7	8	5		22
Dendrocopos leucotos		1				1
Dendrocopos medius		2		2		4
Dendrocopos minor		1		2		3
Dendrocopos sp.		2				2
Dendrocopos syriacus		1		6		7
Dryocopus martius			2		1	3
Jynx torquilla					3	3
Picus canus		3	5	1		9
Picus viridis		3	4	5		12
Picus sp.			1	1		2
Lanius collurio		3	2	44	3	52
Lanius excubitor			1	1		2
Lanius minor			1			1
Oriolus oriolus				3	1	4
Periparus ater	18	2	155	3	2	180
Cyanistes caeruleus	1	14	8	14	2	39
Parus major	3	117	68	140	39	367
Poecile palustris	7	51	131	2	2	193
Parus lugubris			1			1
Alauda arvensis				24		24
Galerida cristata				5		5

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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Habitat	Conifer	Deciduous	Mix	Farmland	Settlement	Total
Lullula arborea			2			2
Delichon urbicum	40	29	30	224	150	473
Hirundo rustica		22	30	279	150	481
Hirundo daurica		2		9		11
Hirundo rupestris			11			11
Riparia riparia			50	33		83
Cettia cetti				5		5
Aegithalos caudatus				10		10
Phylloscopus collybita	14	122	115	30	6	287
Phylloscopus bonelli				2		2
Phylloscopus throchiloides			2			2
Phylloscopus trochilus				4	1	5
Iduna pallida				4		4
Acrocephalus arundinaceus				8		8
Acrocephalus melanopogon				5		5
Acrocephalus schoenobaenus				6		6
Acrocephalus scirpaceus				5		5
Locustella luscinioides				4		4
Sylvia atricapilla		18	6	13	1	38
Sylvia borin		4		2		6
Sylvia communis		1		7	1	9
Sylvia melanocephala		2				2
Sylvia nisoria				1		1
Sylvia sp.			4		1	5
Regulus ignicapillus			5			5
Regulus regulus			11			11
Troglodytes troglodytes	3	47	30	23		103
Sitta europaea		14	9			23
Sitta krueperi	8		13			21
Certhia brachydactyla		4	2			6
Certhia familiaris			1			1
Sturnus vulgaris		35		4551	2040	6626
Turdus merula	9	126	83	119	7	344
Turdus philomelos	3	56	26	8	1	94
Turdus viscivorus			8	1	3	12
Erithacus rubecula	6	77	50	44	3	180
Luscinia megarhynchos		6	1	23	14	44
Muscicapa striata		9	8	6	2	25
Oenanthe oenanthe			11			11
Oenanthe hispanica				2		2
Oenanthe isabellina				2		2
Phoenicurus ochruros	3	3	3	7	4	20
Phoenicurus phoenicurus				1	1	2
Saxicola torquata		1	3	5	1	10
Saxicola rubetra				9		9
Ficedula parva		1	1			2
Prunella modularis		3	1	4		8
Motacilla alba	1	4	22	32	7	66
Motacilla cinerea		9	17	9		35
Motacilla flava			1	3		4
Anthus campestris				3		3
Anthus pratensis				1		1
Anthus spinoletta			1			1
Anthus trivialis				6		6

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75517562

NATURAL RADIOACTIVITY AND RADIOLOGICAL RISK PARAMETERS IN LOCAL AND IMPORTED BUILDING MATERIALS USED IN SUDAN

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(Received 22nd Apr 2020; Accepted 16th Sep 2020)

Abstract. Natural radioactivity levels in selected types of building materials used in Sudan were measured using gamma spectrometry. Radioactivity concentrations were measured in 52 samples including cements, porcelain ware, and ceramic tiles, cement blocks, and red clay bricks. Representative samples were powdered and stored in polyethylene Marinelli containers for four weeks to attain equilibrium between ^{226}Ra and ^{232}Th and their daughters before measurements. The average radioactivity (Bq.kg^{-1}) of ^{226}Ra, ^{232}Th, and ^{40}K, ranged between 12–40, 10–70, and 28–94, respectively in cement; 10–35, 12–28, and 87-143, respectively in cement blocks; 32–132, 26–87, and 285–1070, respectively in red bricks; and 8-527, 18–118, and 129–812, respectively in ceramics and porcelain tiles. The air absorbed dose rates ranged between 12.0±3.0 to 40.5±23.0 nGyh^{-1} in materials used in a superficial amount; 34.4±8.9 to 173.3±52.0 nGyh^{-1} in materials used in bulk. The annual effective doses were varied from 0.06 to 0.85 mSv. Excluding porcelain ware samples. The activity concentration indexes describing external and internal radiation hazards were within the acceptable limits. The study provides important baseline data for setting national regulatory control limits for protection against radiation.
Keywords: *gamma spectrometry, cements and bricks, gamma index, indoor radiation hazards*

Introduction

Building materials contain naturally occurring radioactive materials (NORM) due to the natural radionuclide content in their raw materials or due to the additives used in the manufacturing process, such as zircon sand which contains traces of uranium and thorium and constitutes the radioactive content in ceramic and tiles (IAEA, 2003). The radiological risk from external radiation exposure due to natural radioactivity in building materials is caused by terrestrial gamma radiation from ^{226}Ra, ^{232}Th, and ^{40}K NORM as well as the internal exposure from radon (EC, 1999; UNSCEAR, 2000; Righi and Bruzzi, 2006). Radon in building materials is the major cause of the incidence of fatal cancer in the global population (ICRP, 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

Bearing in mind that certain levels of radiation exposures due to radioactive contents in building materials is inevitable, international radiation protection advisory bodies assert radiation exposure be kept as low as reasonably possible. As people spend nearly 80% of their time indoors, the main motive of radiological studies of building materials has been to limit public radiation exposure due to the natural radioactivity of the constituents of these materials.

Sudan is one of the largest countries in Africa where houses are built using stones and other local materials that are characterized by the local geological sands. This diversity of materials makes it difficult to study samples of materials used all over the country. We found only two studies on this subject. One is by Sam and Abbas (2001), who reported radioactivity in local and imported cement types used in Sudan. The other is by Salih et al. (2014), who studied the radiation exposure of workers in storage areas for building materials. Due to the dynamic nature of the construction industry, the issue of radioactivity in building materials remains an open question.

In this study, we aim to provide a comprehensive survey of radioactivity present in local and imported building materials in Sudan and its impact on the exposure of the public to radiation. Further, we aim to provide up-to-date information on this subject to assist institutions in formulating regulations on the national level to control radiation.

Materials and Methods

Sampling

In total, 52 samples of local and imported building materials of widely distributed origins were collected from stores in the Sudanese capital, Khartoum. A radioactivity measurement in building materials is important for radiation protection purposes. Representative samples of building materials were powdered and stored in polyethylene Marinelli containers for four weeks to allow equilibrium between ^{226}Ra and ^{232}Th and their daughters to be reached.

Measurements of radioactivity

Sample specific activities were determined using the p-type high purity germanium (HPGe) gamma spectrometry with 30% relative efficiency to (NaI)Tl gamma spectroscopy for 1.33 MeV ^{60}Co radiation of 122 and 1332 keV gamma line of 0.875 and 1.850 keV, respectively (Baltic Instrument, Riga, Latvia). Energy and efficiency calibrations were performed using a mixed radionuclide gamma calibration standard (Amersham Buchler B1575). For the radioactivity quantifications, ^{226}Ra activities were determined from ^{214}Pb (351.92 keV) and ^{214}Bi (609.31 keV). The gamma energy lines of ^{212}Bi, ^{212}Pb, and ^{228}Ac were used to determine the ^{232}Th activity. The radioactivity of ^{40}K was determined from the 1460.81 keV gamma line (IAEA, 2003). The activity concentration (A) in Bqkg^{-1} in samples was calculated using the following expression:

$$A = \frac{N}{PE \times \mathcal{E} \times Tc \times M} \quad \text{(Eq.1)}$$

where, M denotes the mass of sample in kg, N the sample net area in the peak, PE the gamma emission probability, Tc the counting time, and $\mathcal{E}$ denotes the photopeak efficiency. Uncertainty in the activity concentration was determined at a 95% confidence level (σ =2) using *eq. (2)*:

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DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

$$\frac{u(C_{sp})}{C_{sp}} = \sqrt{\left(\frac{u(N)}{N}\right)^2 + \left(\frac{u(P_E)}{P_E}\right)^2 + \left(\frac{u(\mathcal{E})}{\mathcal{E}}\right)^2 + \left(\frac{u(T_C)}{T_C}\right)^2 + \left(\frac{\Delta M}{M}\right)^2} \quad \text{(Eq.2)}$$

where, $\frac{u(N)}{N}$, $\frac{u(P_E)}{P_E}$, $\frac{u(\mathcal{E})}{\mathcal{E}}$, $\frac{u(T_C)}{T_C}$, and $\frac{\Delta M}{M}$ are the relative uncertainties in the counting rate, gamma emission probability, photopeak efficiency, counting time, and sample mass, respectively. Uncertainty in the activity concentration was determined by the SpectraLineGP software at 1 σ (Baltic Instrument, Riga, Latvia). The overall uncertainty in the determined activity was the square root of the quadratic sum of different activity parameters at a 95% confidence level (σ =2) according to the ISO standard (ISO, 1995).

Radium equivalent activity (Raeq)

In comparing radioactivity amounts in different samples, the radium equivalent activity (Ra_{eq}) is used (Beretka and Mathew, 1985). Ra_{eq} represents the combined specific activities of ^{226}Ra, ^{232}Th, and ^{40}K and determined according to the following formula:

$$Ra_{eq} = A_{Ra} + 1.43A_{th} + 0.077A_K \quad \text{(Eq.3)}$$

where, A_{Ra}, A_{Th}, and A_K are the specific activity of ^{226}Ra, ^{232}Th, and ^{40}K, respectively.

Air absorbed dose rates

The indoor gamma absorbed dose rates in the air arising from radioactivity in building materials can be estimated using the radionuclide-specific dose rates conversion coefficients (nGy h^{-1} per Bq kg^{-1}). For materials of superficial use, such as ceramic and porcelain tiles, absorbed dose rates are determined as (EC, 1999):

$$D = 0.12.C_{Ra} + 0.14.C_{Th} + 0.0096.C_K \quad \text{(Eq.4)}$$

For bulk use materials such as concrete and red brick, absorbed dose rates are determined as (EC, 1999):

$$D = 0.92.C_{Ra} + 1.1.C_{Th} + 0.08.C_K \quad \text{(Eq.5)}$$

Effective doses

The effective dose is an important dosimetry quantity to estimate radiation risk. It facilitates comparison among different exposure categories. According to UNSCEAR (2000), a value of 0.7 Sv/Gy absorbed dose to effective dose conversion coefficient and 0.8 as an indoor occupancy factor. Therefore, an effective dose can be determined from air absorbed dose rates as follows:

$$E(Svy^{-1}) = D(nGyh^{-1}) \cdot 8760hy^{-1} \cdot 0.8 \cdot 0.7SvGy^{-1} \cdot 10^{-3} \quad \text{(Eq.6)}$$

The gamma indexes

The gamma index (I_γ) is an important single quantity used in determining whether the external absorbed dose originating from radioactive content in building materials are

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DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

within the recommended annual dose limit for the public (EC, 1999): I_γ is determined from the respective radioactivity of the three natural radionuclides as follows:

$$I_\gamma = \frac{C_{Th}}{200\ Bq/kg} + \frac{C_{Ra}}{300\ Bq/kg} + \frac{C_K}{3000\ Bq/kg} \quad \text{(Eq.7)}$$

where, C_{Ra}, C_{Th}, and C_K are the radioactivity of ^{226}Ra, ^{232}Th, and ^{40}K, respectively.

Alpha index (I_α)

This is another parameter used in estimating the internal radiation exposure attributed to radon from uranium isotopes in building materials. With A_{Ra} being radioactive (Bq.kg^{-1}) of ^{226}Ra, I_α, is defined as follows (Righi and Bruzzi, 2006):

$$I_\alpha = \frac{A_{Ra}}{200\ Bq \cdot kg^{-1}} \quad \text{(Eq.8)}$$

Building materials with I_α < 1 corresponding to radium radioactivity of 200 (Bq.kg^{-1}) are considered safe for building constructions (NORDIC, 2000). The gamma index (I_γ) and alpha index (I_α) represent the levels to which the annual dose must be limited. The limits are summarised in *Table 1*.

Table 1. *The gamma index (I_γ) and alpha index (I_α) limits to achieve the recommended annual dose limits*

Dose criterion	0.3 mSv/y	1 mSv/y
Activity concentration index (I_γ)		
Materials used in bulk amount: Concrete, red bricks	I ≤ 0.5	I ≤ 1
Materials used in ssuperficial amount: porcelain and ceramic tiles	I ≤ 2	I ≤ 6
Alpha index (I_α)		
All building materials		I ≤ 1

Statistical analysis

The statistical analysis has been carried out using Pearson correlation and was considered significant at P-value <0.05, which indicates strong evidence against the null hypothesis.

Results and Discussion

Natural radioactivity and radiological risk parameters were measured on 52 types of local and imported building materials used in Sudan. *Table 2* shows the type of building materials, their uses, and their countries of origin.

Radioactivity and radium equivalent activity (Raeq)

Table 3 presents the statistical summary of radioactivity concentrations in selected building materials used in Sudan. The boxplot distribution of activity concentrations ^{226}Ra, ^{232}Th, and ^{40}K natural radionuclides is shown in *Fig. 1*. As shown, mean ^{226}Ra activity concentrations ranged from 31.6–131.7 Bq.kg^{-1} in red bricks to 228.4±172.6 Bq.kg^{-1} in Italian porcelain (IP), whereas the mean ^{232}Th activity

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DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

concentrations of ^{232}Th ranged from 6.2±2.0 (SC) to 82.8±20.3 Bq.kg^{-1} in (CP). For ^{40}K, average activity concentration ranged from 62.2±27.1 (SC) to 721.6±319.5 in red bricks from Sudan. The considerable variation in activity concentrations may be ascribed to the raw materials and industrial by-products of present building materials, as these replicate the geological characteristics of the sites of their origin.

Table 2. *Distribution of building materials, their uses, and the country of origin*

Code	Type	Uses	Sample size	Country of origin
PC	Tiles (Porcelain)	Superficial	5	China
CC	Tiles (Ceramic)	Superficial	8	China
PI	Tiles (Porcelain)	Superficial	7	Italy
CE	Tiles (Ceramic)	Superficial	4	Egypt
TS	Tiles (Ceramic)	Superficial	6	Sudan
CS	Cement	Superficial/Bulk	10	Sudan
BC	Concrete block	Bulk	6	Sudan
RB	Red brick	Bulk	6	Sudan

Table 3. *Statistical summary of the activity concentration and radium equivalent activity (Bq.kg^{-1}) for ^{226}Ra, ^{232}Th, ^{40}K in selected types of building materials used in Sudan*

Type	A_{Ra}		A_{Th}		A_K		Ra_{eq}	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Materials used in superficial amount								
ST	22.9	7.9-29.8	28.2	17.5-38.6	662.1	438.5-812.5	114.2	83.1-128.3
CC	48.2	19.3-84.6	32.6	22.2-41.1	169.4	129.4-238.9	107.9	79.3-161.8
CP	158.5	87.6-215.2	82.8	59.0-113.4	489.8	378.4-607.3	314.6	23.8-41.9
IP	228.4	78.3-526.8	69.9	41.5-101.6	346.5	238.9-428.1	355.1	159.2-646.0
EC	58.0	47.2-69.2	53.7	33.1-80.4	398.3	262.4-515.5	165.4	129.8-209.0
Materials used in bulk amount								
SC	24.6	12.0-39.6	6.2	1.4-8.7	62.8	28.1-93.7	38.2	21.3-51.6
BC	23.1	9.8-35.0	21.9	11.7-28.0	108.8	87.0-143.0	62.7	34.4-86.1
RB	63.9	31.6-131.7	51.6	25.8-86.9	721.6	284.5-1070.0	193.3	120.2-290.8

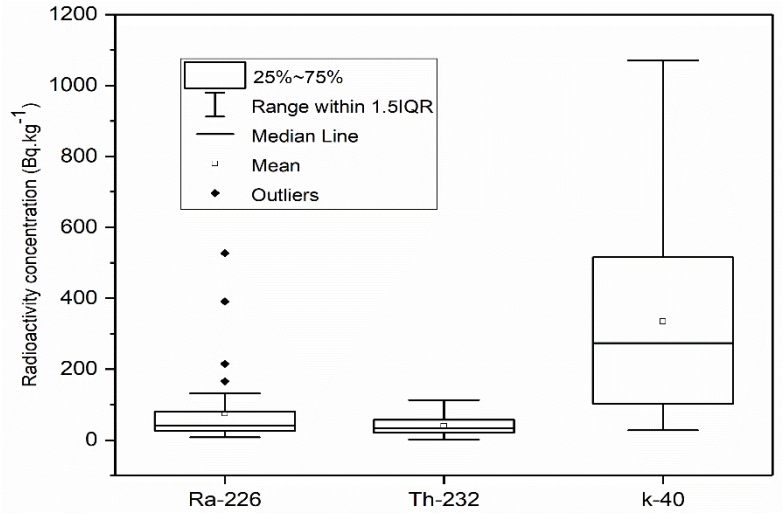

Figure 1. *Boxplot distribution of activity concentrations ^{226}Ra, ^{232}Th and ^{40}K natural radionuclides showing the interquartile range (IQR)(25th to 75th percentile), the range within 1.5IQR, the median and the mean lines and the outliers*

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DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

Of particular interest is the high radioactivity content of the porcelain samples (CP & IP), which show radioactivity approximately one order of magnitude higher than other subjects. Despite their high radioactivity content, as porcelain is used superficially and in small amounts, their relevant radiological risk is expected to be low as compared to other materials that are used in bulk amount. Elevated radioactivity of porcelain samples is related to their zirconium silicate content known for its high natural radionuclide content (IAEA, 2003).

In conformity with the previous observations, the average activity concentrations in building materials obtained in this study and reported in the literature are higher than those found in the earth's crust, being 35, 30, and 400 $Bq.kg^{-1}$ of ^{226}Ra, ^{232}Th, and ^{40}K, respectively (UNSCEAR, 2000).

The activity concentrations of natural radionuclides ^{226}Ra, ^{232}Th, and ^{40}K are not uniform in a given sample, which necessitates setting of a single parameter to represent the total radioactivity, the radium equivalent activity, to be used for comparing the specific activity of materials containing different amounts of ^{226}Ra, ^{232}Th and ^{40}K. The human body treats radium in a similar way to calcium accumulating in human body bones resulting in internal exposure that could cause bone cancer considered to be among the most common radiation cacogenics (NRC, 1988).

As observed in *Table 3*, Ra_{eq} values ranged from 22.9±7.9 to 355.1±195.1 $Bq.kg^{-1}$. These values fall below the maximum permissible value of 370 Bq/kg (UNSCEAR, 2000).

Table 4 shows the comparison of activity concentrations ($Bq.kg^{-1}$) in building materials obtained in this study with those found in the literature. As seen, the activity concentrations in cement found in this study are comparable with or below those observed in previous studies performed in Jordan, Turkey by IAEA and EC (EC, 1999; IAEA, 2003; Mavi and Akkurt, 2010; Salih et al., 2014; Shayeb et al., 2017). The activity concentrations in porcelain and ceramic tiles used in small amounts are comparable with or higher than those reported in the literature.

As portrayed in *Table 4*, there exist wide differences between radioactivity in building materials in our study and those reported by Salih et al. (2014); this could be ascribed to differences in the origin of the studied building materials and this emphasises the importance of regular monitoring radioactivity levels in the materials used for building construction to ensure the safety of the occupants.

Radiation doses

The indoor dose rates from ^{226}Ra, ^{232}Th, and ^{40}K in building materials used in this study (excluding red brick) ranged from 12.0–173.0 $nGyh^{-1}$ (*Table 5*). The determined absorbed doses are comparable to the world average for areas having normal background radiation (55 μGyh^{-1}) (UNSCEAR, 2000). The obtained average annual effective dose values ranged from 0.06 to 0.85 mSv and are, therefore, below the annual dose limit for the public (1.0 mSv).

Radiological risk parameters

The radiological risk to an individual from radioactivity content in building materials is better described using gamma index, I_γ for external exposure and the alpha index (I_α) for internal exposure to radon. I_γ and I_α index limits prescribed for achieving the recommended annual dose limits are summarised in *Table 1* (EC, 1999).

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DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

Table 4. *Comparison of activity concentrations ($Bq.kg^{-1}$) in building materials obtained in this study with the literature*

Country	A_{Ra}	A_{Th}	A_K	Reference
Soil, world average	**35**	**30**	**400**	**UNSCEAR (2000)**
Cement uses in bulk and superficial amount				
Turkey	26	10	130	Mavi and Akkurt (2010)
Sudan	15	33	230	Salih et al. (2014)
Jordan	37-121	54-142	255-621	Shayeb et al. (2017)
IAEA	7–180	7–240	24–850	IAEA (2003)
European Community	50(25–87)	35(10–70)	235(38–587)	Trevisi et al. (2018)
Sudan	25(12±40)	6.1(1.4–8.7)	63(28–94)	This study
Cement block uses in bulk				
Sudan	11.5±1.4	14.98±1.4	378.37±42.0	Salih et al. (2014)
European Community	59(14–272)	34(5–138)	340(17–685)	Trevisi et al. (2018)
IAEA	1–250	1–190	5–1570	IAEA (2003)
Sudan	23(10–35)	22(12–28)	109(87–143)	This study
Red brick uses in bulk				
Turkey	58.9	11.7	248.8	Mavi and Akkurt (2010)
Sudan	20.4 ± 2.1	58.1 ± 3.1	459.6 ± 116.8	Salih et al. (2014)
Jordan	13-19	55-63	160-202	Shayeb et al. (2017)
IAEA	1–200	1–200	60–2000	IAEA (2003)
European Community	51(7–84)	49 (4–102)	555(59–805)	Trevisi et al. (2018)
Sudan	64(32–132)	52(26–87)	722(285–1070)	This study
Tiles /Porcelains/Ceramics used in superficial amount				
Italy	84	54	609	Righi et al. (2009)
Turkey	97	68	471	Turban et al. (2013)
Sudan	31	60	486	Salih et al. (2014)
IAEA	30–200	20–200	160–1410	IAEA (2003)
Sudan	105(8–527)	52(18–118)	393(129–812)	This study

Table 5. *Statistical summary of doses and the radiological hazard parameters: D ($nGyh^{-1}$), E ($mSvy^{-1}$), I_γ and I_α estimated from the radioactivity concentrations in building materials used in Sudan*

Type	D (nGyh-1)		E (mSvy-1)		I_γ		I_α	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
Materials used in superficial amount								
ST	13.1	9.7-14.7	0.06	0.05-0.07	0.43	0.32-0.49	0.57	0.42-0.64
CC	12.0	8.5-18.2	0.06	0.04-0.09	0.41	0.26-0.64	0.54	0.40-0.81
CP	35.3	23.8-41.9	0.17	0.12-0.21	1.23	0.81-1.50	1.57	1.06-1.85
IP	40.5	17.9-75.7	0.20	0.09-0.37	1.49	0.63-2.97	1.78	0.80-3.23
EC	18.3	14.6-22.7	0.09	0.07-0.11	0.60	0.51-0.71	0.83	0.65-1.04
Materials used in bulk amount								
SC	34.4	20.2-46.3	0.17	0.10-0.23	0.16	0.10-0.23	0.19	0.11-0.26
BC	54.0	30.1-74.4	0.27	0.15-0.37	0.22	0.12-0.32	0.31	0.17-0.43
RB	173.3	111.1-253.0	0.85	0.55-1.24	0.73	0.47-1.10	0.97	0.60-1.45

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DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

For ceramics and tiles used in a superficial amount, mean I_γ values ranged from 0.41 to 1.49 and are less than 2, which corresponds with the annual dose limit of 0.3 mSv. These materials can be exempted from regulatory control. For the cement blocks and red bricks, which materials are used in bulk amount, I_γ values ranged from 0.16 to 0.73. These materials exceed the lower limit of I_γ and, therefore, need to be subjected to regulatory control. As presented in *Table 4*, I_α for CP & IP porcelain was 1.57±0.37 and 1.78±0.98, respectively. These levels exceed the prescribed limit of < 1 indicating that the use of this imported decorative material poses risk to the safety of the occupants.

Correlations between ^{226}Ra, ^{232}Th and ^{40}K activities

Table 6 shows the Pearson correlation coefficients of ^{226}Ra, ^{232}Th, and ^{40}K radionuclides. The results are graphically depicted in *Fig. 2* and *Fig. 3*. A significant correlation was observed between ^{232}Th and ^{40}K (R = 0.42, P<0.05); and a highly significant correlation was observed between ^{226}Ra and ^{232}Th (R = 0.68, P<0.001). The correlations observed could be due to the raw materials and industrial by-product of the building materials being of the same origin.

Table 6. *Pearson correlation coefficients between ^{226}Ra, ^{232}Th, and ^{40}K*

Radionuclide	^{226}Ra		^{232}Th		^{40}K	
	Pearson corr.	**p-value**	**Pearson corr.**	**p-value**	**Pearson corr.**	**p-value**
^{226}Ra	1	**	0.68*	<0.001	0.15	0.28
^{232}Th	0.68*	<0.001	1	**	0.42*	0.002
^{40}K	0.15	0.28	0.42*	0.002	1	**

Correlation significant at p<0.05; and highly significant at p<0.001

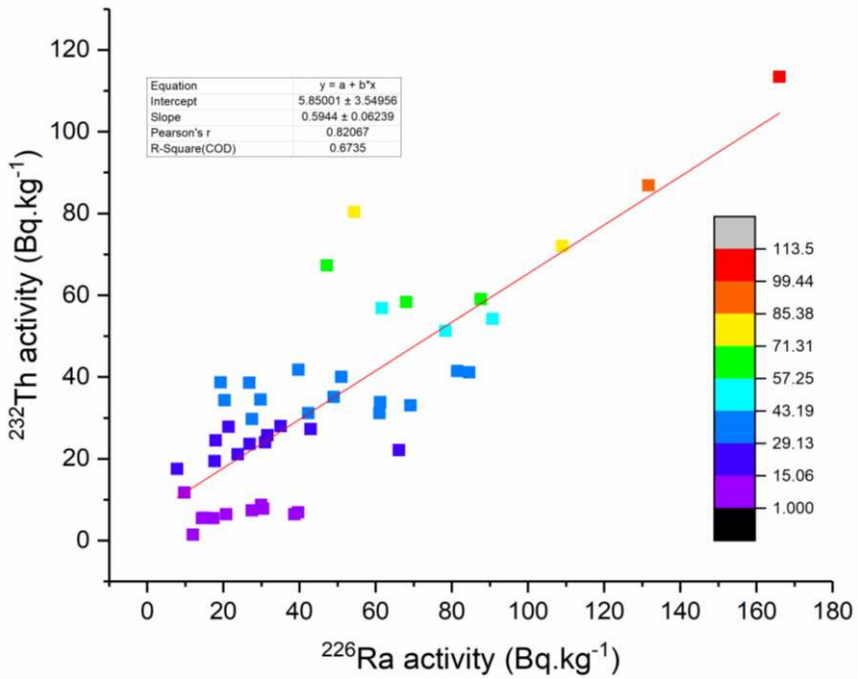

Figure 2. *Correlation between of activity concentrations ^{226}Ra and ^{232}Th natural radionuclides showing colour codes for the corresponding ^{232}Th activity*

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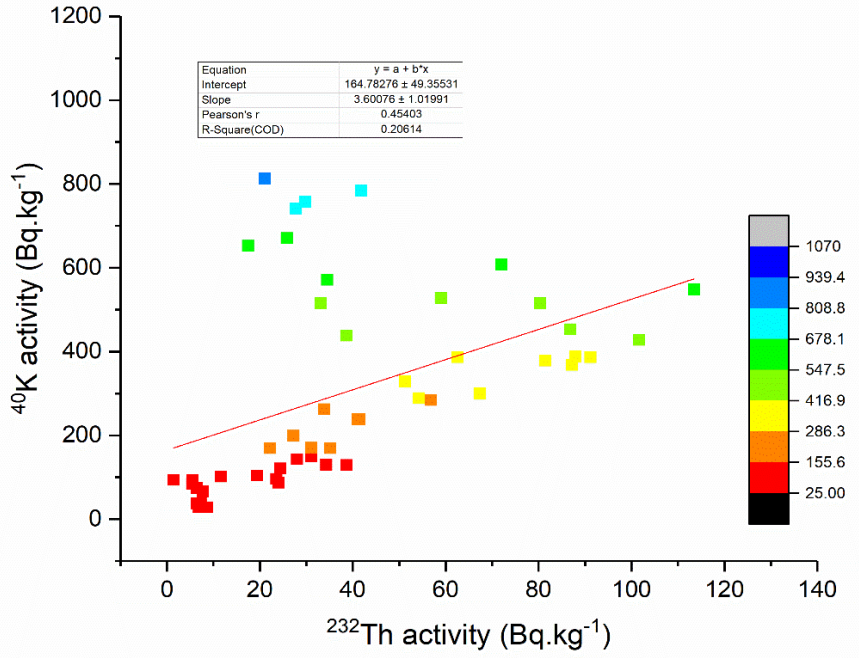

Figure 3. *Correlation between of activity concentrations ^{226}Ra and ^{40}K natural radionuclides showing colour codes for the corresponding ^{40}K activity*

Conclusion

Natural radioactivity levels in the selected building materials used in Sudan were measured using gamma spectrometry. The average values obtained are comparable to those reported in the literature. Continuous demand for both local and imported building materials necessitates regular monitoring of radioactivity levels to protect the occupants of buildings from radiation. The current data provide important radioactivity levels. Their benchmarking is the first step towards acquiring large scale data that would make it possible to set national regulatory control limits for protection of the public from radiation in buildings. These results indicate that considering the various types of dwellings constructed in various states and regions of Sudan and the variety of building materials used in their construction, establishment of a national research project to study radioactivity levels in building materials and evaluate the risk they pose to the population, is strongly recommended. Such a project should also consider investigating radon gas in dwellings and workplaces to establish safety limits to mitigate the radiological risk associated with radioactivity in building materials.

Acknowledgements. The authors would like to thank the building material workhouses for supplying us with the study samples.

Declaration of Conflicting Interests. The authors have no conflict of interests to declare.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75637572

ASSESMENT OF THE SOIL UREASE RESPONSE TO SULFONYLUREA HERBICIDES BASED ON STATISTICAL MODELS

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(Received 23rd Apr 2020; accepted 13th Aug 2020)

Abstract. The aim of this paper was the evaluation of the ecotoxic effects of three sulphonylurea compounds, commonly used as herbicides against weeds in agriculture by mathematical models. The tests were performed both in laboratory and field conditions to study the changes in soil urease, considered a key enzyme for regulating soil nitrogen transformation and a sensitive indicator to herbicides. Mathematical models accurately reproduced the behavior of urease from chemically treated soil samples, based on real, observable processes. Besides, they simplify the view regarding the activity of the selected enzyme, as there are many factors and complicated biogeochemical processes, which might interfere. Overall, we conclude that, for the cambic chernozem model analyzed here, the normal (label-recommended) chlorsulfuron and amidosulfuron doses do not perturb soil urease activity and the former compound is more urease-friendly than either amidosulfuron or tifensulfuron. In the context of the long-term use of these herbicides, our research underlines the importance of mathematical models and the prefiguration of a map for the differentiation of field / laboratory experiments, for the most accurate highlighting of the biochemical imbalances caused by the chemical substances, the risk of overdose and the toxicity risk for soil and environment.
Keyword: *ecotoxic effect, soil enzymes, chlorsulfuron, amidosulfuron, tifensulfuron*

Introduction

Mathematical modeling can be used for developing scientific understanding and to test the effect of changes in a system (Marion and Lawson, 2008). The role of the mathematical models is to replicate the behavior of analyzed samples based on real observable facts (Ledder, 2013). Thus, information on the effect of herbicides on enzymes provides anticipations regarding the management of phytotoxic effects and the quality of soil, surface and groundwater. The researchers use all kinds of mathematical models for predictions related to soil herbicides influence, essential for agronomic purposes and for the protection of the environment (Swarcewicz and Gregorczyk, 2013; McGrath et al., 2019). In this context, information on the reaction of enzymes in the presence of herbicides is essential, as they are catalysts of their degradative processes. Sulfonylurea herbicides are a class of herbicides that have become extremely popular in recent decades due to their broad-spectrum weed control (He et al., 2012) at relatively low doses, i.e., between 20 and 200 g ha^{-1} good crop selectivity, low mammalian toxicity, but they inhibit the enzyme acetolactate synthase (ALS) (Powles and Qin, 2010). These compounds and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75737585

their metabolites can persist for a long time in natural environments, exert a phytotoxic effect (Mehdizadeh et al., 2016), and also affect the activity of soil microorganisms and enzymes (Yang et al., 2007; Radivojević et al., 2014).

However, there is surprisingly little information about the effects of sulfonylurea herbicides on soil enzymes although their measurement can provide us with relevant data about the agroecosystem health and anthropic-induced soil disturbance (Kiss, 2001).

Soil enzymes are intimately involved in organic matter and nutrient cycle in the pedosphere, actively participate in the transformation and decomposition of herbicides at this level, and contribute to the stabilization of soil structure. These enzymes are generally considered as relevant markers for assessing the degree of soil virginity in terms of changes induced by anthropogenic factors (Taylor et al., 2002; Riah et al., 2014). Therefore, the soil enzymatic potential serves as a suitable and subtle indicator of soil biological equilibrium (Lizy Sravanthi et al., 2015), health (Pankhurst, 2006), fertility (Piotrowska-Dlugosz, 2014), and quality (Lone et al., 2014), and is widely used to assess the hazard that herbicides pose on soil biological functioning (Sebiomo, 2011).

The activities of certain soil enzymes, including dehydrogenase, β glucosidase, cellulase, urease, amidase, phosphatase, and arylsulphatase are used alone or coupled to calculate soil health indexes because their measurement involves simple procedures and provides an early warning system for disturbance in soil ecosystems (Shukla and Varma, 2011).

Urease from soil is of microbial and plant origin (Rathore and Nollet, 2012; Hameed et al., 2019), is persistent due to its association with organic and inorganic soil colloids (Baboo et al., 2013) and hydrolyzes urea (commonly used as a nitrogen source for plants).

The European Food Safety Agency has recently emphasized the lack of standardized methods for evaluating the ecotoxicity of herbicides and the pressing need for developing microbial markers sensitive to herbicide exposure (Thiour-Mauprivez et al., 2019). Urease activity is sensitive to herbicide application (Hameed et al., 2019), which in undisturbed soils tends to remain relatively constant over time (Conway and Pretty, 2013). Hence, it can be used for monitoring soil quality (Srinivasulu and Rangaswamy, 2014) and there is also relevant evidence that certain sulfonylurea herbicides can interfere with it (Thiour-Mauprivez et al., 2019).

However, its decrease reduces urea hydrolysis, which is beneficial because it helps keep nitrogen in a form less predisposed to levigation, i.e., ammonium ions (NH_4^+), (Schuster and Schroeder, 1990).

The aim of this study was to investigate the effect of three routinely used sulfonylurea herbicides, that is chlorsulfuron (CLS), amidosulfuron (AMS), and tifensulfuron (TIS), on soil urease activity, by using a mathematical model. In this context, understanding the response of urease activity in presence of herbicides can serve to assess and predict the overdose-related risks in terms of enzymatic potential, biochemical processes, especially the imbalances in the nitrogen circuit in which urease plays a key role, soil microbial biodiversity and toxic effects on the environment. The use of high doses in laboratory models and reporting to field conditions is essential because, according to Sofo et al. (2012), doses above the recommended application rates they can induce long-term disturbance in soil microbial and enzymatic activities, thereby disrupting the natural biochemical balance of the soil.

As a result, a thorough understanding of the effect of sulfonylurea herbicides on soil urease activity is of great interest for optimizing the use of such herbicides, as well as for limiting the potential risks associated with it.

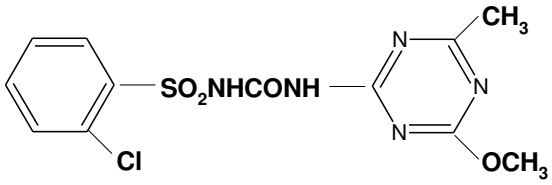
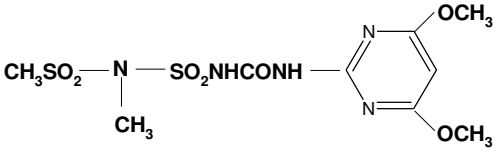
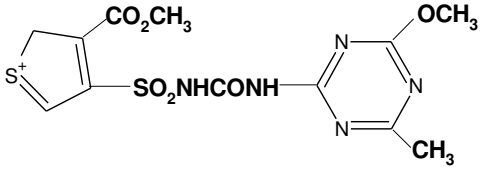
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75737585

Materials and methods

Experimental design

The phytopharmaceutical products considered in this study were: (1) chlorsulfuron (CLS); (2) amidosulfuron (AMS) and (3) tifensulfuron (TIS), (*Table 1*).

Table 1. *Phytopharmaceutical substances used in the experiment (Fluka Chemika-BioChemika 1995/1996, 1995; Manea et al., 2017)*

Herbicide substance	Chemical structure	Manufacturing company
Chlorsulfuron (2-chlor-/N/4-methoxy-6-methyl-1,3,5-triazine-2-il/-aminocarbonyl/-benzosulfonamide)		Du Pont de Nemours, USA
Amidosulfuron (3-(4,6-dimethoxypyrimidin-2-yl)-1-(N-methyl-N-methyl)sulfonyl)urea)		AgrEvo, Germany
Tifensulfuron carboxylate of methyl-2(methoxy-4-methyl-6-triazine-1,3,5 yl-2) amino-carbonyl amynosulfuronyl-3 thiophene		Nemours, SUA

For each herbicide, four doses were considered, irrespective of experimental conditions: (1) control (M); (2) normal dose (ND): chlorsulfuron, 20 g ha^{-1}; amidosulfuron, 60 g ha^{-1}; tifensulfuron, 60 g ha^{-1}; (3) two-fold normal dose (2ND): chlorsulfuron, g ha^{-1}; amidosulfuron, g ha^{-1}; tifensulfuron, g ha^{-1}; (4) five-fold normal dose (5ND): chlorsulfuron, 100 g ha^{-1}; amidosulfuron, 300 g ha^{-1}; tifensulfuron, 300 g ha^{-1}. Such doses have been often reported to occur in arable soils on which these herbicides have been applied. It was considered that the normal dose is the quantity recommended by the manufacturer for agricultural practices.

To provide relevant results, a cambic chernozem was considered in the present work. It is one of the most fertile soil types from Romania (Ianos et al., 1997) and covers important areas in Romania and eastern European countries (Hardarson and Broughton, 2013). The physico-chemical parameters of the 0-20 centimeters (cm) soil horizon, wherein most microbial activity generally occurs (Shukla and Varma, 2011), were: coarse sand - particles less than 2/millimeters (mm) and greater than 0.2 mm in diameter, 0.5%; fine sand – particles between 0.2 mm and 0.02 mm in diameter, 29.2%; silt - particles between 0.02 mm and 0.002 mm in diameter, 29.2%; clay - particles less than 0.002 mm, 41.4%; texture, clay loam; porosity, 49%; hygroscopic coefficient, 9.2%; pH, 6.45; humus content, 4.09%; total nitrogen, 0.156% per gram dry weight soil; assimilable

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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phosphorus content, 21.80 g/100 mg soil; assimilable potassium content, 29.70 g/100 mg soil.

The field study was conducted in the experimental fields of the Banat University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timisoara (BUASVMT). The experimental area (45.45°lat. N, 21.14°long. E, Timisoara, Romania) lies in the Banat silvo-steppe and is characterized by a temperate climate with Mediterranean influences, which is specific to the South-western Romania (Micu et al., 2015). The herbicides were applied in spring (May) according to the manufacturer's recommendations. For each treatment, the experiments have run in triplicate on experimental plots of 18 m^2 each. Five random samples from the 0-20 cm soil horizon were collected using a trowel along a diagonal transect across each plot at 30 days after herbicide application. They were put in self-sealing sterile plastic bags, transported to the lab during the same day and homogenized in batches in a Waring blender (about 1,200 g per each herbicide treatment). Then, the humidity was adjusted to 15-17% by addition of distilled water, following the method described by Stefanic (2006) and the soil samples were maintained under controlled temperature (t = 25°C) for 48 hours to ensure a proper equilibration of the soil microflora.

The laboratory study was performed at the Laboratory of Soil Microbiology from BUASVMT. Before herbicide application, untreated samples collected from the field plots (as shown above) were transported to the lab and treated with herbicides (kept for 7 days), to facilitate soil-microbiota-herbicide interaction (triplicate per each treatment).

Method for determining soil urease activity

At the end of both studies, the soil samples were homogenized in batches (using a Waring blender) and sieved to remove coarse materials (e.g., roots, rocks, macro-organic matter) and particles larger than 2 mm. Next, they were analyzed for determining the soil urease activity by adapting the method routinely used in Romania for this purpose (Stefanic, 2006). Briefly, reaction mixtures consisting 5 g of soil and 10 ml of a 1% urea solution were incubated at 28°C for 24 hours, then treated with 70 ml of a 0.1N potassium sulfate solution, 2 ml of 25% Seignette salt solution and 10 ml of Nessler reagent [7783-33-7, Merck 13-773]. The absorbance was measured at 425 nm and the corresponding calibration curve was created using a 0.002% ammonium chloride solution. The soil urease activity (UA), expressed as micrograms of ammonium nitrogen per kilogram soil dry weigh (μg NH_4^+/g sol d. wt), was calculated according to the formula (*Eq. 1*):

$$\text{U.A.} = \frac{Ep \bullet C \bullet F \bullet 100 \bullet KU}{Eet \bullet V \bullet G} \qquad \text{(Eq.1)}$$

where *Ep* defines the sample extinction, *Eet* the reference solution extinction, *C* the concentration of reference solution (0.01 micrograms of ammonia permilliliter, i.e., μg NH_4^+/ml), *F* the volume of the liquid from the filtrated soil (80 ml), *V* the volume of filtrate transferred in a sterile polyethylene tube (50 ml), *G* the mass of fresh soil (g), and *KU* the adjusted coefficient of soil humidity.

Statistical analysis and mathematical modeling

Data analysis was performed using Excel and the statistical packages PAST software version 2.17 and MVSP version 3.22 (Hammer et al., 2001).

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Planned pair wise comparisons between the reference and different treatments were conducted using Duncan's tests to determine the effect of herbicides on soil urease activity. The *p* values lower than 0.05 were considered significant. The results were expressed as mean values with standard deviation (SD). Finally, we performed a cluster analysis to identify what herbicides and doses exert the lowest impact on soil urease activity. The data were hence classified in four groups, one including the values measured for the control group, and three other groups, one for each herbicide investigated. The constrained Ward's method was used since it allowed us to join the resulting such that increase in within-group variance is minimized.

The role of the mathematical models is to replicate the behavior of analyzed samples based on real observable facts (Ledder, 2013).

Results and discussion

The present study significantly broadens our understanding of side effects associated with the use of sulfonylurea herbicides in agriculture, and moreover, demonstrates for the first time that AMS and TIS can interfere with soil urease activity (*Table 2; Figs. 1-3*). Our results clearly show that the application of normal (label-recommended) dose of CLS and AMS tends not to perturb this enzymatic index in cambic chernozems, irrespective of experimental conditions.

This is supported by other scientists, who have found that, in most cases, the application of herbicides at recommended doses does not affect or has a reduced effect on soil enzymatic activity (Nannipieri, 1994; Utobo and Tewari, 2015). Moreover, this effect can be counteracted over time via absorbtion of herbicides onto soil colloids and stabilization of microbiota structure (Rao et al., 2010).

The measured values for soil urease activity in both experiments were shown in *Table 2.* The application of CLS and AMS under field conditions had no consistent effect on soil urease irrespective of dose (in all cases, $p > 0.05$). In the case of TIS, there was, however, a marked inhibition of soil urease for the ND and 5ND treatments (in both cases, $p < 0.05$), but not for the 2ND treatment (in both cases, $p > 0.05$).

Table 2. *Mean (and SD) for soil urease activity*

Treatment	Field conditions				Laboratory conditions			
		ND	2ND	5ND		ND	2ND	5ND
M	30.09 (1.26)				28.36 (3.29)			
CLS		28.24 (2.86)	30.94 (4.93)	31.90 (4.28)		28.27 (3.48)	32.96 (2.54)*	25.89 (4.83)
AMS		33.61 (5.04)	28.92 (1.02)	26.89 (2.65)		28.00 (1.72)	31.11 (2.06)*	28.27 (5.23)
TIS		27.80 (4.02)*	31.30 (3.04)	28.78 (2.13)*		33.20 (4.74)*	27.37 (4.38)	35.22 (3.29)*

Legend: M-control, CLS = chlorsulfuron, AMS = amidosulfuron, TIS = tifensulfuron, ND - normal dose, 2ND - two-fold normal dose, 5ND - five-fold normal dose. Marked boxes (*) indicate significant differences as compared to the reference group (Duncan's test, $p < 0.05$)

This variations may be the result of the influence of soil physical and chemical properties (Šantric et al., 2018), but also because the microflora and enzymes are mostly absorbed on soil colloids, and direct contact with the herbicide may to be accidental (Stefanic, 1981). For this reason, in order to have a more complete picture of the influence of herbicides,

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experiments were performed both in the field and in the laboratory, testing other doses in addition to the usual dose for agricultural practice. We also consider that spatial and temporal variations of physical, chemical, microbiological, and biochemical proprieties of soil may be involved (Gimsing et al., 2004).

At similar levels, TIS, by contrast, caused significant, but low changes in urease activity for both experiments. Therefore, both CLS and AMS at the recommended dose appear to be more urease-friendly than TIS when dealing with this type of soil.

The variance of soil enzymatic activity in response to herbicide application was constant. This is in line with the results derived from other studies (Rasool et al., 2014; Tomkiel et al., 2014; Kumar et al., 2018). The soil enzymatic response can be also influenced by the presence of biological substrates, such as certain sulphonylureas herbicides, like thifensulfuron-methylor metsulfuron-methyl, and various metabolic products (Belhadj-Tahar et al., 2003).

Under field conditions, different CLS and AMS treatments did not consistently influence soil urease. Several field studies revealed that, depending on the dose, state of the enzyme (intracellular or extracellular enzymes, adsorbed on clay or humic acids), soil type (Šantric et al., 2018), and post-exposure duration, CLS can either inhibit or stimulate the activity of this enzyme (Yang et al., 2006). The measured values returned to normal levels 30 days post-treatment (Sofo et al., 2012), which is consistent with our findings.

Interestingly, TIS tended to significantly inhibit the soil urease activity, thus maintaining nitrogen in a less mobile (levigable) form. As a result, one can expect that field application of this herbicide may temporarily help in preserving the total nitrogen (N) stocks in cambic chernozems. There is also likely that the effect of herbicide application may affect nitrogen biodisponibility in soil (Palma et al., 2016).

When compared to the control group, the use of CLS under laboratory conditions caused a significant increase in soil urease activity for the 2ND treatment ($p < 0.05$), but no consistent effect for the other two treatments ($p > 0.05$). A similar trend was observed for AMS (ND: $p > 0.05$, 2ND: $p < 0.05$, 5ND: $p > 0.05$). In contrast, TIS showed a marked elevation in soil urease activity for the ND treatment ($p < 0.05$) and the 5ND treatment ($p < 0.05$), but no significant effect was found the second highest dose, i.e., the 2ND treatment ($p > 0.05$).

However, in laboratory environments, which are conducive to intense microbial activity by providing ideal temperature and humidity conditions, TIS could be decomposed to thiophene metabolites that can increase the bioavailability of soil nickel, cobalt, and manganese via chelation, and therefore, stimulate soil urease activity. This is in agreement with the laboratory results obtained in this work after TIS application at ND and 5ND doses.

The dendrogram obtained by applying hierarchical cluster analysis to field data (*Fig. 1A*) revealed for AMS the greatest distance to the other groups, whereas the CLS, TIS, and M groups clustered closely together, with CLS appearing to exert the lowest impact on soil urease. However, a different trend was seen under laboratory conditions (*Fig. 1B*). The first cluster contained the measured values for the M, AMS, and CLS groups. A high similitude between CLS and AMS effects on this enzymatic index was observed. The second cluster was distinct and corresponded to soil samples exposed to TIS.

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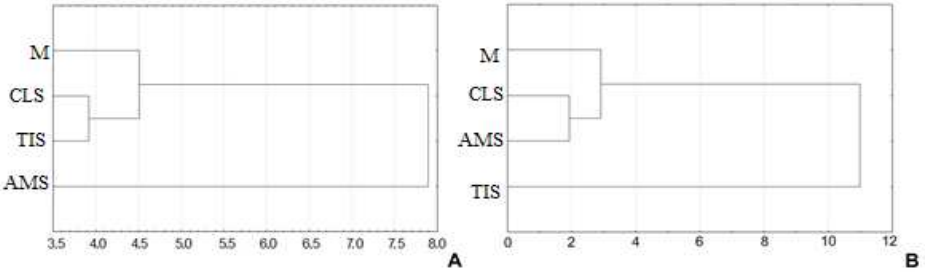

Figure 1. *Cluster analysis using the Ward's method on urease activity in a cambic chernozem model exposed to AMS - amidosulfuron, CLS - chlorsulfuron, TIS - tifensulfuron. Experiments were conducted under field conditions (A) and under laboratory conditions (B), M-control*

Although all herbicides examined have structurally related heterocyclic rings and side groups bound to their positions 4 and 6, we observed different clustering patterns under field and laboratory conditions. Thus, the CLS behavior was similar with that of TIS under field conditions and with that of AMS under laboratory conditions. Field conditions, with considerable swings in humidity and temperature, are expected to induce a more variable response of soil urease to sulfonylurea herbicides as compared to those encountered in controlled laboratory environments. Therefore, the microbiological decomposition of these herbicides may be slower or even follow different pathways under such conditions, and as a consequence, potentially lead to different toxicity manifestations and effects. Such differences seem to occur especially in the case of TIS, which showed a marked inhibitory effect on soil urease in field experiments, but a consistent stimulatory action in laboratory tests.

Overall, our results confirm that these herbicides can either stimulate or inhibit the soil enzymatic activity (Micuti et al., 2018), but significant changes can occur in field conditions only when usual doses are exceeded or when optimal conditions of temperature and humidity promotes soil microbial activity (Pankhurst, 2006).

After cumulating the results of field and laboratory experiments, it was found that, in the case of CLS and AMS overdose, only one out of four treatments caused significant changes in enzymatic activity (as compared to the corresponding controls).

In contrast, such a situation was seen for TIS in two out of four treatments. Moreover, CLS was the only herbicide which in both experiments was clustered the closest to the reference group. As a result, it serves as the most urease-friendly sulfonylurea herbicide among the compounds investigated even in the case of overdose.

Water is essential for preserving the catalytic activity of soil enzymes, while both, soil moisture and temperature serve has the main factors responsible for soil biochemical and microbial characteristics (Giacometti et al., 2013). By applying the generalized linear model mathematical method, we found that urease activities in response to applying CLS (*Fig. 2A*), AMS (*Fig. 2B*) and TIS (*Fig. 2C*) decrease with moisture reduction. This highlights the importance of soil moisture for soil microorganisms, pesticide application (Kavita and Geeta, 2014), and organic matter turnover in soil (Borowik and Wyszkowska, 2016). In fact, it was demonstrated that for soil moisture 20% from the maximum water capacity, the soil enzymatic activity increases for urease, dehydrogenase, acid phosphatase, alkaline phosphatase, β-glucosidase and arylsulfatase. The increase in soil moisture and temperature can improve the mineralisation rate of sulphonylurea compounds, thus reducing their toxicity to soil microbiota, observation confirmed by literature data (Wang et al., 2010). In contrast, any decrease in soil moisture has a negative

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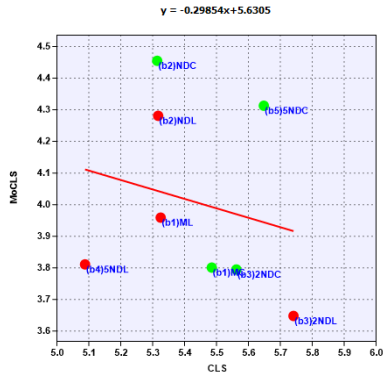
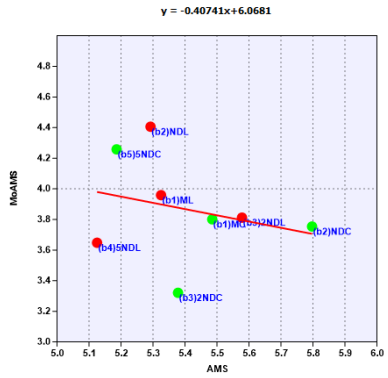
effect on these mineralisation processes (Wang et al., 2007). These data indorse the validity of our mathematical model (*Fig. 2*). Soil humidity equilibrium is essential for maintaining the soil microbiota homeostasis, since both excessive moisture and drought can reduce soil biomass (Landesman and Dighton, 2010).



A. Variation of CLS correlated with samples moisture content using data square-root transformed, normal distribution and identity function

$$y = -0.29854 \cdot x + 5.6305$$

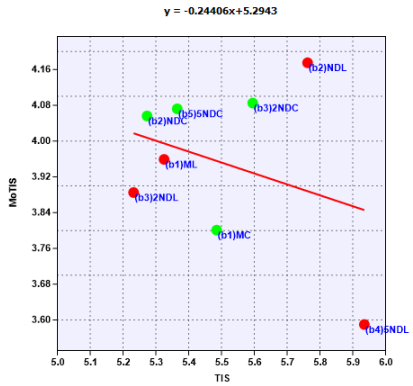
Where x= CLS
y = MoCLS
Slope a: -0.29854
Interc.b: 5.6305; p(slope=0):0.8662



B. Variation of AMS correlated with samples moisture content using data square-root transformed, normal distribution and identity function

$$y = -0.40741x + 6.0681$$

Where x= AMS
y = MoAMS
Slope a: -0.40741
Interc.b: 6.0681; p(slope=0):0.8134



C. Variation of TIS correlated with samples moisture content using data square-root transformed, normal distribution and identity function

$$y = -0.24406 \cdot x + 5.2943$$

Where x= TIS
y = MoTIS
Slope a: -0.24406
Interc.b: 5.2943; p(slope=0):0.8715

Figure 2. *Generalized linear models*
Legend: *red = laboratory data (doses of herbicide used to treat soil samples in laboratory conditions), green = field data (doses of herbicide used to treat soil samples in field conditions), CLS = chlorsulfuron, AMS = amidosulfuron, TIS = tifensulfuron, MoCLS = moisture content in the soil treated with chlorsulfuron chlorsulfuron, MoAMS = moisture content in the soil treated with amidosulfuron, MoTIS = moisture content in the soil treated with tifensulfuron, end termination "L" = laboratory condition, end termination "C" = field condition, (b1)M = control, (b2)ND = normal dose, (b3)2ND = two-fold normal dose, b5(5ND) = five-fold normal dose*

Soil urease is humidity - dependent and as soil moisture increases from θ60 to θ100, the rate of urea hydrolysis decreases by 13.9% -28.7%, even if temperature and nitrogen decomposition rate remain.

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CLS samples show the smallest differences between laboratory and field results, followed by AMS and TIS (*Fig. 3*). The small differences observed here in urease activity for CLS may reflect the adaptation of bacterial communities to CLS, leading to strains capable of degrading this herbicide (Zanardini et al., 2002). Indeed, the last studies of Ergüven (2017), provide support for such a possibility.

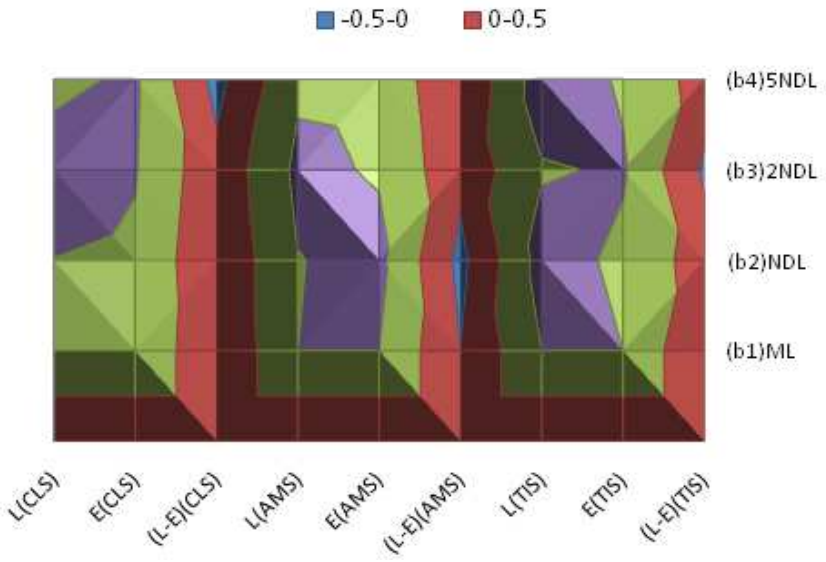

Figure 3. *MAP of differences between Laboratory and Field Experiments*
Legend: *L= laboratory, E = Field Experiment, (L-E) = differences between laboratory data and field experiment data, CLS = chlorsulfuron, AMS = amidosulfuron, TIS = tifensulfuron, end termination "L" = laboratory condition, end termination "C" = field condition, (b1) M = control, (b2)ND = normal dose, b3(2ND) = two-fold normal dose, b5(5ND) = five-fold normal dose*

Based on the current results, we suggest that, among the sulfonylurea herbicides investigated, TIS and CLS have the greatest, and respectively, the lowest potential to affect the soil urease activity. However, further studies are necessary in order to expand on these findings and to elucidate the risk significance of these results. These considerations are also supported by Rachedi et al. (2018) who have recently showed that repeated application of herbicides causes major perturbations of soil biological activities.

Conclusions

In conclusion, the results of the present study suggest that CLS application on cambic chernozems is more urease-friendly than that of either AMS or TIS. It was also found that the CLS-treated soils were always clustered the closest to the untreated soils.

By applying the generalized linear model mathematical method, we found that urease activities in response to applying CLS, AMS and TIS decrease with moisture reduction. In addition, demonstrated that there are slight differences between the activity of urease in soil samples treated with CLS (laboratory and field), followed by AMS and TIS.

The applied statistical models accurately reproduced the urease response in the presence of sulfonylurea herbicides. Their evaluation can predict the risks related to

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overdose, both in terms of the enzymatic potential and the impact on urea function, as well as the consequences on soil and environment.

This approach provides a way for soil and crop management and can be extended to other herbicide classes for the purpose of ecological risk assessment. The study can be extended with the evaluation of herbicide residues in the soil, to know the risks involved in the applied doses and mobility of herbicides and the occurrence of possible accumulations and bio concentrations with risk on the environment and humans.

It is also recommended that further research should extend the trials on several soil types, with the development of databases which include the reaction of these herbicides on specific soil areas, to ensure that farmers have a clear picture and make informed decisions.

Acknowledgments. We are grateful to Professor dr. Gergen Iosif and to Eng. Nica Dragos for constructive comments and recommendations. In addition, the first author (ABB) wishes to thank Professor dr. Stefanic Gheorghe for continuous support throughout her scientific career. The research is supported by the project "Ensuring excellence in the activity of RDI within USAMVBT" code 35PFE, submitted in the competition Program 1 - Development of the national system of research - development, Subprogram 1.2 - Institutional performance, Institutional development projects - Development projects of excellence in RDI.

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DOI: http://dx.doi.org/10.15666/aeer/1806_75737585

COMPARATIVE ANALYSIS OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) GENOTYPES BASED ON SEED AND OIL CHARACTERISTICS

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(Received 25th Apr 2020; accepted 13th Aug 2020)

Abstract. The performance of nine safflower genotypes was estimated for the physical properties of seeds, chemical, and physiochemical characteristics of seed oil. They are with different response to most physical properties, that are considered in machinery designation for handling, harvesting, and oil extraction process. Local and Ardny genotypes had the highest percentage of oleic acid, while linoleic was the highest in Rabiaa, indicating their high usability in food preparation. Al-Shamia is preferable for direct use in food preparation, containing the lowest palmate ratio. Free fatty acid was ranged from 31.59-41.02% for G-2018 and Zaafarani genotypes, respectively. G-2018 had the lowest iodine value at 136.350 g I_2/100 g, providing an oil with a high saturation value. Al-Shamia and Local genotypes had low palmitic and stearic acid (saturated fatty acids) percentages, therefore, a highly stable and healthy edible oil can be extracted from the plants. The principal component analyses attributed high variation among the genotypes. Local and Rabiaa genotypes could be involved in the improvement of oil quality in Kurdistan. Dissimilarity matrix clarified a wide distance between G-2018 and other genotypes based on the traits. Hybridization programs would be reasonable for the oil quality improvement of these genotypes. Thermal stability and frying qualities of oleic and linoleic oils could be studied in the future.
Keywords: *true density, absorption index, protein%, refractive index, fatty acids composition*

Introduction

Safflower (*Carthamus tinctorius* L.) is an annual, highly branched, herbaceous plant, self-pollinated and belongs to the Asteraceae family (Pasandi et al., 2018), has a haploid genome size of about 1.4 GB and 2n = 24 chromosomes (Kumari et al., 2017). Safflower is a minor crop and one of the major oilseed crops worldwide (Gouzy et al., 2016), with a world production of about 627,653 tons in 2018 (FAOSTAT, 2019). There are several global uses of safflower such as dye production, various medicinal utilization, and extraction of edible oil. The extracted oil has a realized effect on reducing blood cholesterol levels (Emongor, 2010), due to containing a higher amount of oleic and linoleic acids than other oilseed crops (Khalid et al., 2017). Safflower seed oil content is ranged between 35 and 50% (Coşge et al., 2007). The oil is flavourless and colourless, and similar in composition to sunflower oil (Kaffka and Kearney, 1998; Han et al., 2009). Considerable attention has been generated in the consumption and development of safflower seed oil as an excellent health care product including the prevention and treatment of hyperlipidemia, arteriosclerosis, and coronary heart disease (Abidi, 2001). Safflower oil is stable and its consistency does not change at low temperatures, making the oil particularly suitable for the preparation of chilled and frozen foods (Ekin, 2005). The high oleic contents of safflower oil make the oil stable during heating and do not give smoke smell during frying (Gyulai, 1996). The oil is not allergenic, making it an ideal material for cosmetics. High polyunsaturated essential fatty acid, linoleic, make the oil to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75877605

be valuable nutritionally and therapeutically for human consumption (Vosoughkia et al., 2011). There is a growing tendency to study new genotypes of safflower with higher seed oil contents (Bart et al., 2010). The objective of this work is to assess the performance and variability of available safflower genotypes in Kurdistan-Iraq for some physical properties, oil characteristics and physicochemical properties of seed oil, and identifying their relationship for efficient selection to increase the oil yield in the safflower breeding program.

Materials and methods

A laboratory experiment was conducted, in the College of Agricultural Engineering Science, University of Sulaimani. Nine varieties (G-2018, Rabiaa, Zaafarani, Al-Mais, Ardny, Iden, Gilla, Al-Shamia and Local) were used in this study. All the genotypes are registered varieties at the Ministry of Agriculture and water resources of Kurdistan- Iraq. Gilla and Al-Mais genotypes were obtained from Erbil Polytechnic University, while the other genotypes were from Bakrajo Agriculture Research Center in Sulaimani, Kurdistan-Iraq. To erase the environmental effects on the genotypes, the varieties were grown in the field of College of Agricultural Engineering Sciences for 2018-2019 growing season in a Complete Randomized Block Design (CRBD) with three replicates. The varieties were grown under rainfed condition of the semi-arid region of Sulaimani-Iraq. The seeds were sown at 6 cm depth and with a plant density 40000 plant/ha as recommended by Ati and Hassan (2016). The collected seeds from these genotypes were used in the analyses. A complete randomized design was applied to examine the study data, with three replicates. Statistical analyses were performed. The source of variance and comparison between the genotypes were determined. The results were expressed as mean, minimum and maximum values with standard deviations (SD). The comparisons of traits' means were made using Duncan's multiple range test at the probability level of 5%. The data were subjected to statistical analysis by using the statistical program package "XLSTAT 2016 software". The one-way analysis of variance (ANOVA) followed by Duncan multiple range tests was employed. Principal component analysis (PCA) was performed in order to discriminate between different genotypes on the basis of different studied properties. Cluster analysis based on squared Euclidean distance was also performed for the genotypes.

Studied characteristics included physical characteristics of seeds, chemical and physiochemical properties of the extracted safflower seed oil. The examinations were carried out as follows:

A. Physical properties of seeds

Seed volume: a total of 100 seeds were selected randomly from each replicate and spilled into a calibrated cylinder containing a certain amount of water to cover the entire surface of the seeds. For each replication, two volumes of water were recorded; water volume before and after inserting the seeds into the cylinder. The numerical difference between the two readings were recorded as the seed volume (Mohsenin, 1986).

True density: seed weight was divided by its volume and it was expressed based on g/ml (Mohsenin, 1986).

Water absorption capacity (g): a total of 100 seeds from each replicate were weighed and then poured into a flask containing water and adjusted at temperature 22 °C for 12 h until they completely soaked and swelled, then surface water of turgidity seeds was

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removed by absorbent paper and weighed again, and then the numerical difference between the two readings was divided by 100 (Mohsenin, 1986).

Water absorption index (WAI): obtained by dividing the water absorption capacity by the main size (g), as followed by Williams et al. (1983).

1000 seed weight (g): one thousand seed weight was obtained using an electronic balance with an accuracy of 0.001 (MODEL: AND,HR-200, Serial no.12317438, Japan)

Geometric mean diameter (mm): The geometric mean diameter values (Gmd) were calculated according to the following formula (Song and Litchfield, 1991):

$$Gmd = (a * b * c *)^{1/3} \quad \text{(Eq.1)}$$

where *a* is the length, *b* is the width and *c* is the thickness of safflower seeds in mm.

To determine the length, width, thickness, weight, 10 seeds were selected randomly for each replicate. Dimension properties were carried out by using a Vernier Calipers with an accuracy of ± 0.01.

Sphericity %: according to Mohsenin (1986) sphericity of safflower seeds was calculated with the following equation by values of length (L), width (W) and thickness (T):

$$S = ((LWT)^{1/3})100 \quad \text{(Eq.2)}$$

B. Chemical properties of safflower seed oil

Protein%: The Kjeldahl method (BUCHI K-424, Germany) was used to determine protein concentration. A conversion factor *(F)* was used to convert the measured nitrogen concentration to a protein concentration. A conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) was used for this application, however, this is only an average value, and each protein has a different conversion factor depending on its amino-acid composition (Van Dijk and Houba, 2000).

Oil %: seed oil percentage was determined according to William (2000) using Soxhlet apparatus (BUCHI Extraction System B-811 Buchi Labrotechnik AG, CH-9230 Fkawil, Switzerland).

Fatty acid composition (%): the percentage of saturated and unsaturated fatty acids, namely Oleic %, Linoleic %, Linolenic %, Palmitic % and Stearic % were determined using Gas-Liquid Chromatography (Typ: TS 606/3-I, Ser. Nr.:06410001, Liebherr Kuhlsystem FKS 2600, Bruttogehalt 260, Typ:200051), by the method of the International Union of Pure and Applied Chemistry (Paquot, 2013).

Percentage of free fatty acid (FFA%): it was determined in the oil by standard AOCS method (AOCS, 1980).

C. Physicochemical properties of safflower seeds oil

Refractive index, specific gravity, iodine value, pH and peroxide value for safflower seeds oil were determined by method s described by AOCS (1998).

Results and discussion

The analysed results indicated a highly significant variance among the nine safflower genotypes (at 1% and 5% levels of probability) for all physical properties of the seeds,

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except true density. *Table 1* indicates the reasonable diversity between the studied safflower genotypes.

Table 1. *Analysis of variance for physical properties of safflower seed*

Source	df	Mean square						
		Seed volume (ml)	True density (g/ml)	Water absorption capacity (g)	Water absorption index	1000 kernel weight (g)	Geometric mean diameter (mm)	Sphericity %
Genotype	8	0.001**	5732.944	0.0001**	0.058*	82.051**	0.070**	12.382**
Error	18	0.0001	1802.435	0.0001	0.005	1.126	0.167	16.764
Minimum		0.010	43.889	0.009	0.235	32.800	4.369	57.796
Maximum		0.090	328.000	0.026	0.785	49.100	6.117	75.091
Mean		0.048	103.972	0.018	0.436	42.494	4.977	65.201
Std. deviation		0.018	54.880	0.005	0.145	5.102	0.370	3.926

There were also variable significant effects for chemical properties of safflower oil, as indicated in the result of analyzed variance (*Table 2*). Highly significant differences were recorded for oleic%, palmitic%, oil% and protein%, while significant differences at a 5% level of probability were obtained for stearic%, and non-significant difference for linolenic% was indicated for the seed oil from the nine safflower genotypes. The variance analysis results here indicating the presence of significant genetic variability for seed oil characteristics among different safflower genotypes under study.

Table 2. *Analysis of variance for chemical properties of safflower seed oil*

Source	df	Mean square							
		Oleic %	linoleic %	Palmitic %	Stearic %	Linolenic %	Free fatty acid%	Oil %	Protein %
Genotype	8	0.708**	2.414*	0.981**	0.059*	0.007	26.198**	5.517**	0.204**
Error	18	0.002	0.033	0.022	0.023	0.023	0.007	0.009	0.010
Minimum		8.240	72.490	5.480	2.030	0.120	31.150	30.980	16.010
Maximum		9.810	75.990	7.710	2.650	0.750	41.030	34.610	16.980
Mean		9.227	74.166	6.933	2.321	0.397	33.291	32.805	16.361
Std. deviation		0.468	0.875	0.563	0.185	0.133	2.840	1.305	0.265

A similar pattern was also realized for the analysis of physiochemical characteristics, giving high significant variances for peroxide value and pH, while non-significant difference was identified for refractive index and specific gravity, from data obtained from the seed oil of the nine safflower genotypes, as indicated in the result of analyzed variance in *Table 3*.

Table 3. *Analysis of variance for physiochemical properties of safflower seed oil*

Source	df	Mean square				
		Peroxide value	pH	Iodine value	Refractive index	Specific gravity
Genotype	8	0.035**	0.077**	13.430**	0.006	0.0001
Error	18	0.004	0.002	0.009	0.007	0.001
Minimum		1.990	6.830	136.150	0.530	1.434
Maximum		2.370	7.410	142.510	0.986	1.434
Mean		2.220	7.094	139.885	0.924	1.434
Std. deviation		0.115	0.158	2.034	0.081	1.434

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Physical properties

Physical properties measurements of seed are important actions to be considered in the designation of machines and equipment used for the postharvest processing of agricultural products. Seeds from different safflower genotypes had a considerable variable effect on the studied characteristics, except geometric mean diameter and sphericity% (*Table 4*). A maximum seed volume of 0.073 ml was recorded for G-2018, and it was significantly different from all other genotypes. Influencing seed size by genotypes, environment and management practices has been confirmed by researchers (Kaya and Day, 2008; Robinson, 1978). Minimum seed volume was found to be 0.020 ml and recorded for Iden genotype. The seed size has a variable effect on plant growth and postharvest processing. It is realized that smaller seed size to medium has better germination and seedling vigour (Farhoudi and Motamedi, 2010).

Table 4. *Effect of genotypes on physical properties of safflower seed*

Genotype	Seed volume (ml)	True density (g/ml)	Water absorption capacity (g)	Water absorption index	1000 seed weight (g)	Geometric mean diameter (mm)	Sphericity %
G-2018	0.073 a	58.846 c	0.023 a	0.567 b	40.600 bc	4.772 a	62.571 a
Rabiaa	0.048 bc	96.541 bc	0.019 ab	0.409 c	46.533 a	5.137 a	68.505 a
Zaafarani	0.040 cd	96.323 bc	0.020 ab	0.529 b	37.060 d	4.886 a	66.948 a
Al-Mais	0.057 abc	85.238 bc	0.017 b	0.372 c	47.200 a	5.027 a	66.113 a
Ardny	0.060 ab	79.333 bc	0.018 b	0.370 c	47.600 a	5.024 a	66.753 a
Iden	0.020 e	204.33 a	0.024 a	0.704 a	33.800 e	5.117 a	64.923 a
Gilla	0.053 bc	91.211 bc	0.017 b	0.348 cd	48.333 a	4.743 a	62.643 a
Al-Shamia	0.030 de	142.100 ab	0.009 c	0.241 d	39.120 c	5.147 a	64.479 a
Local	0.052 bc	81.818 bc	0.016 b	0.382 c	42.200 b	4.936 a	63.878 a

Different letters for the traits data indicated significant differences according to comparison analysis of Dunkin's for the traits data at a 95% confidence interval

Seeds from Iden genotype had maximum values for true density, water absorption capacity and water absorption index, while for both traits of seed volume and 1000 seed weight this genotype recorded minimum values. True density is negatively associated with a range of moisture content while positively correlated with the increase in porosity of a grain bed (Baümler et al., 2006). The result obtained here for 1000 seed weight is important and in general, it surpassed results from other studies. Pasandi et al. (2018) obtained a range of 28.29-30.33 g when Esfahan cultivar is grown in Iran under full irrigation, while Aktas et al. (2006) identified a higher 1000 seed weight of safflower seed compared to this research. Sphericity% ranged from 62.571 to 68.505%, however, no significant difference was observed between the studied genotypes. A similar trend was observed for geometric mean diameter. Comparable results of 66.03 to 64.43% of sphericity for safflower seed was obtained by Martins et al. (2017).

Chemical properties

In all the genotypes safflower seed oil varied in chemical properties, except for Linolenic acid (*Table 5*). The highest oleic% and palmitic% were recorded for the Local genotype. A high percent of linoleic acid (75.69) was referred to Rabiaa genotype, while Zaafarani had the maximum amount of free fatty acid%, however, this is slightly lower than the percentages obtained by Mihaela et al. (2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75877605

Table 5. *Effect of genotypes on chemical properties of safflower seed oil*

Genotype	Oleic%	Linoleic%	Palmitic%	Stearic%	Linolenic%	FFA%	Oil%	Protein%
G-2018	8.260 h	74.480 b	5.680 d	2.190 b	0.320 a	31.590 f	33.690 bc	16.250 cd
Rabiaa	9.120 e	75.690 a	6.990 b	2.350 ab	0.450 a	32.960 b	32.360 d	16.350 bc
Zaafarani	8.880 g	73.580 cd	6.790 b	2.440 ab	0.350 a	41.020 a	31.250 e	16.020 e
Al-Mais	9.230 d	72.590 e	7.410 a	2.393 ab	0.410 a	32.130 e	33.580 c	16.410 bc
Ardny	9.760 a	73.450 d	7.350 a	2.590 a	0.360 a	31.250 g	31.000 f	16.130 de
Iden	8.980 f	74.580 b	6.903 b	2.330 ab	0.380 a	32.480 d	33.690 bc	16.470 b
Gilla	9.560 b	74.550 b	7.340 a	2.170 b	0.450 a	32.740 c	33.850 b	16.350 bc
Al-Shamia	9.470 c	74.690 b	6.490 c	2.250 b	0.410 a	32.770 c	34.580 a	16.950 a
Local	9.780 a	73.880 c	7.440 a	2.180 b	0.440 a	32.680 c	31.247 e	16.320 bc

Different letters for the traits data indicated significant differences according to comparison analysis of Dunkin's for the traits data at a 95% confidence interval

Higher amount of oleic and linoleic acid in the safflower oil under study, compared to other results, indicate their high usability in food preparation (Khalid et al., 2017), causing the decrease of fat accumulation rate and the diminishing of bodyweight (Norris et al., 2009). A high level of linoleic acid, an essential fatty acid, makes the oil a premium edible oil, because of its nutritional advantages and potential therapeutic properties in the prevention of coronary heart disease and cancer, however the presence of the large amounts of linoleic acid makes the oil quite sensitive to oxidation (Oomah et al., 2000). Palmitic acid was the major saturated fatty acid ranged from 5.680-7.440%, followed by stearic acid (2170.2.590%). Linoleic acid is the principal fatty acid having the range of 72.590-75.690%, followed by oleic acid as the second main fatty acid (8.260-9.770%). Al-Shamia recoded the minimum ratio of palmate compared to all other genotypes, which is considered to be preferable for direct using in food preparation, as low intakes of saturated fatty acids have been associated with decreased blood cholesterol levels. High blood cholesterol level is one of the factors associated with heart diseases (Al Surmi et al., 2016). There is no significant difference between the genotypes for linolenic acid, their ranges varied from 0.32-0.45%.

The ration of linoleic acid, palmitic acid and stearic acids in this study are in accordance with those obtained by Tinctorius (2011) when four common safflower cultivars were studied in Iran, however the oleic acid of the current genotypes ranged less compared to the Iranian cultivars. Similar results of chemical composition were also identified by other researchers who worked on two cultivars of high oleic safflower seeds (Salaberría et al., 2016).

Fatty acid compositions of safflower oils analysed here are similar to those indicated by some other researchers (Rafiquzzaman et al., 2006; Bozan and Temelli, 2008; Yeilaghi et al., 2012), however, oleic acid has less rate compared to 13.75% which obtained by Katkade et al. (2018). The greater amount of linoleic acid increased the oil quality as it can facilitate digestion and blood de-aggregation (Aşkın, 2018).

The fatty acid composition of vegetable oil is the main factor affecting its commercial uses. It is influenced by genotype and environmental conditions (Gecgel et al., 2007). In addition, fatty acid composition affects the taste and chemical quality of the oil (Aşkın, 2018). The free fatty acid percentage of the current study ranged from 31.59% to 41.02 for G-2018 and Zaafarani genotypes, respectively. Fatty acid composition of the oil varies with plant species, cultivar, and growing conditions (Kostik et al., 2013; Sabzalian et al., 2008). Free fatty acid is also a critical acid value to

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estimate the quality of oil, affecting the oil implication for industrial and domestic uses. It is preferable to be at a low rate, as it indicates the extent of triglyceride hydrolysis to produce mono and di-glyceride (Khalid et al., 2017). The ratios obtained here for different genotypes are lower compared to the ration indicated by other researchers (Ben Moumen et al., 2013). The functionality of oilseeds in industrial, pharmaceutical and food products depends upon their fatty acid composition. In the current study, the highest oil percent of 34.58 refers to Al-Shamia. This could be due to decreasing the shell ration in this genotype making the oil content increase (Applewhite, 1966). The observed values for oil contents in this study were in close range to those reported previously (Çamaş et al., 2007; Esendal et al., 2008; Ashrafi and Razmjoo, 2010; Pasandi et al., 2018), however obtained oil content value for the safflower genotypes were much less than 61.50% that obtained by Salazar Zazueta and Price (1989). Oil percent is ranging from medium to high percent (20-45%) based on the genotype and environmental condition (Liu et al., 2016).

Al-Shamia recorded the highest protein ratio (16.950). Protein ratio was also reasonable in other genotypes such as Iden, Al-Mais, Rabiaa genotypes. In general protein percent of the current study is reasonable for safflower genotypes and it is in accordance to what was obtained by others ranging from 14.70% to 16.21% (Al Surmi et al., 2016).

Physiochemical properties

Peroxide values (PV) for different safflower seed oil samples ranged from 2.00 and 2.360 meq O_2/Kg (*Table 6*). The highest value was 2.360, which was recorded for genotype G-2018. Peroxide value is a crude indicator of the amount of primary oxidation of lipids (Vossen, 2007). The PV is, in fact, a measure of the amount of the hydroperoxide formed through oxidation during storage (Cosio et al., 2006). Peroxide determination is important to set the degradability of raw material for biofuel production (de Oliveira et al., 2018). It is a measure of oxidation during storage and the freshness of the lipid matrix. High peroxide value is an indicator of oxidation level, the greater the peroxide value, the more oxidized oil is present (Atinafu and Bedemo, 2011).

Table 6. *Effect of varieties on physicochemical properties of safflower seeds oil*

Varieties	Peroxide value	pH	Iodine value	Refractive index	Specific gravity
G-2018	2.360 a	6.980 de	136.350 h	0.984 a	1.466 a
Rabiaa	2.237 c	7.023 d	137.560 g	0.924 ab	1.465 a
Zaafarani	2.000 d	6.870 f	141.360 c	0.928 ab	1.467 a
Al-Mais	2.140 c	7.120 c	142.480 a	0.927 ab	1.467 a
Ardny	2.350 ab	7.210 b	139.690 d	0.931 ab	1.455 a
Iden	2.250 bc	7.220 b	139.470 e	0.942 ab	1.458 a
Gilla	2.213 c	7.360 a	141.407 c	0.949 ab	1.459 a
Al-Shamia	2.230 c	7.150 bc	138.580 f	0.815 b	1.469 a
Local	2.200 c	6.913 ef	142.070 b	0.912 ab	1.454 a

Different letters for the traits data indicated significant differences according to comparison analysis of Dunkin's for the traits data at a 95% confidence interval

The pH of the seed oil is also varied significantly for the different safflower genotypes, Gilla oil had the highest pH value, while Zaafarani had the lowest record. There is an

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effect of pH on the hydrolysis of safflower oil, that is considered during oil extraction. The increased pH value will certainly decrease the degree of hydrolysis (Aziz et al., 2015), by breaking down the substrate (Goswami et al., 2009), through modifying the ionization state of enzyme and altering the activity of an enzyme (Serri et al., 2008).

Iodine value ranged from 136-142 g I_2/100 g oil). The highest iodine value was recorded for Al-Mais (142.480), whereas the lowest iodine value (136.350) was recorded for the genotype G-2018. The obtained results were agreed with the range of 130-150 g I_2/100 g oil which was reported previously by Nagraj (1995) and the range of 136-148 g I_2/100 g oil that was reported by Alimentarius (2013). Higher iodine value indicates a lower degree of saturation and vice versa. This value could be used to quantify the number of double bonds present in the oil, which signifies the susceptibility of oil to oxidation (Dim et al., 2013).

Refractive index and specific gravity measurements are not providing sufficient information for quantitative detection of a pure analyte, however they are highly useful to check oil contamination and/or adulteration (Bhavsar et al., 2017). The refractive index is used mainly to measure the change in the unsaturation of the oil. The refractive index ranged from 0.984 to 0.815 and specific gravity ranged from 1.454 to 1.469. These results are similar to those obtained by Nagraj (1995) and Alimentarius (2013) for safflower seeds oil, but in the current study the oil has a lower refractive index compared to the ranges obtained by Katkade et al. (2018).

Principal component analysis

In addition to exploring the variation between different genotypes based on different physical, chemical and physiochemical characteristics, principal companion analysis was performed to estimate the relative importance and contribution of each genotype to the total variance and illustrate their relatedness. Superiority of safflower seed from the nine genotypes for various traits was explained in the PCA diagram for some physical properties (*Fig. 1*).

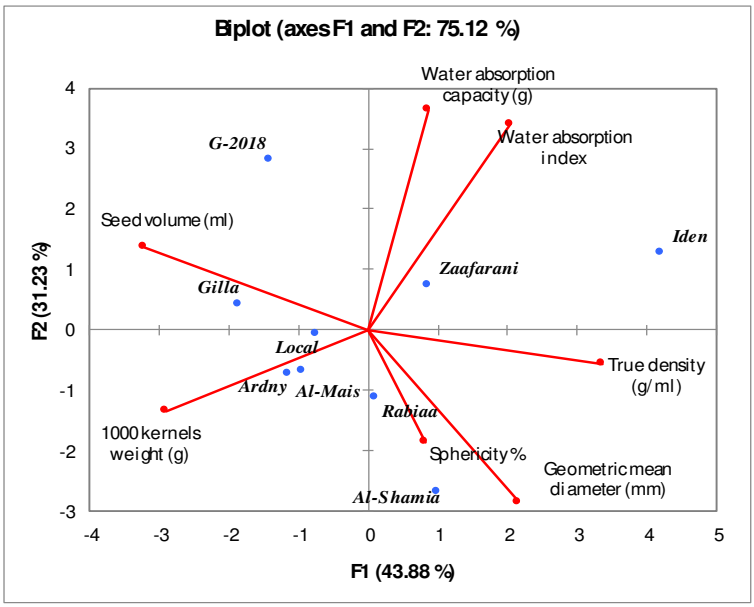

Figure 1. *Biplot diagram of principal component analyses of the first and second components for the distribution of 9 safflower genotypes, based on some physical properties*

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The first two principal components were accounted for 75.12% of the total variations between different genotypes. The biplot indicates the variation derived from the first and second factors (F1 and F2) for seed physical data of all nine safflower genotypes. Al-Shamia and Rabiaa seed oil were superior in sphericity % and geometric mean diameter (mm), while for Gilla and G-2018 seed oil low value was recorded for these two traits, as positioned away from the scatter point of these traits. Iden on the diagram showed a high contribution to true density while it reversely indicates low seed volume, being preferable for the seed germination and manufacturing processes. The attributed variations among these genotypes might partially reflect their different backgrounds at a genetic level (Mohsenin, 1986).

In studying the chemical characteristics of seed oil from different safflower genotypes, the principal component analysis was accounted for 67.1% of the total variation for different genotypes under study (*Fig. 2*). Al-Shamia was found to be superior in protein percent 16.950% followed by the seed oils from Gilla and Rabiaa genotypes. Zaafarani has recorded a minimum value for protein content, however it has the maximum FFA%. In addition, the Local genotype is superior in oleic% and palmitic%, recording maximum values of 9.780% and 7.440%, respectively (*Table 5*). While G-2018 has the minimum value for oleic%, unsaturated fatty acid, and palmitic%, saturated fatty acid. Linoleic acid was superior in Rabia genotype, followed by Al-Shamia, and Iden. Percentage of palmitic and stearic acids in safflower oil are among the factors to determine its quality, however oleic and linoleic acids are significantly affecting oil quality more than the others, due to their direct effects on human health. Fatty acid composition is varied according to genotypes and environmental condition of their growing (Kostik et al., 2013; Pasandi et al., 2018; Oz, 2016; Yorulmaz et al., 2019).

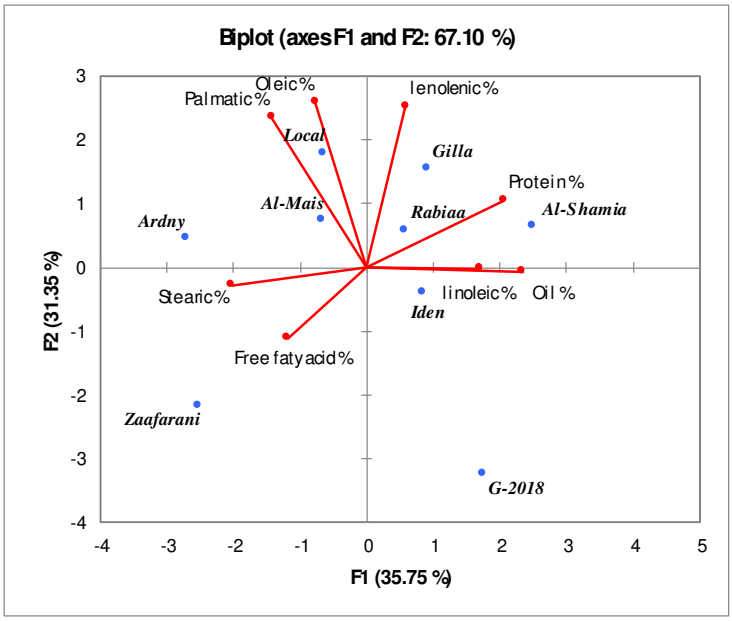

Figure 2. *Biplot diagram of principal component analyses of the first and second components for the distribution of 9 safflower genotypes, based on some chemical properties*

PCA findings, variations in oleic and linoleic acid contents emphasized the possibility of improving oil quality through breeding or cultivation programs for the

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specified genotypes. As fatty acid ratios are fundamental for the market value of safflower, it is reasonable to suppose that genotype with the highest content of oleic and linoleic acids, such as Local and Rabiaa could be considered as valuable genetic material for the improvements in safflower oil quality in Kurdistan-Iraq region.

Biplot-PCA for physiochemical properties indicates the presence of high genetic variations among different oil seeds from the nine safflower genotypes (*Fig. 3*). According to the result of principal component analysis, variable components accounted for 65.63% of the total variation for physiochemical properties. Oil from the seed of Iden genotype is superior in refractive index and pH, followed by Ardny and Gilla while Al-Shamia has recorded lower value for both refractive index and pH of the oil. In addition, Al-Shamia shows a maximum value for specific gravity while the minimum value was identified for Gilla. The result of the present study could be exploited in planning and execution of the future breeding programs of safflower genotypes.

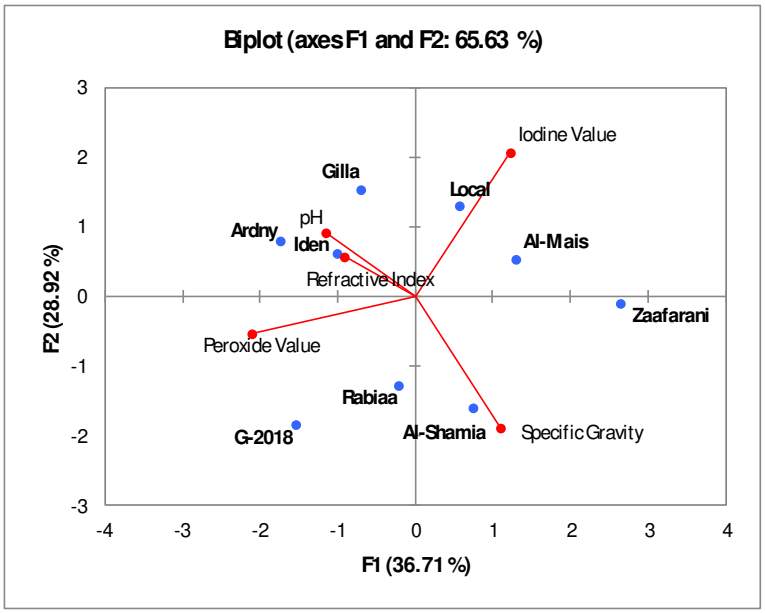

Figure 3. *Biplot diagram of principal component analyses of the first and second components for the distribution of nine safflower genotypes, based on some physiochemical properties of the seed oil*

Agglomerative hierarchical clustering using physical, chemical and physiochemical data

Pairwise comparisons were made between all the nine safflower genotypes and average dissimilarity values were calculated based on the characteristics data. All physical, chemical and physiochemical characteristics data from the safflower genotypes were utilized to evaluate the relationship between the genotypes.

For the physical characteristics of the seed oil, the dissimilarity matrix revealed variable values ranged from 6.604 (between Local and Al-Mais) to 145.665 (between Iden 157 and G-2018), as given in *Table 7*. This wide range of dissimilarity between different genotypes indicates the presence of reasonable variability among the genotypes under study. Hybridization program would be reasonable if conducted between the genotypes with high dissimilarity value.

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Table 7. *Dissimilarity matrix among the seeds of nine safflower genotypes based on physical properties, following Euclidean distance proximity of Ward's method analysis*

	G-2018	Rabiaa	Zaafarani	Al-Mais	Ardny	Iden	Gilla	Al-Shamia	Local
G-2018	0								
Rabiaa	38.619	0							
Zaafarani	37.898	9.607	0						
Al-Mais	27.436	11.573	15.048	0					
Ardny	22.052	17.330	19.996	5.953	0				
Iden	145.665	108.602	108.079	119.853	125.773	0			
Gilla	33.277	8.134	13.107	7.006	12.593	114.076	0		
Al-Shamia	83.290	46.334	45.891	57.457	63.378	62.464	51.750	0	
Local	23.066	16.031	15.693	6.458	6.604	122.808	11.288	60.364	0

For better estimation of the distance between the seed oil of all the safflower genotypes Euclidean distance and unweighted pair-group average were followed to estimate dissimilarity (*Table 8*). The dissimilarity values varied from the lowest value of 0.816 (between Gilla and Iden) to the highest value of 9.87 (between Zaafarani and G-2018).

Table 8. *Dissimilarity matrix among nine safflower genotypes based on chemical properties of safflower seed oil, following Euclidean distance proximity of Ward's method analysis*

	G-2018	Rabiaa	Zaafarani	Al-Mais	Ardny	Iden	Gilla	Al-Shamia	Local
G-2018	0								
Rabiaa	2.760	0							
Zaafarani	9.870	8.419	0						
Al-Mais	2.808	3.462	9.279	0					
Ardny	3.692	3.232	9.831	2.928	0				
Iden	1.699	1.811	8.951	2.103	3.319	0			
Gilla	2.414	1.979	8.787	2.111	3.439	0.816	0		
Al-Shamia	2.202	2.590	9.040	2.652	4.275	1.240	1.285	0	
Local	3.598	2.294	8.429	2.787	1.584	2.734	2.700	3.630	0

The dissimilarity matrix for physiochemical properties shows lower different distances between the safflower genotypes (*Table 9*). The lowest dissimilarity distance value was 0.242 (between Iden and Ardny), while the highest distance value was 6.136 (between Al-Mais and G-2018). It is identified that G-2018 shows a wide distance fromother genotypes based on all physical, chemical and physiochemical properties.

Hierarchical cluster analysis (HCA) is an unsupervised technique utilized to cluster genotypic data. HCA was applied to cluster seed oil from nine safflower genotypes available in Kurdistan Region according to different characteristics of physical, chemical and physiochemical properties. Genetic divergence was investigated successfully in safflower genotypes using cluster analysis based on some agronomic and oil content characteristics (Atole et al., 2018; Shinwari et al., 2014; Sabaghnia et al., 2018).

Clustering analysis, based on physical properties, revealed two major groups at the dissimilarity based on the threshold value for physical properties (*Fig. 4*). These groups had a variance decomposition between the classes and variance within the class. The first cluster comprised of the genotypes; G-2018, Local, Al-Mais, Ardny, Zaafarani, Rabiaa and Gilla). While in the second group Iden and Al-Shamia were

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clustered together. This will clarify the variation between the genotypes for any improving program in the future to select the right genotypes based on the purpose of development.

Table 9. *Dissimilarity matrix among nine safflower genotypes based on physiochemical properties of safflower seed oil, following Euclidean distance proximity of Ward's method analysis*

	G-2018	Rabiaa	Zaafarani	Al-Mais	Ardny	Iden	Gilla	Al-Shamia	Local
G-2018	0								
Rabiaa	1.218	0							
Zaafarani	5.024	3.810	0						
Al-Mais	6.136	4.922	1.156	0					
Ardny	3.348	2.141	1.740	2.799	0				
Iden	3.131	1.920	1.938	3.014	0.242	0			
Gilla	5.074	3.862	0.537	1.102	1.729	1.942	0		
Al-Shamia	2.247	1.034	2.806	3.903	1.124	0.902	2.838	0	
Local	5.723	4.512	0.739	0.464	2.403	2.619	0.801	3.500	0

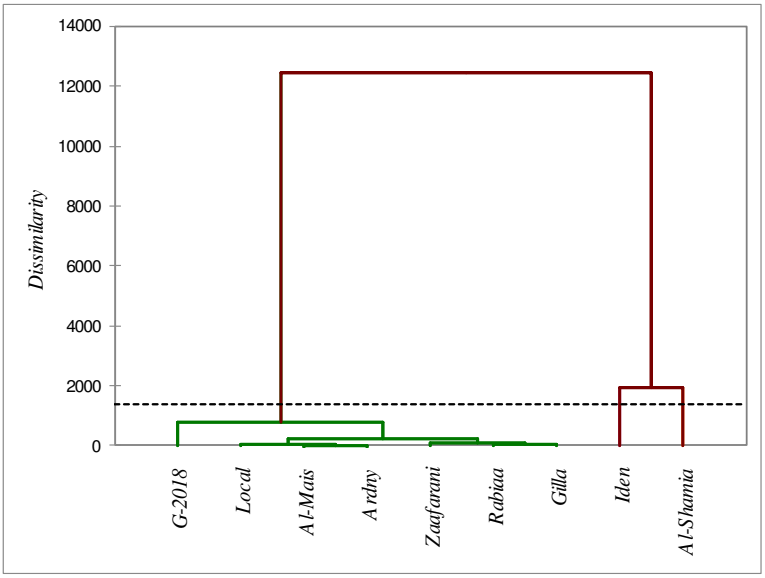

Figure 4. *Dendrogram generated using Unweighted Euclidean distance proximity of Ward's method analysis of dissimilarity, showing the distance between safflower genotypes using physical data of safflower seed. Dissimilarity values are present at the left side of the dendrogram*

The dendrogram was generated by UPGMA clustering pattern of nine safflower genotypes using chemical properties (*Fig. 5*). The dendrogram clearly revealed four clusters based on the threshold value. Zaafarani is different from the others occupying a separate group. Ardny and Local genotypes were clustered together making another group. Al-Mais made a third group, while the fourth cluster comprised of the genotypes; Rabiaa, G-2018, Al-Shamia, Iden and Gilla. Research institutes should take

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consideration of the necessity for the collection, conservation, and utilization of local genotypes. The necessity of such action has been highlighted in this study by giving a reasonable extension to the safflower gene pool through the contribution of indigenous genetic resources such as the Local genotype.

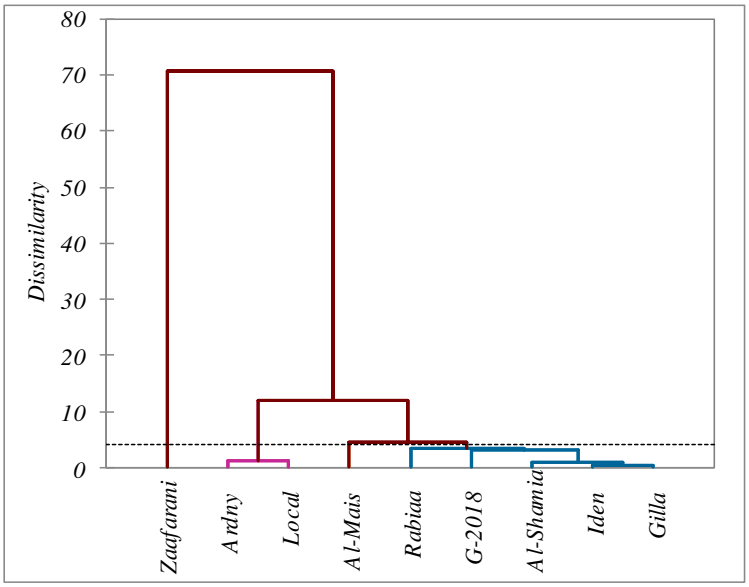

Figure 5. *Dendrogram generated using Unweighted Euclidean distance proximity of Ward's method analysis of dissimilarity, showing the distance between safflower genotypes using chemical data of safflower seed oil. Dissimilarity values are present at the left side of the dendrogram*

Pairwise comparisons were made between all the nine genotypes and the average dissimilarity values were calculated based on the physiochemical properties, three groups in the dendrogram were clustered based on the threshold level (*Fig. 6*). The first group contains Al-Mais, Local, Zaafarani and Gilla, while both Ardny and Iden made the second group, the third cluster consists of the genotypes; G-2018, Rabiaa and Al-Shamia.

The analyses accomplished here indicate the genetic distinction between the safflower genotypes distributed over different clusters, implying high potential of the genotypes in improvement programs, as some genotypes showed superiority in most of the studied parameters. The distance between safflower genotypes using physical, chemical and physiochemical data of safflower seeds oil indicate the extension of genetic bases for the genotypes under study to be manipulated in their utilizing based on the required purposes for the seed oil in safflower. To reduce the risk of bottlenecking the diversity in safflower, adopting further genotypes with wide diversities have to be involved in the future improvement programs of this crop.

No distinct regional grouping patterns of these safflower genotypes were clearly identified group clusters, because the origins of the entire genotypes are not presented herein this investigation. The results obtained from the genotype clustering would be valuable for plant breeders, whereby the most promising genotypes in the population could be selected from different clusters for the improvement of safflower based on the required characteristics.

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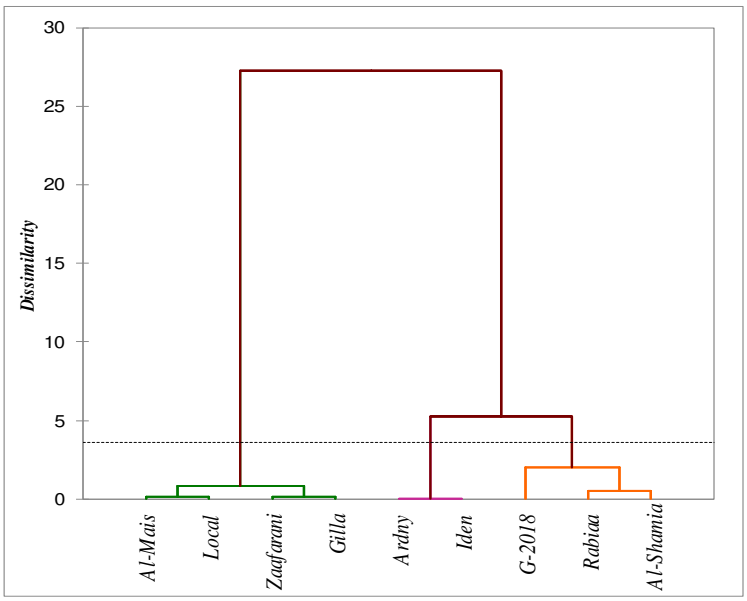

Figure 6. *Dendrogram generated using unweighted Euclidean distance proximity of Ward's method analysis of dissimilarity, showing the distance between safflower genotypes using physiochemical data of safflower seed oil. Dissimilarity values are present at the left side of the dendrogram*

Conclusions

The analyses of variance indicated highly significant variances (at 5% and 1% level of probability for most of the studied traits (physical, chemical and physiochemical). Safflower genotypes are different in their response to physical properties of seed except for geometric mean diameter and sphericity%. A maximum seed volume of 0.073 ml was recorded for G-2018, which was significantly different from all other genotypes. Physical properties of safflower seeds are essential for designing types of equipment for handling, harvesting, storing and oil extraction process. These properties are affected by numerous factors such as genotypes and environmental conditions of growing.

The different safflower genotypes varied in their chemical properties for seed oil, except for linolenic acid. The highest oleic% was identified for Local genotype and Ardny (being non significantly different), while high percent of linoleic acid was referred to Rabiaa genotype, indicating their high usability in food preparation because they can decrease fat accumulation rate and diminish body weight. Al-Shamia is considered to be preferable for direct use in food preparation, due to containing the lowest ratio of palmate. Free fatty acid content was variable and ranged from 31.59% to 41.02 for G-2018 and Zaafarani genotypes, respectively. This is a critical value to estimate the quality of oil for industrial and domestic uses. Protein percentage was reasonable in different genotypes such as; Al-Shamia, Iden, Al-Mais and Rabiaa. Peroxide value as a measure of oxidation during storage ranged from 2.00 and 2.360 meq O_2/Kg. There are also significant differences in pH and iodine values for the different genotypes. G-2018 had the lowest iodine value of 136.350 g I_2/100 g oil, which means that the oil has a high saturation value. Palmitic and stearic acid were identified to be low in Al-Shamia and Local genotypes. The lower saturated fatty acid form in these two genotypes indicates a high stability oil with superior quality for edible

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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purpose and commercial applications. The oil obtained from safflower varieties is a relatively rich source of various important functional nutrients.

The presence of variability is important for genetic studies and consequently improvement and selection. Principal companion analysis was performed to estimate the variability of genotypes and their contribution to total variance based on the traits studied. The first two principal components were accounted for 75.12%, 67.1% and 65.63% of the total variation based on physical, chemical and physiochemical properties. The attributed variations among the genotypes based on physical properties might partially reflect their genetic construction. The variation realized via PCA for oleic and linoleic acid content could make a possibility of improving the oil quality through the development programs of safflower. Focusing on Local and Rabiaa genotype, will be reasonable, in the future improvement program of seed oil quality in Kurdistan-Iraq.

Dissimilarity matrix specified G-2018 genotype with a wide distance from other genotypes based on all the physical, chemical and physiochemical properties. Hybridization program would be reasonable for oil quality improvement if conducted between the genotypes with high dissimilarity value. Genetic divergence was investigated successfully in the current safflower genotypes based on hierarchical cluster analysis. With the help of clustering pattern and genetic relationship, breeders could identify the diverse genotype with least similarity from clusters and employ them in future breeding programs of safflower, because despite the nutritional value of safflower seed oil there is an increased potential in medicinal purposes of seed oil at global level. Based on the current study it is recommended to include wider genotypes of safflower in the improvement program of the seed oil and its quality based on the recommended references. Different field trial and agricultural practices are recommended before evaluating the oil quality parameters. In addition, oleic and linoleic oils have to be investigated in the future for thermal stability and frying qualities.

Acknowledgments. The authors are grateful for the College of Agricultural Engineering Sciences, the University of Sulaimani for providing all laboratories facilities and for providing land to conduct the research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_75877605

OPTIMIZATION OF PO_4^{2-} AND NO_3^{2-} CONCENTRATION IN BENTHIC EPILITHIC DIATOM CULTURES AS BIOLOGICAL INDICATORS IN PREDICTING THE TROPHIC STATUS OF THE SWARTPRUIT RIVER, SOUTH AFRICA

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(Received 30th Apr 2020; accepted 11th Aug 2020)

Abstract. A study probing the optimization of PO_4^{2-} and NO_3^{2-} concentrations in benthic epilithic diatom cultures as biological indicators in predicting the trophic status and water quality conditions of the Swartpruit River was undertaken. Diatom materials were collected from November 2014 – February 2015 from the river and cultivated in an optimized growth medium with 0.003, 0.005, 0.01, 0.015, 0.2, 0.025, 0.05, 0.1, 0.15, 0.2, .025, 0.3, 0.4 mg/L PO_4^{2-} and 0.1, 0.3, 0.5, 1.0, 2.0, 2.5, 5.0, 7.0, 9.0, 10, 12, 13 mg/L NO_3^{2-}) amounts, respectively. The results revealed that optimization of PO_4^{2-} and NO_3^{2-} concentrations in the growth media encouraged corresponding fluctuations in the taxonomic profiles and abundance of diatom assemblage cultures and was plausibly applied in determining the trophic status and water quality condition of the river. Principal component analysis was used to evaluate the relationship between diatom diversity and optimized phosphate and nitrate concentrations in an ordination biplot revealed 45.62% and 37.93 % variations in the data. We anticipate that the methodology and data analysis presented in this report could be applied in predicting the trophic status and water quality of any inland freshwater resource like the Swartspruit River, which undergoes seasonal harmful algal bloom.
Keywords: *diatom assemblages, water quality, harmful algal bloom, taxonomic profiles, inland freshwater resource*

Introduction

Diatoms are pivotal in the food web and have a tight link to biochemical fundamental processes like photosynthesis and respiration in aquatic environments (Wong, 2014; Torres et al., 2014; Barinova et al., 2019; Zhang et al., 2020). They are important primary producers and represent an important source of food for aquatic grazing organisms (Maria et al., 2011; Estifanos et al., 2013; Shinneman et al., 2016; Trobaja and Mann, 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76077625

Diatoms occupy a unique niche in the aquatic ecosystem, requiring nutrients for growth and reproduction (Dong et al., 2012; Muhid et al., 2013; Barinova et al., 2019). They are widely diverse and abundant in the freshwater environments as some species might require different nutrient compositions from others (Lynn et al., 2000; Taylor et al., 2007; Kociolek et al., 2014; Trobaja and Mann, 2019; Zhang et al., 2020) as all the resources required for the growth of diatom assemblage cells are restricted to specific biochemical roles (Wear et al., 2015; Barinova et al., 2019). The trophic status of a freshwater resource refers to the total weight of algal biomass. As a consequence, algal biomass has been identified as the most important effect that determines the composition of diatom species in freshwater ecosystems (Poulíčková et al., 2004; Klose et al., 2012; Barinova et al., 2019).

The concentration of nutrients like nitrate (NO_3^{2-}) and phosphate (PO_4^{2-}) are important variables, which aid the growth of diatoms (Kwon et al., 2013; Shinneman et al., 2016; Trobaja and Mann, 2019; Zhang et al., 2020). These are major factors which regulate the trophic status in aquatic ecosystems (Mensah et al., 2013; Trobaja and Mann, 2019). Phosphates are amongst the limited nutrients in the freshwater ecosystems and causes significant growth amongst diatoms (Winter and Duthie, 2000; Shinneman et al., 2016; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020). The bio-accumulation in aquatic ecosystems has been reported to stem from animal wastes, fertilizers, sewage, detergents and other land use patterns (Walsh and Wepener, 2009). On the other hand, the bio-accumulation of nitrates in aquatic ecosystems originate from common forms of nitrogen available in the freshwater ecosystems. Reports (Giles, 2005) suggest that they are made available in the freshwater environment by the conversion of nitrogen gas (N_2) by algae or legumes into nitrate (NO_3^{2-}) or by oxidation of ammonium gas (NH_4^+) into nitrite (NO_2^-) and to nitrate by bacteria. The depletion of phosphates and nitrates in freshwater ecosystems brings about perturbation in diatom communities which directly affect their productivity (Kwon et al., 2013; Shinneman et al., 2016; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020). Diatoms are frequently used as biological indicators of trophic status and water quality conditions in rivers (Bellinger et al., 2006; Shinneman et al., 2016; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020). Their application as bio-indicators in artificial or controlled environment has received little or no attention from researchers in recent years. The current study aims at monitoring the growth pattern of sampled benthic epilithic diatom assemblages in controlled optimized (PO_4^{2-} and NO_3^{2-}) amounts in algal growth media to ascertain if their diversity provides a better insight to their response to different nutrient concentrations. The study also aims at predicting the trophic status and water quality conditions of a freshwater resource like the Swartspruit River in the North West Province, South Africa by the controlled optimization of phosphate and nitrate (PO_4^{2-} and NO_3^{2-}) concentrations in benthic epilithic diatom assemblage cultures under laboratory conditions. To the best of our knowledge, little or no relevant information currently exist in the literature that reports the controlled optimization of PO_4^{2-} and NO_3^{2-} amounts in benthic epilithic diatom assemblage cultures in predicting the trophic status and water quality conditions of this surface freshwater reservoir. Our findings demonstrates that controlled optimization of PO_4^{2-} and NO_3^{2-} amounts in diatom assemblage cultures can be applied in predicting the trophic status and water quality conditions of the Swartpruit River, which is a very important inland freshwater resource available to inhabitants in the Greater Hartebeespoort Metropolitan Area in North West Province, South Africa. We are of the anticipation that the findings presented in this report can have a significant impact in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76077625

integrating new knowledge with existing knowledge (if any) to gain a better insights into the state of water quality and safety around and downstream of the Swartspruit River, North West Province, South Africa.

Materials and Methods

Study Site

The Swartspruit River is located in Greater Hartbeespoort Metropolitan Area, North West Province, South Africa. The study sites include four station points, two located near the upstream water intake of the Jukskeispruit tributary JKSTT (-25º74'9426"S +27º90'4568"E), the upstream water intake of the Rietvlei stream RTVLS (-25º75'1059" +27º90'2755"E), the upstream water intake of the Kaalspruitfontein stream KALTS (25º74'9426"S +27º90'4568"E) and the upstream water intake of the Modderfontein stream MDFTS (-25º74'8338"S +27º90'6078"E). The site selection were based on; (a) areas in the river that could easily be reached by either boat or by walking along the banks, (b) relative distance to fishing and animal conservation area, (c) relative distance to nearby water treatment facility, and (d) proximity to the Reinting waste water treatment plant. The sites were also chosen to be not less than ten meters (10 m) apart from one another and were located on areas with cobbles and boulders that are potential habitats for benthic epilithic diatoms (Desrosiers et al., 2013).

Sample Collection of Diatom Materials

Benthic epilithic diatom assemblage materials were collected in November 2014 - February 2015 from the river. Samples were collected not more than 10 meters from banks/littoral zone of the river. Water sample of about one litre was collected from each sample location in glass bowl. Five cobles/boulders within diameters $> 64 \leq 256$ mm were sampled for benthic epilithic diatom materials. Other debris attached to the stones were cautiously removed by agitating the stone in the water. With the aid of a brush, attached diatoms were brushed into the water sample placed on the bowl. Diatom material samples were then transferred to sample bottles and labelled accordingly with sample name, location and date.

Preparation of WC Growth Media

WC trace element solution was prepared by dissolving 0.436 g Na_2EDTA and 0.315 g of $FeCl_3$ in thirty milliliter (30 mL) of distilled water. Other micronutrients were added according established protocols. The solution was then transferred to a 100 mL volumetric flask and made up to volume with distilled water. For the preparation of each vitamin solutions, 2.4 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was dissolved in 200 mL of deionized water. Subsequent amount (in grams) of vitamins; B_{12} (0.027), Thiamine (0.067) and Biotin (0.005) were added and allowed to completely dissolve (Geren et al., 2020).

Cultivation of Diatom Assemblages in Control Optimized PO_4^{2-} and NO_3^{2-} Growth Media

For the preparation of one liter (1L) WC medium, approximately 900 mL of deionized water was measured in a measuring cylinder and transferred to a beaker. Then 1 mL each of the components ($NaNO_3$, $CaCl_2$, $MgSO_4$, $NaHCO_3$, $NaSiO_3$, K_2HPO_4 and H_3BO_3) with

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76077625

the exception of vitamins which were added in an optimized order while stirring. The pH of the medium was adjusted to 7.8 accordingly. The volume of the solution was made up to one liter with deionized water. The vitamin solutions were added with a dropper and the mixture was allowed to mix properly. The medium was filter sterilized using a 0.45 μm Millipore filter. Diatoms were then cultured in a volume of 20:180 mL of inoculum to medium. The diatom cultures were incubated for seven days at 20 ± 2°C under illumination. Cultures from the control experiment was then used as inoculum in the optimized PO_4^{2-} and NO_3^{2-} media. The cultures were in triplicates, in a volume of 20:180 mL of inoculum to medium. The diatom cultures were incubated for seven days at 20 ± 2°C under illumination before characterization. The amounts of phosphate and nitrate used in the P1 –P12 and N1– N12 treatments were (0.003, 0.005, 0.01, 0.015, 0.025, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4) and (0.1, 0.3, 0.5, 1.0, 2.0, 2.5, 5, 7, 9, 10, 12, 13) milligram per liter, respectively.

Morphological Characterization of Sampled and Cultivated Diatoms

The collected diatoms material samples were initially examined with the microscope to establish if the samples contained live diatom cells prior to cleaning. Aliquots of about ten millilitre (10 mL) of samples were used for microscopic characterization. The diatoms cells were cleaned using hot HCl and $KMnO_4$ in order to completely remove organic matter present in sample and to dissolve any residual calcium carbonate (Trobajo et al., 2019). Cleaned diatom samples were mounted for microscopic characterization using Naphrax mounting medium (refractive index 1.69) obtained from Brunel microscope Ltd (Shinneman et al., 2016). Diatom valves mounted on slides were identified and counted using an oil immersion lens at 1000x magnification with a light microscope (Motic BA210) equipped with incident light. The field of view was used as the area defining the limits of the counts. All diatoms visible in the field of view were identified and counted before moving to the next field of view (Garvetto et al., 2018). Each individual specimen was counted as a single unit, without differentiation between a valve and frustules (Moorhouse et al., 2018). A minimum of 400 valves were counted and identified for each samples. The morphological identification of diatoms was done according to established protocols (Taylor et al., 2007; Zimmermann et al., 2015).

Statistical Analysis

Using XLSTAT software version 2016 (Addinsoft to Microsoft Excel 2016, New York, USA) principal component analysis (PCA) was performed to determine the relationship between optimized PO_4^{2-} and NO_3^{2-} amounts and diatom assemblage community compositions. Interpretation was done in angles between variables and between variables and PCA dimensions. Narrow angles in the biplots represented positively linked variables, right angles depicted variables that were unrelated to each other and obtuse angles represented negative relationship.

Results

Taxonomic Profiles and Abundance of Diatom Assemblages in Control Growth Media

Figure 1 denotes the taxonomic compositions and abundance of diatom assemblages in control culture. Benthic epilithic diatom specific counts and overall abundance of assemblages morphologically identified in the control culture are represented in the table.

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A total of 47 different species belonging to twenty 25 genera were identified in the control culture. The composition of individual species varied amongst taxa. *Nitzchia linearis*, *Nitzchia perspicua, Rhopalodia operculata* and *Triblionella gracillis* species had the minimum composition while *Aulacoseira granulata var.augustissima* had the maximum counts.

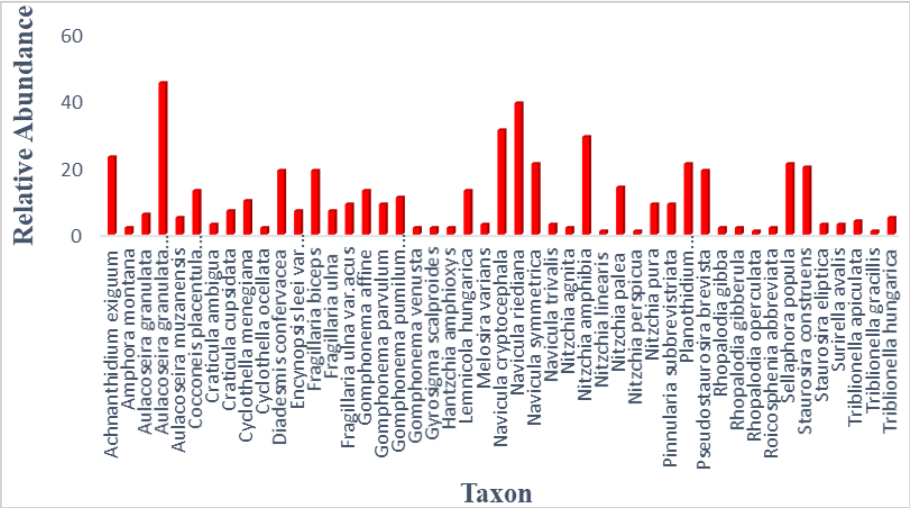

Figure 1. *Taxonomic profiles and abundance of diatom assemblages in control growth media*

Taxonomic Profiles and Abundance of Diatom Assemblages in Optimized PO_4^{2-} Growth Media

Figures 2 and 3 depicts the functional relative taxonomic profiles and abundance of diatom assemblages in optimized PO_4^{2-} growth media and heat map of 25 genera in the optimized PO_4^{2-} growth media, respectively. The 25 dominant genera in the optimized PO_4^{2-} growth media were selected and compared with taxonomic profiles and abundance of benthic epilithic diatom assemblage cultures in the control growth media. The results obtained from P1 culture showed that the most prevalent species were *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Diademis confervacea, Navicula cryptocephala, Navicula rediana, Nitzchia amphibia, Nitzchia palea, Pseudostaurosira brevista* and *Sellaphora popula.* The result also revealed that benthic epilithic diatoms assemblages that were depleting in the P1 culture include *Cyclothela ocellata, Nitzchia agnita, Rhopalodia spp., Staurosira eliptica* and *Triblionella gracillis*. The data obtained from P2 culture showed that the abundant diatom species were *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Fragillaria biceps, Navicula cryptocephala, Navicula rediana, Planothidiun frequentissimum,* and *Sellaphora popula*. The data also indicated that benthic epilithic diatom species that were depleting includes *Aulacoseira muzanensis, Nitzchia linearis, and Rhopalodia gibberula*. P3 culture data revealed that *Aulacoseira granulata var. angustissima, Navicula cryptocephala, Navicula rediana, Navicula symmetrica, Nitzchia pura, Pseudostaurosira brevista, Sellaphora popula* and *Staurosira construens* were the dominant diatom species. *Gyrosigma scalproides, Melosira varians, Navicula trivalis* and *Nitzchia agnita* were depleting in this culture. Data obtained from P4 cultures showed that diatom species like *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Fragillaria biceps, Navicula cryptocephala,*

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DOI: http://dx.doi.org/10.15666/aeer/1806_76077625

Navicula symmetrica, Nitzchia amphibia, Planothidiun frequentissimum and *Sellaphora popula* were dominant. Depleting benthic epilithic diatom species were *Craticula ambigua, Cyclothella ocellata, Gyrosigma scalproides, Navicula trivalis, Rhopalodia gibberula, Rhopalodia operculata, Staurosira eliptica and triblionella gracillis*. P5 culture showed *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Diademis confervacea, Fragillaria biceps, Navicula cryptocephala, Navicula rediana, Navicula symmetrica, Nitzchia amphibia, Nitzchia pura, Pseudostaurosira brevista* and *Staurosira construens* as the dominant diatom species while species like *Gomphonema venusta, Nitzchia agnita, Nitzchia perspicua* and *Staurosira eliptica* were all depleting in this culture. In the P6 culture, Aulacoseira granulata*, Aulacoseira granulata var. angustissima, Navicula cryptocephala, Navicula rediana, Navicula symmetrica, Nitzchia amphibia, Nitzchia pura,* and *Planothidiun frequentissimum* diatom species showed abundant composition while *Craticula ambigua, Cyclothella ocellata, Navicula trivalis, Nitzchia agnita* and *Rhopalodia operculata* species displayed depleting trends. P7 cultures exhibited the abundance of diatom species like *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Fragillaria biceps, Fragillaria ulna var. acus, Navicula cryptocephala, Navicula symmetrica, Nitzchia amphibia, Nitzchia pura, Planothidiun frequentissimum* and *Sellaphora popula*. Depleting diatom species observed in this culture include *Gomphonema venusta, Gyrosigma scalproides, Rhopalodia gibba and Rhopalodia operculata*. P8 species composition showed that *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Navicula cryptocephala, Planothidiun frequentissimum* and *Sellaphora popula* species were dominant. The data also showed that *Hantzchia amphioxys*, *Lemnicola hungarica*, *Navicula trivalis*, *Nitzchia agnita*, *Nitzchia linearis*, *Rhopalodia gibba* and *Rhopalodia gibberula* were depleting. Results obtained from P9 culture showed that diatom species which dominated were *Achnantidiun exiguum,* Aulacoseira granulata*, Aulacoseira granulata var. angustissima, Navicula cryptocephala, Nitzchia pura, Planothidiun frequentissimum* and *Sellaphora popula*. Accordingly, species composition revealed that *Gomphonema venusta, Nitzchia agnita, Nitzchia linearis, Rhopalodia operculata* and *Staurosira eliptica* were depleting. Diatom species composition analysis clearly showed that in P10 culture, *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Diademis confervacea, Navicula cryptocephala* and *Planothidiun frequentissimum* were the dominant diatom species, while *Craticula ambigua, Gomphonema pumilum var. rigidum, Gomphonema venusta, Hantzchia amphioxys, Navicula trivalis, Nitzchia agnita, Rhopalodia gibba and Rhopalodia gibberula* diatom species were depleting. Diatom species composition analysis in P11 culture showed that *Achnantidiun exiguum, Aulacoseira granulata, Aulacoseira granulata var. angustissima, Navicula cryptocephala, Navicula reidiana, Planothidiun frequentissimum* and *Sellaphora popula* were dominant while Gomphonema *pumilum var. rigidum, Gomphonema venusta, Lemnicola hungarica*, *Nitzchia agnita*, *Nitzchia perspicua*, *Pinnularia subbrevistriata*, *Pseudostaurosira brevista* and *Rhopalodia spp* all displayed depleting trends. P12 cultures clearly shows that diatom species like *Achnantidiun exiguum*, *Aulacoseira granulata var. angustissima, Gomphonema parvulu*, *Navicula cryptocephala*, *Planothidium frequentissimum* and *Sellaphora popula* were dominant while diatom assemblages like *Cocconeis placentula var euglipta, Craticula ambigua*, *Gyrosigma scalproides*, *Lemnicola hungarica*, *Rhopalodia gibba*, *Rhopalodia operculata*, *Staurosira eliptica* and *Triblionella hungarica* species showed depleting trends.

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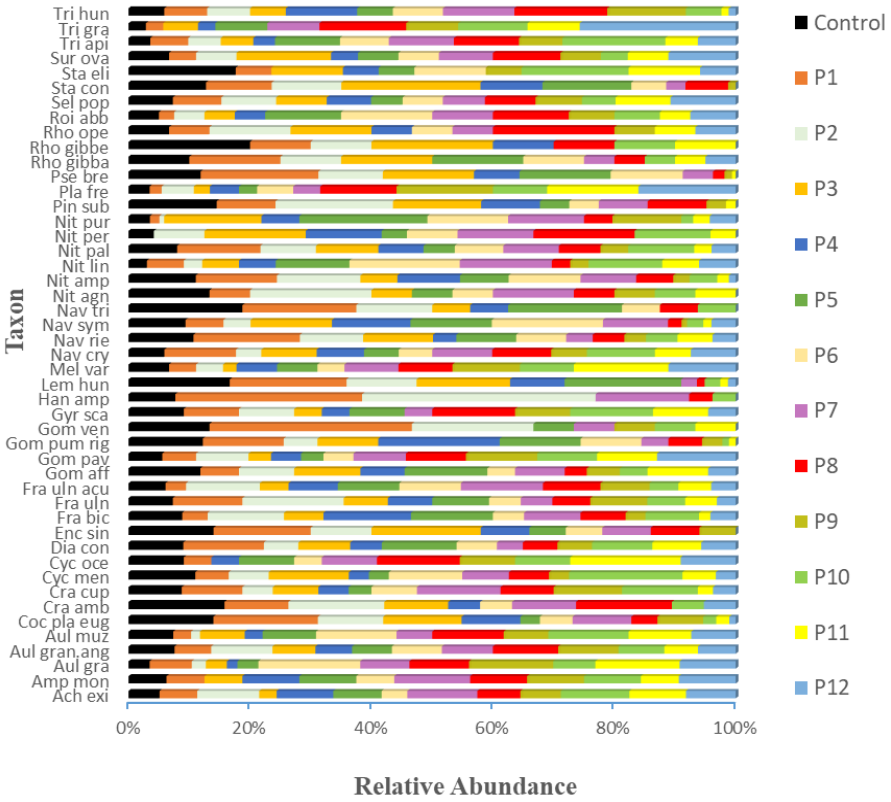

Figure 2. *Functional relative taxonomic profiles and abundance of diatom assemblages in optimized PO_4^{2-} growth media*

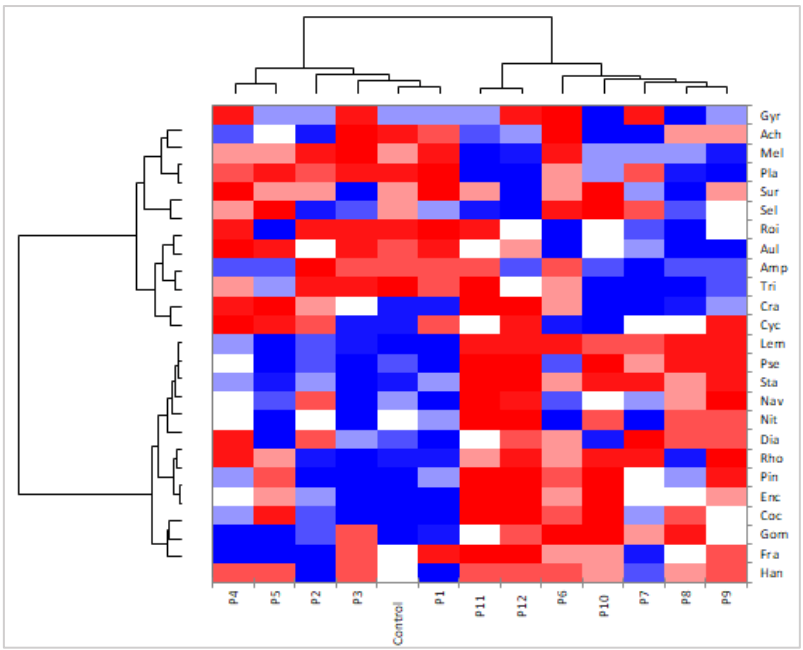

Figure 3. *Heat map depicting 25 genera in the optimized PO_4^{2-} growth media. The 25 dominant genera in the optimized PO_4^{2-} growth media were selected and compared with taxonomic profiles and abundance of benthic epilithic diatom assemblage cultures in the control growth media*

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Figure 4 is a PCA ordination biplot depicting the relationship between the taxonomic diversity profiles and abundance of the benthic epilithic diatom assemblages and optimized PO_4^{2-} concentration in the growth media. The biplot portrays 45.62% of variations in the data with the F1 axis representing 31.56 % and the F2 axis denoting 14.06 %. The data illustrated that there was a definite separation among cultures on the F1 axis. The separation showed P1 - P6 on the positive margin and P7 - P12 on the negative. The P1 - P6 had PO_4^{2-} amounts which ranged from 0.003 – 0.05 mg/L, indicating that higher PO_4^{2-} amounts were mainly dominated by the presence of *Gomphonema pavulum*, *Planothidium frequentissimum*, *Melosira varians*, *Sellaphora popula*, *Aulacoseira* spp., *Cyclothella* spp., *Triblionella* spp. and *Roicosphenia* species. Lower phosphate concentrations (P1 - P5) favoured the growth of *Fragillaria*, *Nitzchia*, *Encynopsis*, *Ropalodia*, *Navicula* and *Gomphonema* species. The P1 - P5 cultures representing a non-impacted water quality conditions encouraged a wide diversity of benthic epilithic diatom assemblages. Similarly, P7 – P12 cultures representing an impacted water quality conditions / higher trophic status indicates that only taxa with preference for the optimized PO_4^{2-} amounts were present.

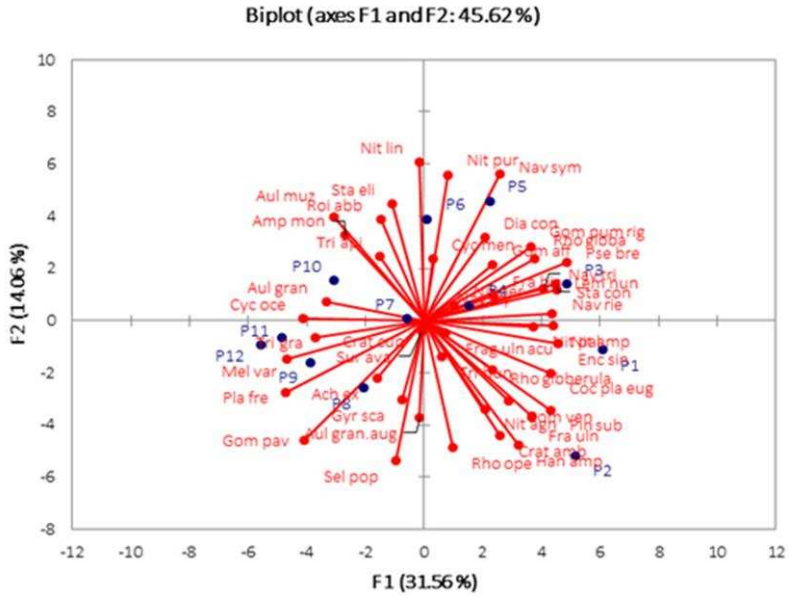

Figure 4. *PCA biplot illustrating the corelation between taxonomic compositions and abundance of benthic epilithic diatom assemblages in optimized PO_4^{2-} growth media*

Taxonomic Profiles and Abundance of Diatom Assemblages in Optimized NO_3^{2-} Growth Media

Figures 5 and 6 depicts the functional relative taxonomic profiles and abundance of diatom assemblages in optimized NO_3^{2-} growth media and heat map depicting 25 genera in the optimized NO_3^{2-} growth media. The 25 dominant genera in the optimized NO_3^{2-} growth media were selected and compared with taxonomic profiles and abundance of benthic epilithic diatom assemblage cultures in the control growth media. The data obtained from N1 culture showed that the most prevalent species were *Achnantidium exiguum*, *Aulacoseira granulata*, *Cocconeis placentula var. Euglypta*, *Fragillaria ulna var. acus*, *Navicula crytocephala*, *Navicula reidiana*, *NItzchia amphibia*, *Nitzchia palea*,

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Pseudostaurosira brevista, *Sellaphora popula* and *Staurosira construens*. The only diatom species observed exhibiting a depleting trend was *Rhopalodia gibba*. The data from N2 culture revealed that the abundant diatom species were *Achnanthidium exiguum*, *Aulacoseira granulata*, *Navicula cryptocephala* and *Nitzchia palea.* Depleting species included *Gomphonema pumilum var. rigidum*, *Navicula trivalis* and *Staurosira eliptica* species. N3 culture showed that *Achnanthidium exiguum*, *Aulacoseira granulata*, *Fragillaria ulna var.acus*, *Navicula reidiana* and *Sellaphora popula* dominating. The only diatom species observed depleting was *Gyrosigma scalproides*. Results obtained from N4 cultures showed that diatom species like *Achnanthidium exiguum*, *Diademis confervacea*, *Fragillaria ulna var. acus*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana* and *Nitzchia palea* were dominant. Benthic epilithic diatom species observed depleting were *Craticula ambigua*, *Navicula trivalis*, *Nitzchia linearis*, *Nitzchia perspicua*, *Pinnularia subbrevistriata*, *Rhopalodia operculata*, *Staurosira eliptica* and *triblionella hungarica*. N5 culture showed that *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var. angustissimum*, *Fragillaria ulna var. acus*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana* and *Nitzchia amphibia*, *Planothidium frequentissimum*, *Pseudostaurosira brevista* and *Sellaphora popula* were dominant. Diatom species depleting in this culture were *Aulacoseira muzzanensis*, *Craticula ambigua*, *Cyclothella ocellata*, *Gyrosigma scalproides*, *Melosia varians*, *Navicula trivalis*, *Rhopalodia operculata* and *Surrirella avalis*. In the N6 culture, *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var. angustissimum*, *Diademis confervacea*, *Fragillaria ulna var. acus*, *Navicula cryptocephala*, *Navicula reidiana*, *Navicula symmetrica*, *Nitzchia amphibia*, *Nitzchia palea*, *Planothidium frequentissimum* and *Pseudostaurosira brevista*. Diatom species like *Gyrosigma scalproides*, *Hantzchia amphioxys*, *Navicula trivalis*, *Rhopalodia gibberula*, *Rhopalodia operculata* and *Triblionella hungarica* displayed depleting trends. N7 cultures exhibited the abundance of diatom species like *Achnanthidium exiguum*, *Aulacoseira granulata*, *Navicula symmetrica* and *Pseudostaurosira brevista*. Diatom species which were depleting are *Encynopsis leei var. sinnensis*, *Gomphonema venusta*, *Nitzchia linearis*, *Nitzchia perspicua*, *Rhopalodia gibba*, *Staurosira construens* and *Triblionella gracillis*. N8 species composition showed that *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var. angustissimum*, *Fragillaria ulna*, *Gomphonema parvulum*, *Navicula reidiana*, *Navicula symmetrica*, *Nitzchia amphibia*, and *Planothidium frequentissimum* were dominant. Depleting species were *Nitzchia linearis*, *Nitzchia perspicua*, and *Staurosira eliptica*. The N9 culture showed that diatom species which dominated were *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var. angustissimum*, *Fragillaria ulna var. acus*, *Navicula cryptocephala Navicula reidiana*, *Navicula symmetrica*, *Planothidium frequentissimum* and *Pseudostaurosira brevista*. The depleting species were *Craticula ambigua*, *Gomphonema venusta*, *Gyrosigma scalproides*, *Navicula trivalis*, *Nitzchia agnita*, *Rhopalodia operculata*, *Triblionella gracillis* and *Triblionella hungarica*. Diatom species composition analysis clearly showed that in N10 culture, *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var. angustissimum*, *Fragillaria ulna var. acus*, *Gomphonema parvulum*, *Navicula cryptocephala*, Navicula *reidiana*, *Nitzchia amphibia*, *Planothidium frequentissimum*, *Pseudostaurosira brevista* and *Sellaphora popula* were dominant. *Gomphonema pumilum var. rigidum* was the only depleting diatom species. Diatom species composition analysis in N11 culture showed that *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var.*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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angustissimum, *Diademis confervacea*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana*, *Nitzchia amphibia*, *Planothidium frequentissimum* and *Pseudostaurosira brevista* were dominant. The diatom species observed depleting were *craticula cupsidata*, *Gyrosigma scalproides*, *Nitzchia agnita*, *Nitzchia perspicua*, *NItzchia pura*, *Rhopalodia gibberula*, *Rhopalodia operculata*, *Staurosira eliptica*, *Triblionella gracillis* and *Triblionella hungarica*. N12 cultures clearly shows that diatom species like *Achnanthidium exiguum*, *Aulacoseira granulata*, *Aulacoseira granulata var.* angustissimum, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana*, *Nitzchia amphibia*, and *Planothidium frequentissimum*. Diatom species observed depleting were *Encynopsis leei var. sinnensis*, *Gyrosigma scalproides*, *Lemnicola hungarica*, *Nitzchia agnita*, *Nitzchia linearis*, *Pinnularia subbrevistriata*, *Rhopalodia*, *Staurosira* and *Triblionella spp*.

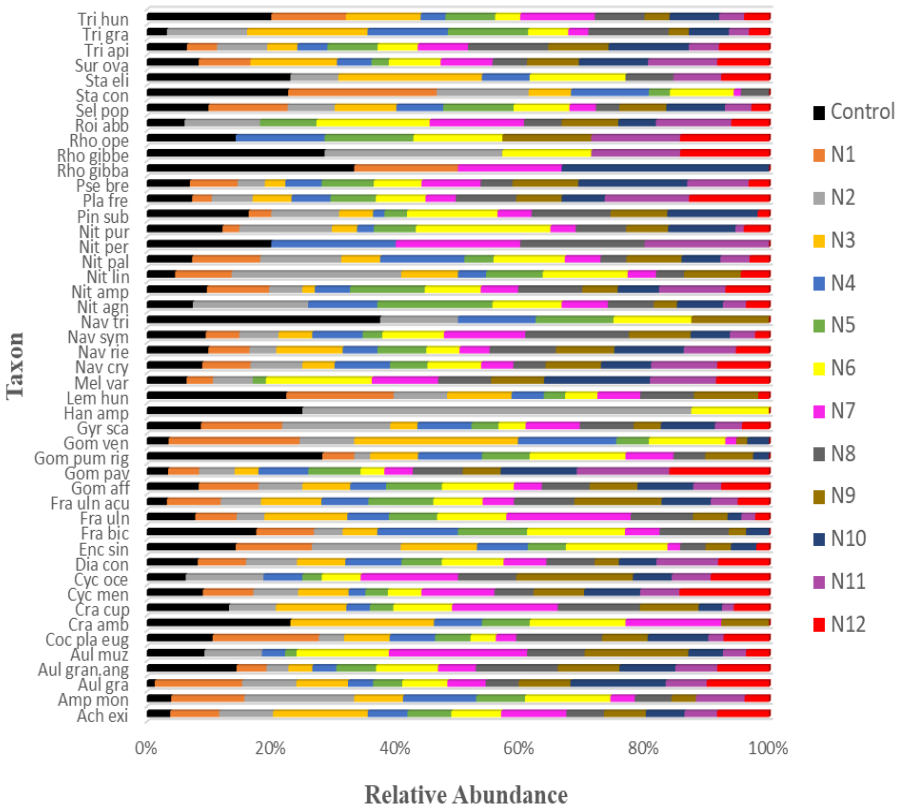

Figure 5. *Taxonomic profiles and abundance of diatom assemblages in optimized NO_3^{2-} growth media*

Figure 7 is a PCA biplot denoting the relationship between the taxonomic diversity profiles and abundance of benthic epilithic diatom assemblages and optimized NO_3^{2-} concentrations in the growth media. The biplot describes 37.93 % of variations in the data with the F1 axis describing 21.72 % and the F2 axis denoting 16.21 % of variations. The data demostrated that N4 - N6 cultures were more correlated with *Encynopsis, Gyrosigma*, *Nitzchia*, *Hantzchia*, *Navicula*, *Diademis*, *Amphora* and *Rhopalodia* diatom species. N7–N12 cultures favoured the growth of *Roicosphenia*, *Navicula*, *Gomphonema, Aulacoseira*, *Cyclothela*, *Rhoalodia*, *Pseudostaurosira*, *Surrirella* and *Triblionella* species.

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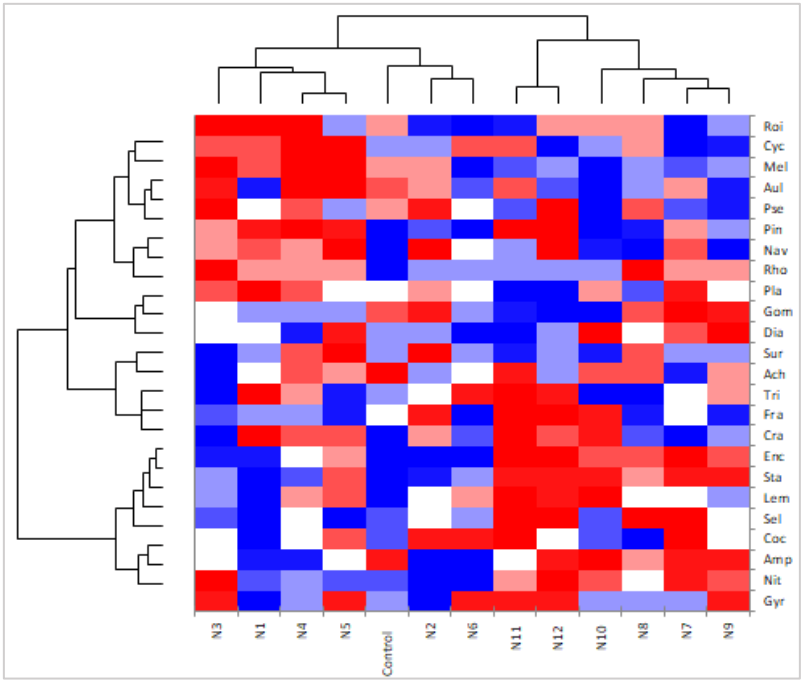

Figure 6. *Heat map depicting 25 genera in the optimized NO_3^{2-} growth media. The 25 dominant genera in the optimized PO_4^{2-} growth media were selected and compared with taxonomic profiles and abundance of benthic epilithic diatom assemblage cultures in the control growth media*

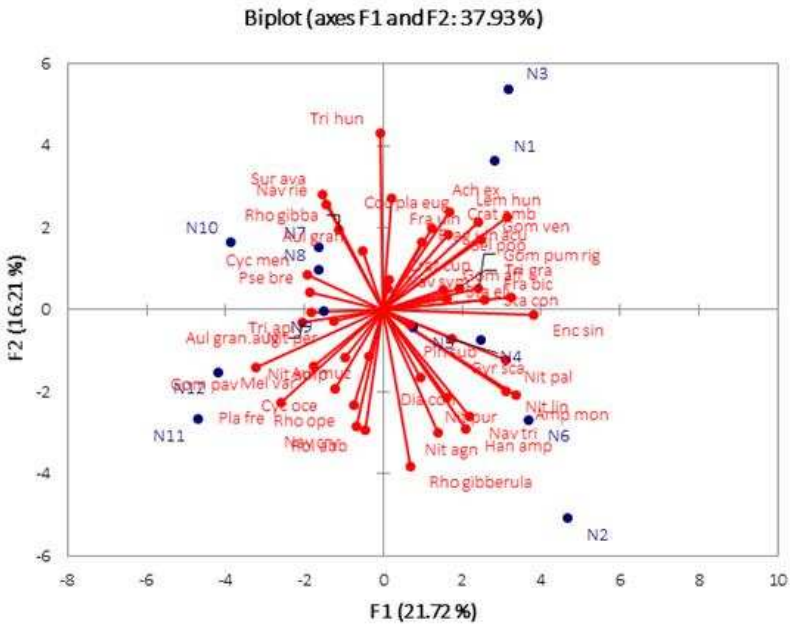

Figure 7. *PCA biplot depicting the corelation between taxonomic diversity profile and abundance of benthic epilithic diatom assemblages in optimized NO_3^{2-} concentration growth media*

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Discussion

The results clearly indicated that the prevalence of benthic epilithic diatom assemblages could be related to phosphate and nitrate trophic status in a controlled environment. The amount of PO_4^{2-} and NO_3^{2-} in the control WC media was 0.0087 mg/L and 0.0851 mg/L, respectively. These concentrations are within the mesotrophic and oligitrophic condition which is consistent with a non-polluted (good water quality) trophic status (*Tables 1 and 2*).

Table 1. *Actual nutrient concentrations that determine the trophic level of river systems*

Physicochemical variables	**Trophic level**					
	V. low (1)	Low (2)	Moderate (3)	High (4)	V. high (5)	Ex. High (6)
Phosphate (µg/L)	< 20	20 - 60	60 - 100	100 - 200	200 - 1000	> 1000
Nitrate (mg/L)	< 5	5 - 10	10 - 20	20 - 30	30 - 40	> 40
Dissolved oxygen (% saturation)	80	70	60	50	20	< 20

Table 2. *Trophic levels of inland freshwater ecosystem*

Trophic category	**Variables**	
	Nitrate (mgL^{-1})	Phosphate (μgL^{-1})
Oligotrophic	< 0.5	> 5.0
Mesotrophic	0.5 – 2.5	5 - 25
Eutrophic	2.5 10	25 - 250
Hypertrophic	> 10	> 250

A total of 47 different species belonging to twenty 25 genera were identified in the control culture. The composition of individual species varied amongst taxa (*Figure 1*). *Aulacoseira granulata var.augustissima* had the maximum counts suggesting that the species can florish in oligo- mesotrophic conditions corroborating earlier reports by Taylor et al. (2007) who reported that *Aulacoseira granulata var.augustissima* can be found in eutrophic rivers. Similar conditions existed in the river when environmental samples were collected suggesting that this species can florish in both polluted and non polluted environments. Comparatively *Nitzchia linearis*, *Nitzchia perspicua, Rhopalodia operculata* and *Triblionella gracillis* species were depleting in this culture medium indicating that these species do not florish very well under oligo-mesotrophic conditions. Such findings corroborates earlier studies by Bere et al. (2013), Shinneman et al. (2016), Trobaja and Mann (2019), Barinova et al. (2019) and Zhang et al. (2020) which reported that *Nitzchia linearis* is tolerant of polluted condition. P1 and P2 cultures (0.003 and 0.005 mg/L PO_4^{2-}) represents oligotrophic water condition (*Tables 1 and 2*). The data obtained from these cultures showed that the most prevalent species were *Achantidiun exiguum, Aulacoseira granulata var. angustissima, Diademis confervacea, Fragillaria biceps, Navicula cryptocephala, Navicula rediana, Nitzchia amphibia, Nitzchia palea, Planothidiun frequentissimum Pseudostaurosira brevista* and *Sellaphora popula*. These species could prevail and dominate in non-polluted environments corroborating reports (Taylor et al., 2007; Shinneman et al., 2016; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020) who have reported that these species are prevalent in different

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trophic water levels including eutrophic and extremely polluted waters. The depletion of benthic epilithic diatom species (*Figures 2 and 3*) like *Aulacoseira muzanensis, Cyclothela ocellata, Nitzchia agnita, Nitzchia linearis, Rhopalodia spp., Staurosira eliptica* and *Triblionella gracillis* in the P1 and P2 cultures plausibly indicated that these species do not flourish in oligotrophic conditions. Literature reports (Shinneman et al., 2016; Moorhouse et al., 2018; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020) describing the prevalence of *Aulacoseira muzanensis, Cyclothela ocellata* and *Nitzchia linearis* in meso-eutrophic waters supports this assertion. P3, P4 and P5 cultures with 0.01, 0.015 and 0.025 mg/L PO_4^{2-} respectively represents a mesotrophic water quality condition (*Tables 1 and 2*). Species abundance analysis of these cultures (*Figures 2 and 3*) showed *Achnantidiun exiguum, Aulacoseira granulata var. angustissima, Diademis confervacea, Fragillaria biceps, Navicula cryptocephala, Navicula rediana, Navicula symmetrica, Nitzchia amphibia, Nitzchia pura, Planothidiun frequentissimum Pseudostaurosira brevista Sellaphora popula* and *Staurosira construens* were the dominant diatom assemblages. These species could also prevail and dominate in non-polluted environments also corroborating reports (Harding et al., 2005) who has reported that these species are prevalent in diverse freshwaters resources including eutrophic and extremely polluted surface waters. Our assumption is that the depletion of diatoms species (*Figures 2 and 3*) like *Craticula ambigua, Cyclothella ocellata, Gomphonema venusta, Gyrosigma scalproides, Melosira varians, Navicula trivalis*, *Nitzchia agnita*, *Nitzchia* perspicua, Rhopalodia *gibberula, Rhopalodia operculata, Staurosira eliptica* and *Triblionella gracillis* in the P3, P4 and P5 cultures may be an indication that these species do not flourish in mesotrophic conditions. This is due to the fact that published reports (Shinneman et al., 2016; Moorhouse et al., 2018; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020) describing the prevalence of *Craticula ambigua, Cyclothella ocellata, Gomphonema venusta, Gyrosigma scalproides, Melosira varians, Navicula trivalis*, *Nitzchia agnita*, *Nitzchia* perspicua, Rhopalodia *gibberula, Rhopalodia operculata, Staurosira eliptica* and *Triblionella gracillis* in meso-eutrophic waters supports this proposition. P6, P7, P8, P9 and P10 cultures having 0.05, 0.1, 0.15, 0.2 and 0.25 mg/L PO_4^{2-} respectively signifies a eutrophic water quality condition (*Tables 1 and 2*). Again species abundance analysis (*Figures 2 and 3*) of these cultures indicated that *Achantidiun exiguum, Aulacoseira granulata var. angustissima,* Aulacoseira granulata*, Diademis confervacea, Fragillaria biceps, Fragillaria ulna var. acus, Navicula cryptocephala Navicula rediana, Navicula symmetrica, Nitzchia amphibia, Nitzchia pura, Planothidiun frequentissimum Sellaphora popula* were the dominant diatom species. The data also showed diatom species like *Craticula ambigua, Cyclothella ocellata, Gomphonema pumilum var. rigidum, Gomphonema venusta, Gyrosigma scalproides, Hantzchia amphioxys*, *Lemnicola hungarica*, *Navicula trivalis, Nitzchia agnita Nitzchia linearis*, *Rhopalodia gibba Rhopalodia gibberula Rhopalodia operculata* and *Staurosira eliptica* were depleting under eutrophic conditions. The depletion of these species under similar water trophic status corroborates previous reports (Lacerda et al., 2004; Della Bella et al., 2007) who have hypothesized the depletion of some of the species in eutrophic waters. P11 and P12 cultures (0.3, 0.4 mg/L PO_4^{2-}) presented a hyper-eutrophic water conditions (*Tables 1 and 2*). Diatom species composition analysis in these cultures revealed that *Achnantidiun exiguum, Aulacoseira granulata var. angustissima, Aulacoseira granulata, Gomphonema parvulu, Navicula cryptocephala, Navicula reidiana, Planothidium frequentissimum sellaphora popula* were the dominant species. The revelation reasonably indicates that these species are

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DOI: http://dx.doi.org/10.15666/aeer/1806_76077625

tolerant of hyper-eutrophic to extremely polluted conditions (Leelahakriengkrai and Peerapornpisal, 2010). The data also revealed that species like *Cocconeis placentula var euglipta*, *Craticula ambigua*, *Gomphonema pumilum var. rigidum, Gomphonema venusta, Gyrosigma Scalproides*, *Lemnicola hungarica*, *Nitzchia agnita*, *Nitzchia perspicua*, *Pinnularia subbrevistriata*, *Pseudostaurosira brevista*, *Rhopalodia spp. Staurosira eliptica* and *Triblionella hungarica* all displayed depleting trends under hyper-eutrophic conditions corroborating previous reports (Leland and Porter, 2000; Leira and Sabater, 2005; Salomoni et al., 2006). However, the depletion of *Craticula ambigua* and *Triblionella hungarica* under hyper-eutrophic conditions is in disagreement with Taylor et al. (2007) who has reported that these species are tolerant of critically polluted waters. An analysis to evaluate the correlation between diatom diversity and optimized PO_4^{2-} amounts in the growth media was done using principal component analysis (*Figure 4*). The results portrays 45.62% of variations in the data. The F1 axis describes 31.56 % and the F2 axis describes 14.06 % plausibly suggesting that the the optimization of PO_4^{2-} concentrations encouraged corresponding fluctuations in the diversity and abundance of diatom assemblages. P1 – P6 cultures (oligo-mesotrophic conditions) displayed separation on the F1 axis, and favoured enormous diversity of diatoms which could be attributed to non-polluted water trophic status. The oligo-mesotrophic water conditions were mainly dominated by *Fragillaria*, *Nitzchia*, *Encynopsis*, *Ropalodia*, *Navicula* and *Gomphonema* species. The prevalence of these species in moderate PO_4^{2-} amounts have been reported in streams and rivers (Bellinger et al., 2006; Moorhouse et al., 2018). According to Bellinger et al. (2006), diatoms react profusely to even minor concentrations of phosphate. P7 – P12 cultures with higher phosphate concentrations (eutrophic – hyper-eutrophic conditions) had lower diversity of diatoms. The eutrophic – hyper-eutrophic water quality conditions were mainly dominated by presence of *Gomphonema pavulum*, *Planothidium frequentissimum*, *Melosira varians*, *Sellaphora popula*, *Aulacoseira* spp., *Cyclothella* spp., *Triblionella* spp. and *Roicosphenia* species suggesting that only taxa with preference to optimized phosphate concentrations were present.

Similarly, N1 and N2 cultures (0.1 and 0.3 mg/L NO_3^{2-}) represents the oligotrophic water condition (*Tables 1 and 2*). The data obtained from these cultures revealed that the most prevalent species were *Achnanthidium exiguum*, *Aulacoseira granulata*, *Cocconeis placentula var. euglypta*, *Fragillaria ulna var. acus*, *Navicula cryptocephala*, *Navicula reidiana*, *Nitzchia amphibia*, *Nitzchia palea*, *Pseudostaurosira brevista*, *Sellaphora popula* and *Staurosira construens*. *Navicula cryptocephala*, *Pseudostaurosira brevista* and *Staurosira construens* species could prevail and dominate in oligotrophic environments corroborating reports (Taylor et al., 2007). The depletion of benthic epilithic diatom species (*Figures 5 and 6*) like *Gomphonema pumilum var. rigidum*, *Navicula trivalis*, *Rhopalodia gibba* and *Staurosira eliptica* in N1 and N2 cultures practically indicated that these species do not flourish in oligotrophic conditions (Trobajo et al., 2019). N3, N4, N5 and N6 cultres (0.5, 1.0, 2.0, 2.5 mg/L NO_3^{2-}) represents a mesotrophic water condition (*Tables 1 and 2*). Species abundance analysis of these cultures (*Figures 5 and 6*) showed that *Achnanthidium exiguum*, *Aulacoseira granulata var. angustissimum*, *Aulacoseira granulata*, *Diademis confervacea*, *Fragillaria ulna var. acus*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana*, *Navicula symmetrica*, *Nitzchia amphibia*, *Nitzchia palea*, *Planothidium frequentissimum*, *Pseudostaurosira brevista* and *Sellaphora popula* were dominant. The results showed that *Achnanthidium exiguum*, *Diademis confervacea*, *Fragillaria ulna var. acus*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Planothidium frequentissimum*,

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Pseudostaurosira brevista and *Sellaphora popula* could prevail and dominate in mesotrophic environments corroborating reports (Taylor et al., 2007; Shinneman et al., 2016; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020). However, the dominant presence of species like *Aulacoseira granulata var. angustissimum*, *Aulacoseira granulata*, *Navicula reidiana*, *Navicula symmetrica*, *Nitzchia amphibia* and *Nitzchia palea* in mesotrophic water conditions is in disagreement with the findings of Bere et al. (2013) who reported that these diatom species persist mostly in eutrophic and extremely polluted environments. In constrast, the depletion of diatoms species (*Figures 5 and 6*) like *Aulacoseira muzzanensis*, *Craticula ambigua*, *Cyclothella ocellata*, *Gyrosigma scalproides*, *Hantzchia amphioxys*, *Melosira varians*, *Navicula trivalis*, *Nitzchia linearis*, *Nitzchia perspicua*, *Pinnularia subbrevistriata*, *Rhopalodia gibberula*, *Rhopalodia operculata*, *Staurosira eliptica*, *Surrirella avalis* and *Triblionella hungarica* in these cultures presumably implies that these species do not flourish in mesotrophic conditions (Chia et al., 2011; Trobajo et al., 2019). N7, N8, N9 and N10 (5.0, 7.0, 9.0, 10 mg/L NO_3^{2-}) represents a eutrophic water condition (*Tables 1 and 2*). Species abundance analysis of these cultures showed that diatom species like *Achnanthidium exiguum*, *Aulacoseira granulata var. angustissimum*, *Aulacoseira granulata*, *Fragillaria ulna*, *Fragillaria ulna var. acus*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana*, *Navicula symmetrica*, *Nitzchia amphibia*, *Planothidium frequentissimum*, *Pseudostaurosira brevista* and *Sellaphora popula* were dominant in these cultures. The data credibly suggest that these species could prevail and dominate in eutrophic freshwater environments. The depletion of diatoms species like (*Figures 5 and 6*) *Craticula ambigua*, *Encynopsis leei var. sinnensis*, *Gomphonema pumilum var. rigidum*, *Gomphonema venusta*, *Gyrosigma scalproides*, *Navicula trivalis*, *Nitzchia agnita*, *Nitzchia linearis*, *Nitzchia perspicua*, *Rhopalodia gibba*, *Rhopalodia operculata*, *Staurosira construens*, *Staurosira eliptica*, *Triblionella gracillis*, *Triblionella hungarica* in constrast may again insinuate their intolerance to eutrophic freshwater conditions. Equally, the depletion of *Encynopsis leei var. sinnensis*, *Gomphonema venusta* and *Staurosira construens* under eutrophic water conditions surprisingly is in disagreement with Harding et al. (2005) and Barinova et al. (2019) who are of the opinion that these species can flourish and dominate in eutrophic freshwater conditions. N11, N12 and N13 (11, 12, 13 mg/L NO_3^{2-}) denote a hyper-eutrophic water condition (*Tables 1 and 2*). Again species abundance analysis of these cultures (*Figures 5 and 6*) showed that diatom species like *Achnanthidium exiguum*, *Aulacoseira granulata var. angustissimum*, *Aulacoseira granulata*, *Diademis confervacea*, *Gomphonema parvulum*, *Navicula cryptocephala*, *Navicula reidiana*, *Nitzchia amphibia*, *Planothidium frequentissimum* and *Pseudostaurosira brevista* were dominant in these cultures. The data undoubtedly implied that these species could prevail and dominate in hyper-eutrophic environments. It is worth mentioning that diatom species like *Aulacoseira granulata var. angustissimum*, *Aulacoseira granulata*, *Navicula reidiana* and *Pseudostaurosira brevista* have been reported to flourish under hyper-eutrophic environments (Della Bella et al., 2007). The depletion of benthic epilithic diatom assemblages (*Figures 5 and 6*) *Craticula cupsidata*, *Encynopsis leei var. sinnensis*, *Gyrosigma scalproides*, *Lemnicola hungarica*, *Nitzchia agnita*, *Nitzchia linearis*, *Nitzchia perspicua*, *Nitzchia pura*, *Pinnularia subbrevistriata*, *Rhopalodia, Staurosira* and *Triblionella spp* suggest that these species could flourish well in waters of good quality (Della Bella et al., 2007). Correspondingly, an analysis to evaluate the correlation between diatom diversity and optimized NO_3^{2-} amounts in the growth media was done using principal component analysis (*Figure 7*). The data

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illustrated 37.93 % of variations in the data. The F1 axis describes 21.72 % and the F2 axis describes 16.21% of variations, revealing that the optimization of NO_3^{2-} amounts within mesotrophic to hyper-eutrophic conditions displayed correlation with specific diatom species. The PCA ordination biplot illustrated that N1 – N3 cultures (oligotrophic – slightly mesotrophic conditions) did not have any correlation with the distribution of diatom species. N4 - N6 cultures (mesotrophic condition) were more correlated with *Amphora, Diademis*, *Encynopsis, Gyrosigma*, *Hantzchia*, *Navicula*, *Nitzchia* and *Rhopalodia spp*. According to Taylor et al. (2007), *Encynopsis leei var. sinensis* species can be found in oligo-mesotrophic water condition. The PCA ordination biplot also showed that N7–N12 cultures (eutrophic – hyper-eutrphic conditions) favoured the growth of *Roicosphenia*, *Navicula*, *Gomphonema, Aulacoseira*, *Cyclothela*, *Rhoalodia*, *Pseudostaurosira*, *Surrirella* and *Triblionella* species. These species have been reported to grow on enhanced NO_3^{2-} concentrations (Bellinger et al., 2006; Shinneman et al., 2016; Trobaja and Mann, 2019; Barinova et al., 2019; Zhang et al., 2020). It should be noted that diverse benthic epilithic diatom assemblages responded differently to controlled optimizations in nutrient concentrations. The diversity and abundance of benthic epilithic diatom species were influenced by the loadings of phosphate and nitrate in water (Shinneman et al., 2016; Moorhouse et al., 2018; Barinova et al., 2019; Trobaja and Mann, 2019; Zhang et al., 2020). At moderate PO_4^{2-} and NO_3^{2-} concentrations, benthic epilithic diatom assemblages displayed a wide of diversity. However, at higher concentrations there was reduction in the diversity in some of the diatom species. Our findings clearly indicates that benthic epilithic diatom assemblages are more dependent on phosphate than nitrates concentrations in a controlled environment. The result showed that diatoms growth were impacted by even reduced concentrations of phosphate compared to higher nitrate concentrations. These findings are in agreement with early works of Han et al. (2016) who reported that diatom cells have higher reflection on the concentrations of phosphate than nitrate. The data clearly demonstrates that optimization of PO_4^{2-} and NO_3^{2-} trophic levels on benthic epilithic diatom assemblage cultures could plausibly be employed in predicting of trophic status and water quality conditions of a surface freshwater impoundment. This is due to the fact that PO_4^{2-} and NO_3^{2-} concentrations are important variables which aid the growth of diatom assemblage cultures in aquatic ecosystems (Shinneman et al., 2016; Moorhouse et al., 2018; Barinova et al., 2019; Trobaja and Mann, 2019; Zhang et al., 2020).

Conclusion

In conclusion, the present study set out to investigate the controlled optimization of PO_4^{2-} and NO_3^{2-} concentrations in benthic epilithic diatom assemblage cultures in predicting the trophic status and water quality conditions of the Swartpruit River, North West Province, South Africa. The findings of the study has obviously revealed that monitoring the growth pattern of benthic epilithic diatom assemblage cultures in a controlled optimized PO_4^{2-} and NO_3^{2-} amounts growth media to ascertain taxonomic compositions and abundance of diatom culture can be used in predicting the trophic status and freshwater quality conditions of a freshwater ecosystem. The data presented in this study have highlighted the fact that the trophic status the trophic status and water quality conditions of a very important surface freshwater resource like the Swartspruit River, South Africa can be plausibly predicted by controlled optimization of PO_4^{2-} and NO_3^{2-} amounts in the growth media. The researchers are of the anticipation that the information

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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presented in this report will be of precise significance to the Greater Haartebeesport Metropolitan Authorities, researchers and the general public concerning the risk assessments, health consequences and the seasonal occurrence and prevalence of pathogenic algal species in this very important drinking freshwater reservoir. Our findings have provided probable and useful insights to the risk that may be associated with the occurrence and prevalence of toxigenic benthic epilithic diatom assemblage blooms in this freshwater reservoir which is predominantly used for agricultural, domestic, industrial and recreational activities by the inhabitants of the Greater Haartebeesport Metropolitan Area in the North West Province, South Africa. We anticipate that the findings presented in this report can have a significant impact in integrating new knowledge with existing knowledge (if any) to gain a better insights into the state of water quality and safety around and downstream of this very important inland freshwater resource in South Africa.

Acknowledgements. The authors are grateful to the University of South Africa (UNISA) and the University of Johannesburg, South Africa for the financial support. We also thank the Agricultural Research Council-Institute for Soil, Climate and Water (ARC-ISCW), South Africa for their support.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76077625

ATMOSPHERIC DEPOSITION IN MATURE FIR (*Abies nordmanniana*) STANDS IN ALADAĞI-BOLU, TURKEY

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(Received 30th Apr 2020; accepted 11th Aug 2020)

Abstract. The aim of this study was to determine the atmospheric deposition in fir forests (*Abies nordmannianna* (Steven)) in the Bolu Aladağ region. The study area selected was located in the mountainous Aladağ region stretching along the south of Bolu Province in the Western Black Sea Region of Turkey. Four elevation zones (1000, 1200, 1400, and 1600 m above sea level) were designated on the closed-canopy fir forest-dominated northern slopes of the mountain. Sampling plots were established for each elevation zone, eight on randomly located sites under the canopy and three on adjacent open sites. For each elevation zone, open site, throughfall, and stemflow deposition samples were collected between April and November for two years (2013-2014). Chemical analysis of the samples was performed in the ICP laboratory located within the Aegean Forestry Research Institute in İzmir. One-way ANOVA was carried out for each variable to determine the differences in the sampling plots. Sulfate, nitrogen, potassium, and calcium were the main ions deposited in the fir forests in the 1000-1600-m elevation zones in Aladağ. The acid neutralization capacity of the deposition samples collected in the open site plots was high for all elevation zones.
Keywords: *atmospheric pollution, fir forest, Level II site, ICP-Forests, Turkey*

Introduction

Atmospheric pollution caused by intense agricultural practices, industrial activities, and increased use of fossil fuel has become one of the main agents disrupting forest ecosystems. The challenge of environmental problems goes beyond the jurisdiction of states, and their solution requires international cooperation. From the 1960s onward, international efforts for environmental protection have been initiated, with the UN in particular playing a major role in organizing international efforts. The 1979 Convention on Long-Range Transboundary Air Pollution and its seven protocols have emphasized international cooperation as a solution. As stated in the Rio Declaration Principle 10: "Environmental issues are best handled with the participation of all concerned parties."

After the increase in forest losses beginning from the second half of the 20th century until the 1980s, a number of studies were initiated to investigate the impact of air pollution on European forests (Bussotti and Ferretti, 1998; Erisman et al., 2008; Novotný et al., 2016; Kosonen et al., 2019; Schmitz et al., 2019; Thimonier et al., 2019; Etzold et al., 2020). A network was established in the early 1980s to monitor and evaluate the status of forests in Europe (Augustin et al., 2005). Most of these studies concentrated on spruce, fir, and beech forests in central and northern Europe. The International Cooperation Program for the Monitoring and Evaluation of the Impact of Air Pollution on Forests (ICP-Forests) was launched in 1985 within the context of the United Nations Economic Commission for Europe (UNECE) Decision on Long-Range Transboundary Air Pollution. Currently, 42 countries in and around Europe are included in the program

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76277641

(Anonymous, 2010). Within the scope of the study, 800 intensive monitoring plots were established and atmospheric deposition data were collected in 540 of these plots. Turkey joined the study in 2007 and established monitoring plots in main forest types across the country. In this context, Turkey has established 54 Level II intensive monitoring plots. One of the subjects studied in the intensive observation sites is atmospheric deposition. Most of the studies dealing with air pollution effects on forests have been conducted in central and northern Europe. Data from other parts of Europe, the Balkans, and surrounding areas including Turkey are limited. Therefore, the aim of the current study was to determine the atmospheric deposition in fir forests [*Abies nordmannianna* (Steven) Spach subsp. *Equi-trojani* (Asch. & Sint. ex Boiss.) Coode & Cullen] located in the Bolu Aladağ region where one of the ICP-Level II plots was established (Anonymous, 2015). Thus, national data from these fir stands will contribute to the evaluation of the forest conditions affected by transboundary air pollution.

Materials and Methods

Study area

The study area was selected in the mountainous Aladağ region that stretches along the south of Bolu Province in the Western Black Sea Region of Turkey. The geographical position of the area lies between 30º 32’ – 32º 36’ east longitudes and 40º 06’ – 41º 01’ 40º 06 ' 41º 01' north latitudes. The mean annual temperature of the area is 13 °C and it receives more than 800 mm average annual precipitation (Kantarcı, 1980; Şahin, 2017). The region has a mild, semi-humid maritime climate generated from the Black Sea on the north and under the influence of the Central Anatolian steppe climate from the south. The prevailing winds in the area are in the north, north-east, and north-west directions. The fir zone above 1300-m elevation is characterized by fog; therefore, a more humid and cooler climate prevails above this elevation (Kantarcı, 1980). The Bolu meteorological station is the only meteorological station in the vicinity of the sampling sites, which are located about 20 km to the south at about 900 m above sea level (asl) (*Fig. 1*).

The vegetation on the northern slope of Aladağ is primarily composed of +100-year-old Turkish fir trees. Oak and beech species are mixed in the canopy cover in some locations. The soil texture ranges from loamy-clay to clay-loam and rarely, sandy loam. The soil is relatively deep, well drained, and derived from basaltic-andesite material from the volcanic and pyroclastic rocks of the lower and middle Miocene period. Soil in the region has been classified as gray-brown forest soil according to the old soil classification. Fir stands, covering about 670 thousand ha, compose almost 3% of the forests in Turkey (Mayer and Aksoy, 1998). Fir species have established forests in moist vegetation zones 650-2000 m asl in the northwest part of Anatolia (Kantarcı, 1980). The fir zone in Aladağ stretches for 1000 m along the north-facing slope of the mountain at an elevation of 1660 m. The south-facing slope is dominated by Scots pine (*P. sylvestris*) forests.

Methods

Four elevation zones (1000, 1200, 1400, and 1600 m asl) were designated on the northern slopes of the mountain dominated by closed-canopy fir forests with leaf area index between 3 and 5. At each elevation zone, sampling plots 20 × 30 m in size were established under the forest canopy and on nearby open sites, following the ICP-Forests

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DOI: http://dx.doi.org/10.15666/aeer/1806_76277641

Manual guidelines. The sampling sites were located near a Level II ICP-Forests site established for Turkish fir stands. For each elevation zone, open site, throughfall, and stemflow deposition samples were collected from April through November for two years (2013 and 2014). Throughfall and stemflow data were subtracted from the total precipitation collected on the open site plots to calculate the interception rates. Samples were not collected between December and March because roads were closed due to snow. Based on 60 years of climatic data gathered from the Bolu station, about 39% of the precipitation occurs between December and March. In this study, since precipitation was sampled between April and November, we were able to calculate the total annual precipitation by increasing the collected precipitation data by about 39%. Based on these calculations, the sampling sites at 1000-1200 m altitudes received about 75% higher precipitation than the meteorological station average for 900 m asl.

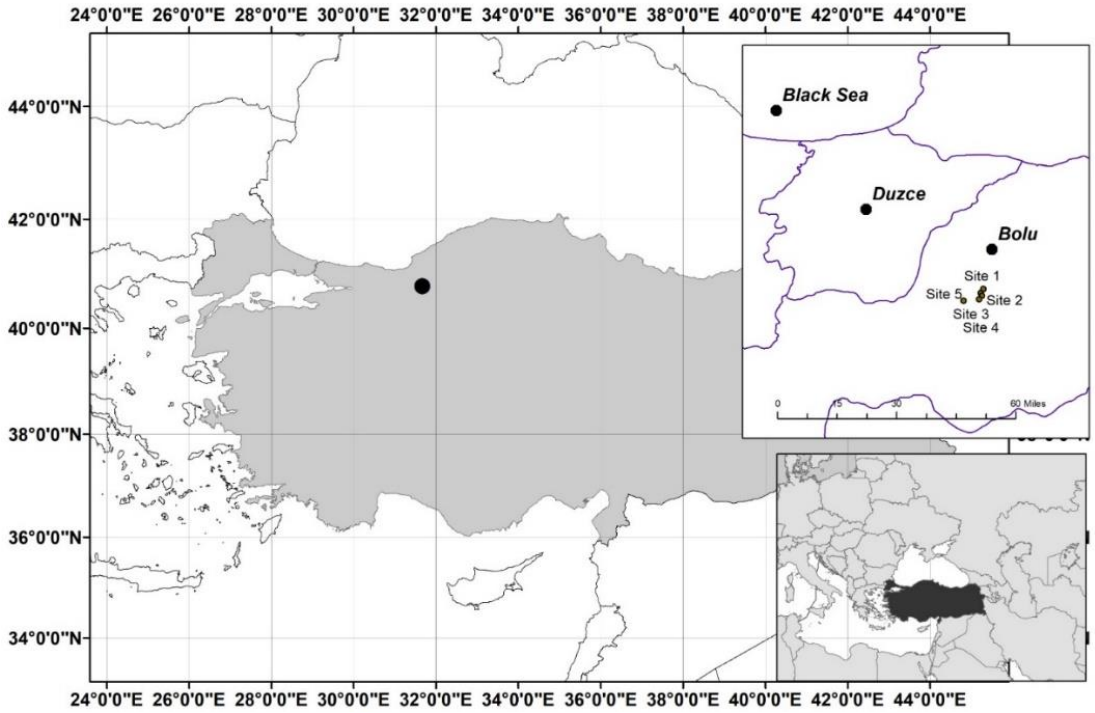

Figure 1. *Location of sampling sites for deposition study in Aladağ fir forest*

For each elevation zone, sampling plots were established, eight on randomly located spots under the canopy and three on adjacent open sites (20-50 m distant). Deposition samples were collected using 500-mL polyethylene (PE) bottles connected to funnels having a 100-cm^2 opening. All sample containers were numbered and placed on top of a PVC pole 1 m above ground. In order to collect stemflow, on each sampling plot, three Grade-3 (diameter class) fir trees were chosen to represent the stand, and spiral collectors were installed on the tree stems at a height of 0.5-1.5 m from the ground (*Fig. 2*).

The collectors were tightly attached to the stem to prevent leakage and containers were placed near the tree. The deposition samples were collected weekly and processed according to Part XIV of the ICP-Forests Manual: "Sampling and Analysis of Deposition, Methods and Criteria for Harmonized Sampling". The average sample at each collection was formed by proportional mixing of samples from each collector in

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order to represent the variability. Each week, the field was visited and samples were collected, placed in boxes containing ice batteries, brought to the laboratory, and stored at 4 °C until they were analyzed.

Figure 2. *Throughfall, open site and stemflow precipitation collection in Aladağ fir forest*

Laboratory analysis

Chemical analyses of the samples were made in the ICP laboratory established within the Aegean Forestry Research Institute in İzmir. The samples were filtered through a 0.45-µm pore membrane before analysis. First, the solution pH (Thermo Scientific™ Orion™ 3-Star Benchtop pH Meter) and electrical conductivity (EC) were measured, and then the ion, anion, and cation concentrations of the samples were analyzed via liquid chromatography (Thermo Scientific Dionex™ ICS-5000+ DC). The mandatory variables required by the ICP-Forests Manual for Level-II sites were analyzed for each sample. For this, each deposition sample was analyzed for fluorine (F^-), chlorine (Cl^-), nitrite (NO_2^-), bromine (Br^-), phosphate (PO_4^{-3}), sulfate (SO_4^{-2}), nitrate (NO_3^-), lithium (Li^+), sodium (Na^+), potassium (K^+), calcium (Ca^{+2}), magnesium (Mg^{+2}), and ammonium (NH_4^+) concentrations.

Statistical analysis

For each variable, one-way analysis of variance (ANOVA) was performed to determine the differences in the sampling plots. The Tukey mean separation test was used for variables where the ANOVA results differed. The non-parametric Kruskal-Wallis test was applied to the total amount of PO_4^{-3}, Cl^-, and NH_4^+ deposition in the open sites and the monthly stemflow NO_2^- and Na^+ data because they did not show normal distribution. The SAS (1996) package program was used in the analysis, with results considered to be different at the level of $\alpha = 0.05$.

Results

The sampling plots at 1400 and 1600 m received about 17% more rainfall than those of the sites at 1000 and 1200 m during the 2013-2014 sampling period. For each sampling site, about 30% of the total precipitation was intercepted by the canopy crown. Stemflow accounted for about 1% of the precipitation (*Fig. 3*).

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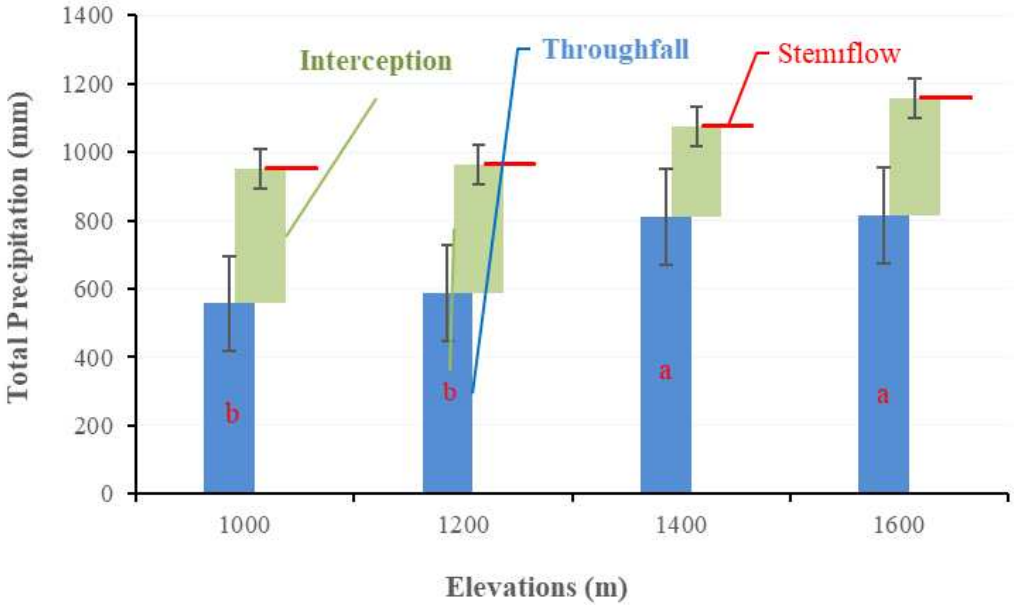

Figure 3. *Means ± standard deviation of annual precipitation (mm) in Aladağ fir forest (means followed with the same letter are not significantly different at α = 0.05*

Since the EC values across the sampling sites were below 0.1 dS m^{-1}, there was no salinity problem in the depositions. The pH values of the deposition samples averaged 6 and were not significantly different among sampling sites.

Anions

Nitrate concentration in both the open site and throughfall depositions were reduced by about 15% and 75%, respectively, above the 1000-m elevation zone. Throughfall NO_3^- concentrations were 600% and 200% greater than in open site samples at 1000 m and other sites, respectively. Sulfate concentrations did not show significant differences among elevation zones. However, SO_4^{-2} concentrations in throughfall samples were 300% higher compared to those of open sites. Stemflow SO_4^{-2} at 1000-m elevation was 200% higher than at the other elevation zones. Phosphate concentrations in both open site and throughfall samples showed significant variation within each sampling plot. Open site Cl^- concentrations were not significantly different among elevation zones; however, they increased 200-300% in throughfall samples compared to those of open sites across all sampling sites. Stemflow Cl^- concentration at 1600 m was 65%, which was 40% lower than at 1000 m and the averages of the 1200 and 1400 m elevation zones. Open site NO_2^- concentration decreased by 75% at 1600 m compared to the other elevation zones. Throughfall NO_2^- concentrations were 600%, 200%, and 1200% higher than those of open sites at 1000-m, 1400-m, and 1600-m elevation zones, respectively. Neither open site nor throughfall F^- concentrations changed significantly among the elevation zones. However, throughfall F^- concentrations were about 250% higher compared to the adjacent open sites for each elevation zone. Stemflow F^- concentration at 1200- and 1400-m elevation zones decreased to half of the values recorded at 1000 m, and decreased again by 50% at 1600 m compared to the previous elevation. Open site, throughfall, and stemflow Br^- concentrations showed significant variation within each sampling plot (*Table 1*).

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Table 1. *Means (mg l^{-1}) ± standard deviation of anion concentrations of throughfall, stemflow, and open site bulk depositions collected at different elevations (m) in Aladağ fir forest*

Elevation (m)		1000	1200	1400	1600
NO_3^-	Throughfall	6.9 ± 3.5aA	1.8 ± 1.01bA	1.6 ± 1.1bA	1.7 ± 1.06bA
	Open site	1.1 ± 0.8aB	0.87 ± 0.6bA	0.79 ± 0.49bA	0.84 ± 0.73bA
	Stem flow	0.7 ± 0.2aB	0.74 ± 0.25aA	0.99 ± 0.35aA	0.27 ± 0.25aA
SO_4^{-2}	Throughfall	13 ± 9aB	13.8 ± 8.8aB	10 ± 8.1aB	14.9 ± 8.7aB
	Open site	3.9 ± 3.1aC	4.3 ± 3.5aC	3.5 ± 2.9aC	3.6 ± 3.2aC
	Stem flow	169 ± 2aA	83 ± 8bA	89 ± 1.1bA	94 ± 1.5bA
PO_4^{-3}	Throughfall	0.6 ± 0.5aA	1.51 ± 1.14aA	0.38 ± 0.21aA	0.07± 0.02aA
	Open site	0.17 ± 0.07aA	0.27 ± 0.23aA	0.08 ± 0.05aA	0.05± 0.02aA
	Stem flow	17 ± 14.5aA	4.1 ± 3.4aA	1.8 ± 1.5aA	2.6 ± 1.8a
Cl^-	Throughfall	3.2 ± 3a	3 ± 2a	2.1 ± 1.1a	2.8 ± 1.9a
	Open site	0.9 ± 0.7a	0.9 ± 0.7a	1 ± 0.89a	0.9 ± 0.8a
	Stem flow	33 ±17aA	21± 13bA	20 ± 16bA	12 ± 10cA
NO_2^-	Throughfall	0.43 ± 0.27aA	0.19 ± 0.14bA	0.15 ± 0.11bA	0.25± 0.16bA
	Open site	0.07 ± 0.004aB	0.11 ± 0.09aA	0.08 ± 0.03aB	0.02 ± 0.01bB
	Stem flow	0.29 ± 0.14aA	0.09 ± 0.05aA	0.27 ± 0.19aA	0.06 ± 0.04aB
F^-	Throughfall	0.13 ± 0.11aA	0.14 ± 0.12aA	0.11 ± 0.08aA	0.11± 0.06aA
	Open site	0.05 ± 0.037aB	0.057 ± 0.036aB	0.044 ± 0.035aB	0.04± 0.036aB
	Stem flow	0.95 ± 0.79aA	0.44 ± 0.39aA	0.46 ± 0.36aA	0.26 ± 0.21aA
Br^-	Throughfall	0.002 ± 0.0004aA	0.006 ± 0.002aA	0.002 ± 0.0009aA	0.003 ± 0.001aA
	Open site	0.003 ± 0.0026aA	0.001 ± 0.0002aA	0.001 ± 0.0008aA	0.003 ± 0.001aA
	Stem flow	0.014 ± 0.012aA	0.078 ± 0.054aA	0.073 ± 0.071aA	0.04 ± 0.03aA

For each row, variable means followed by the same lower-case letter are not significantly different at $\alpha = 0.05$. For each column, variable means followed by an upper-case letter are not significantly different at $\alpha = 0.05$

For stemflow, no correlation was detected between anion concentrations and tree diameter.

Cations

None of the open site, throughfall, or stemflow K^+ concentrations were significantly different among elevation zones. However, throughfall K^+ concentrations were 300-500% higher than those of open site plots across the elevation zones. Stemflow K^+ concentrations were 400–700% higher than those of throughfall at each elevation zone. Open site NH_4^+ concentrations at 1200 m were 250% higher than those at 1000-m elevation, but this value decreased by 75% at the 1400- and 1600-m elevation zones compared to values at 1200 m. However, throughfall and stemflow NH_4^+ concentrations were not significantly different from those of open site plots at any of the elevation zones. Neither open site nor throughfall Ca^{+2} concentrations were significantly different among elevation zones. However, throughfall Ca^{+2} concentrations at 1000-m elevation zones were 150% higher compared to the adjacent open site plots. Stemflow Ca^{+2} concentrations at 1600 m decreased to 50% of those at other elevation zones. Stemflow Ca^{+2} concentrations were about 500% higher than those of the open site plots at each elevation zone. Neither open site nor throughfall Na^+ concentrations varied significantly among elevation zones. However, throughfall Na^+ concentrations were about 150% higher than

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those of adjacent open site plots at 1000-m elevation zones. Stemflow Na^+ concentrations were about 400% higher than those of throughfall at each elevation zone. Stemflow Na^+ concentrations at 1600 m decreased by about 30% compared to the other elevation zones.

Neither open site nor throughfall Mg^{+2} concentrations significantly differed among elevation zones. However, stemflow Mg^{+2} concentrations at 1600 m decreased by about 50% compared to those at the other elevation zones. Throughfall Mg^{+2} concentrations were about 250% higher than those of the adjacent open site plots at each elevation zone. Stemflow Mg^{+2} concentrations were 300%–1000% higher than those of throughfall at each elevation zone. Neither open site nor throughfall Li^+ concentrations showed significant differences among elevation zones. Throughfall Li^+ concentrations were also similar to those of the adjacent open sites at each elevation zone; however, stemflow Li^+ concentrations at 1200-, 1400-, and 1600-m elevation zones were almost 400% higher than open site and throughfall concentrations (*Table 2*).

Table 2. *Means (mg l^{-1}) ± standard deviation of cation concentrations of throughfall, stemflow, and open site bulk depositions collected at different elevations (m) in Aladağ fir forest*

Elevation (m)		1000	1200	1400	1600
K^+	Throughfall	10 ± 6.3aB	15 ± 9.6aB	8.5 ± 4.8aB	8.9 ± 3.2aB
	Open site	2.1 ± 1.2aC	2.1 ± 1aC	2.9 ± 2.5aC	1.9 ± 0.9aC
	Stemflow	76.5 ± 55aA	61 ± 45aA	58 ± 43aA	42 ± 23.19aA
NH_4^+	Throughfall	1.3 ± 1.01aA	2.1 ± 1.8aA	1 ± 0.7aA	0.86 ± 0.71aA
	Open site	1.13 ± 1.01bA	2.7 ± 1.9aA	0.9 ± 0.8bA	0.97 ± 0.78bA
	Stemflow	3.18 ± 3.09aA	2.2 ± 2aA	2.8 ± 2.5aA	3.1 ± 2.7aA
Ca^{+2}	Throughfall	6.9 ± 4.6aB	6.4 ± 3.7aB	5.2 ± 3.5aB	5.7 ± 2.7aB
	Open site	3.4 ± 2.8aC	3.9 ± 3.2aB	3.2 ± 2.1aB	3.1 ± 1.9aB
	Stemflow	37 ± 20aA	27 ± 20aA	33 ± 20aA	17 ± 13bA
Na^+	Throughfall	1.6 ± 1.1aB	1.56 ± 1.4aB	1.2 ± 0.9aB	1.4 ± 1.1aB
	Open site	0.7 ± 0.5aC	1 ± 0.76aB	0.7 ± 0.5aB	0.68 ± 0.47aB
	Stemflow	6.2 ± 4.3aA	6.1 ± 5.5aA	7.3 ± 5.9aA	3.9 ± 3.8bA
Mg^{+2}	Throughfall	1.14 ± 0.91aB	1.2 ± 0.9aB	0.8 ± 0.5aB	1 ± 0.8aB
	Open site	0.32 ± 0.19aB	0.4 ± 0.3aB	0.4 ± 0.3aB	0.26 ± 0.19aB
	Stemflow	8.6 ± 5.4aA	6 ± 4.3aA	7.3 ± 5aA	3.6 ± 2.5bA
Li^+	Throughfall	0.001 ± 0.001aA	0.001 ±0.001 aB	0.001 ± 0.001aB	0.001 ± 0.001aB
	Open site	0.0036 ±0.001 aA	0.0010 ± 0.001aB	0.0010 ± 0.001aB	0.003 ±0.001 aB
	Stemflow	0.0014 ± 0.001bA	0.0057 ± 0.001aA	0.0044 ± 0.001aA	0.005 ± 0.001aA

For each row, variable means followed by the same lower-case letter are not significantly different at α = 0.05. For each column, variable means followed by an uppercase-letter are not significantly different at α = 0.05

For stemflow, no correlation was detected between cation concentrations and tree diameter.

Total deposition

The total amount of ion deposition was calculated as kg ha^{-1} yr^{-1} using the precipitation and the concentrations for each sample. The annual SO_4^{-2} deposition in the open site plots was higher than the sum of the other anions at each elevation zone (*Fig. 4*). The amount of NO_2^-, F^-, and Br^- depositions in the open site plots remained below 1 kg across the elevation zones.

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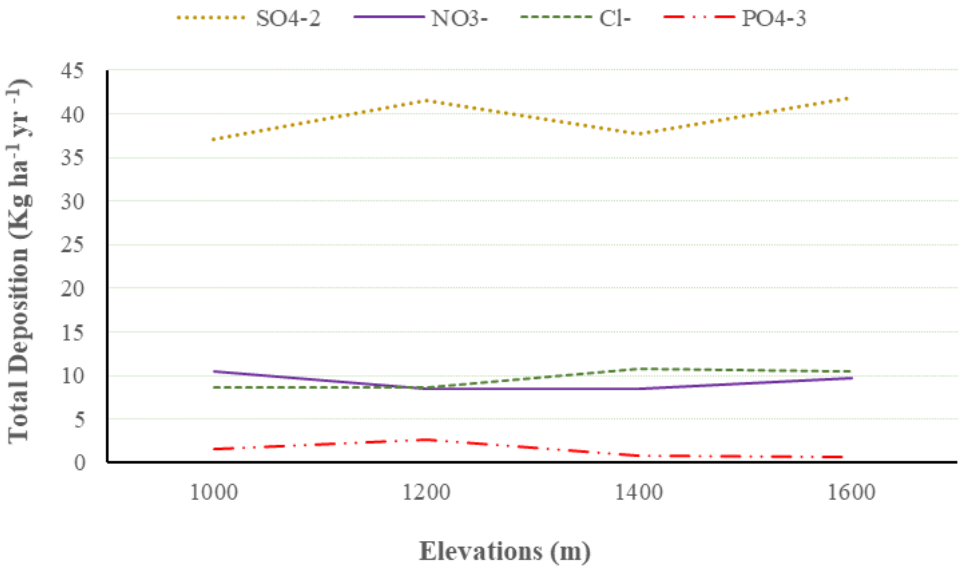

Figure 4. *Means ± standard deviation of open site total anion deposition (Kg ha^{-1} yr^{-1}) collected at different elevations (m) in Aladağ fir forest*

The open site Ca^{+2} and K^{+} depositions were higher than the other cations at each elevation zone. However, the open site NH_4^{+} deposition at 1200 m was about twice as high as that at the other elevation zones (*Fig. 5*).

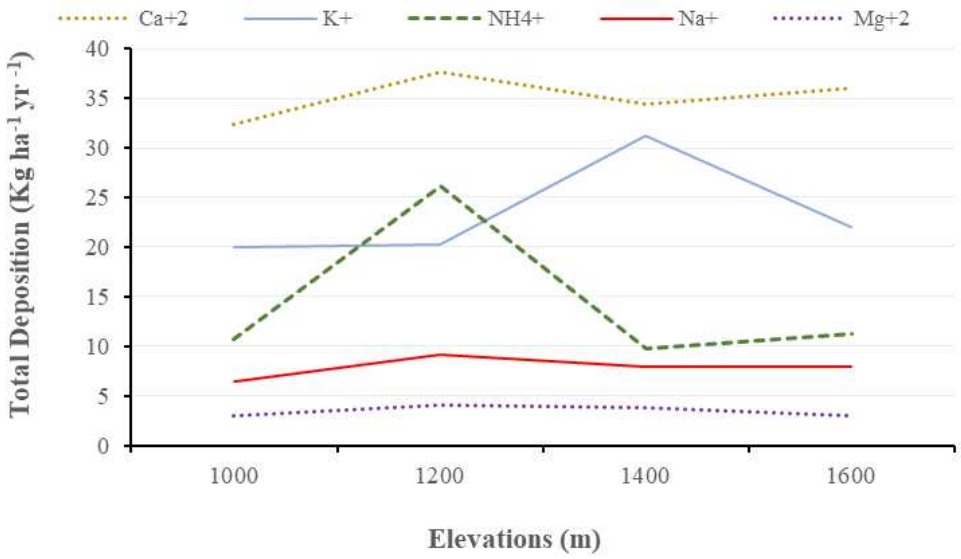

Figure 5. *Means ± standard deviation of open site total cation deposition (Kg ha^{-1} yr^{-1}) collected at different elevations (m) in Aladağ fir forest*

Throughfall SO_4^{-2} deposition was 50% higher at 1600 m compared to that at the other elevation zones. Throughfall NO_3^{-} deposition decreased by 75% above the 1000-m elevation zone (*Fig. 6*). Throughfall SO_4^{-2}, NO_3^{-}, Cl^{-}, and PO_4^{-3} deposition values were about 200%, 300%, 300%, and 400% higher than those of the adjacent open site plots at each elevation zone (*Fig. 6*).

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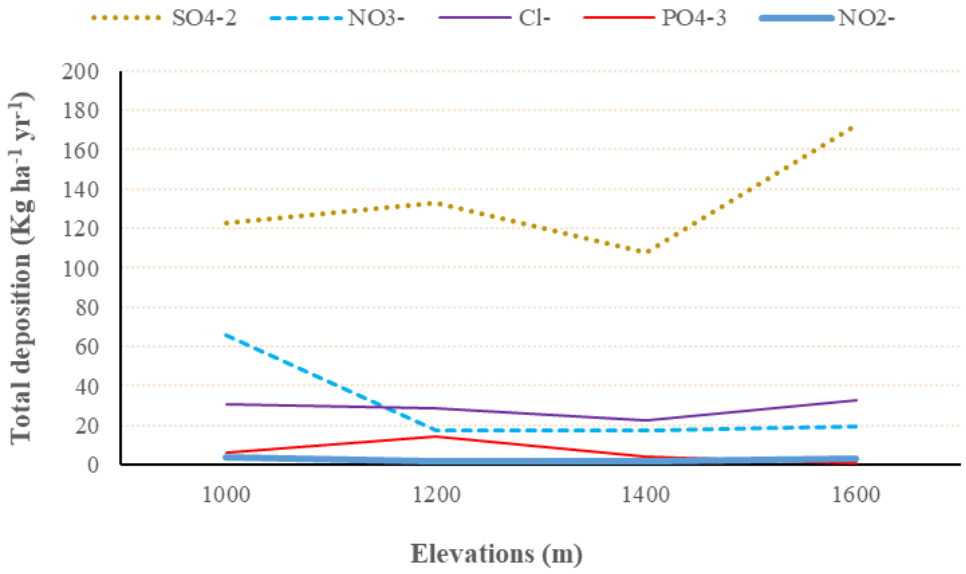

Figure 6. *Means ± standard deviation of throughfall total anion deposition (Kg ha^{-1} yr^{-1}) collected at different elevations (m) in Aladağ fir forest*

Throughfall K^+ deposition at 1200 m was about 40% higher than that at the other elevation zones (*Fig. 7*).

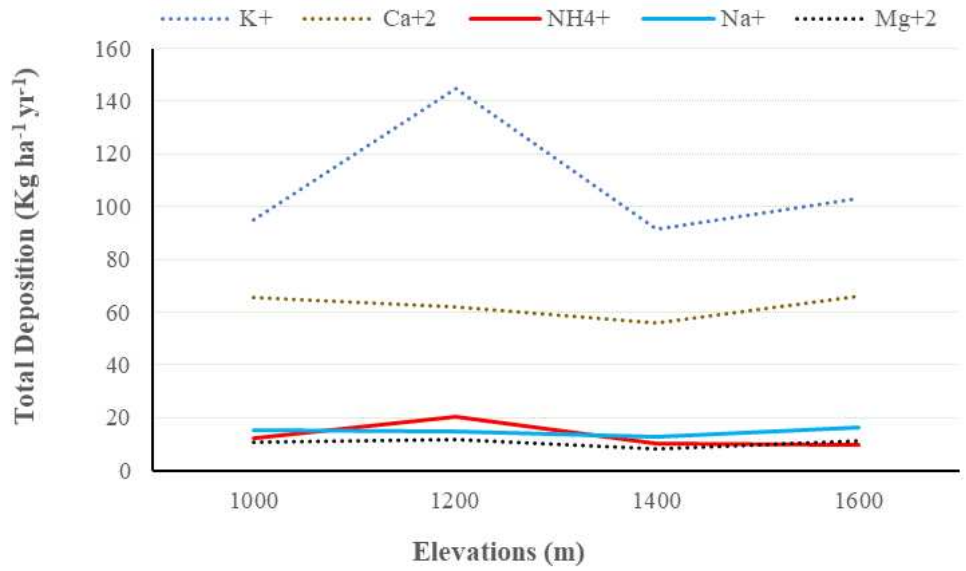

Figure 7. *Means ± standard deviation of throughfall total cation deposition (Kg ha^{-1} yr^{-1}) collected at different elevations (m) in Aladağ fir forest*

Throughfall K^+, Mg^{+2}, Na^+, Ca^{+2}, and NH_4^+ deposition values were about 400%, 300%, 200%, and 200% higher, respectively, than those of the adjacent open site plots at each elevation zone (*Fig. 7*).

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Discussion

Canopy interception rate is related to the amount and intensity of precipitation, its distribution, and the stand type. In the current study, about 29% of the rainfall was intercepted by the fir canopy. In a study conducted by Garces et al. (2012) in the humid forests of Colombia having approximately 1500 mm of rainfall, the corresponding rate was about 19%. The differences between the current study and that of Garces et al. (2012) may be attributed to the stand type since the current study was carried out in a closed-canopy fir forest. Krupová et al. (2017) reported that the pH of precipitation collected between 2008 and 2010 was 5.5 and 6 in the spruce and fir forests of Slovakia, respectively. They reported a lower pH value in throughfall deposition compared to that of the open site plots. The higher pH value in the present study can be partially explained by the high base cation concentrations of the depositions.

In the current study, either the open site or the throughfall ion concentrations recorded at four elevation zones were within the specified ranges presented in the ICP-Forests Manual. However, the SO_4 and NO_3 anion and K and Ca cation values were higher compared to the other ions in the current study (*Table 3*).

Table 3. *Limits for some of the deposition variables in Part XIV of the ICP-Forests Manual and corresponding values in bulk depositions collected at different elevations (1000-1600 m) in Aladağ fir forest*

Variables	ICP-Min.	ICP-Max.	1000 m	1200 m	1400 m	1600 m
Reaction (pH)	2.5	9.4	6	6	6	6
EC (µS/cm)	1	10000	<100	<100	<100	<100
K (mg L^{-1})	0.002	250	8.2	11.8	7.07	7.2
Ca (mg L^{-1})	0.001	275	6.1	5.8	4.7	5.1
Mg (mg L^{-1})	0.0025	100	0.95	1	0.66	0.82
Na (mg L^{-1})	0.003	500	1.4	1.41	1.06	1.25
NH_4 (mg L^{-1})	0.002	175	1.3	2.3	0.96	0.89
Cl (mg L^{-1})	0.002	800	2.7	2.5	1.8	2.4
NO_3 (mg L^{-1})	0.002	175	5.5	1.55	1.36	1.52
SO_4 (mg L^{-1})	0.01	500	10.8	11.4	8.4	12.03
PO_4 (mg L^{-1})	0.0017	1000	0.49	1.21	0.31	0.07

Even though nitrogen is one of the most important plant nutrients in terrestrial ecosystems, until the 1980s its deposition was considered one of the main destructive agents in a majority of European forest. In addition to nitrogen, sulfate is also one of the most important air pollutants. Over-loaded NO_x and SO_x emissions from increased burning of fossil fuels and intensive agricultural and industrial activities had negatively affected European forest for several decades (Fisher et al., 2007; Erisman et al., 2008; Novotný et al., 2016; Thimonier et al., 2019). Following the framework agreement signed in 1979 to reduce the growing air pollution, atmospheric pollutants such as sulfur and nitrogen were targeted to remain within certain limits with the protocols of Aarhus 1998, Geneva 1984, Helsinki 1985, Soa 1988, and Gothenburg 1999. While some European countries have rapidly complied with the requirements of the agreement by preparing critical precipitation threshold maps, other countries have not been able to contribute sufficiently to reducing pollutants (Grennfelt et al., 2020). In Turkey, measures

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concerning the release of industrial facility pollutants into the atmosphere were not as strictly regulated as in European countries. In addition, coal was used as fuel in most settlements in the Western Black Sea Region until 2005. Wood and coal are still used as the main fuel in rural areas. Although some air pollution variables are measured in terms of public health in city centers, there is insufficient data on the effects of these pollutants on forests, agriculture, and aquatic ecosystems, and the legal legislation to reduce them is either limited or inadequate. Therefore, pollutants released from settlements, intensive agricultural practices, and industrial activities can be transported by atmospheric movement and deposited on natural ecosystems in the vicinity of the source of the pollutants.

Research conducted in beech, spruce, and fir forests in central and western Europe has revealed that nitrogen deposition above 15-20 kg ha^{-1} may seriously affect above- and belowground ecosystem properties including biological diversity (Bobbink and Hettelingh, 2011; Schmitz et al., 2019). Waldner et al. (2015) stated that human-induced atmospheric sulfur and nitrogen deposition after the second half of the 20th century had acidified most of the forest soil. Schleppi et al. (2017) reported that 10-15 kg ha^{-1} annual nitrogen deposition in many European forests may accelerate nitrate leaching and result in water pollution. In the current study, the total open site and throughfall nitrogen deposition (NH_4 + NO_3) values were 200% and 300% more than those reported by Bobbink and Hettelingh (2011). Annual open site SO_4^{-2} deposition in the current study was about 40 kg/ha. Throughfall deposition was 300% higher than that of open site plots at 1000–1400-m elevation zones. The fate of the nitrogen deposited on forest soils depends on the interaction of many variables. The impact of nitrogen deposition on fir forests in the current study may not follow the same direction as many research findings in central and northern European forests. Excessive nitrogen deposition may lead to nutrient imbalance in saturated soils and it also increases plant sensitivity to insect and fungal diseases. Although there has been a problem of nitrogen saturation for many years in central and western European forests, each increment in nitrogen deposition has a negative impact on these forests (Schmitz et al., 2019; Etzold et al., 2020). On the other hand, poor soils may benefit from the fertilizing effect of nitrogen deposition (Waldner et al., 2015). Soil nutritional imbalance not only may impede the growth and physiological condition of plants, but it may also render forest ecosystems vulnerable to insect attacks. Yildiz et al. (2007a) reported that the fir-bark beetle (*Pityokteines curvidens* (Germ.)) and small fir-bark beetle (*Cryphalus piceae* (Ratz.)) cause a significant amount of tree loss in the Western Black Sea fir forests where the current study was conducted. They also reported that insect attacks decreased when the needle calcium content increased.

The acidifying effect of atmospheric depositions on soils varies depending on soil properties. For example, some soils with abundant base (K, Ca, and Mg) cations may display higher buffering capacity (Yildiz et al., 2007a). Yildiz et al. (2007b) reported that the soil in the region contains 400 kg ha^{-1} Ca, 160 ha^{-1} K, and 160 ha^{-1} Mg in the first 20-cm depth of the soil. The soil in the region has a substantial amount of acid neutralizing capacity for incoming atmospheric deposition. The anion and cation balance of the atmospheric deposition may also determine its acidifying capacity. By simply calculating the acid neutralization capacity (ANC) of the deposition at each sampling site using the *equations 1 and 2*:

$$(ANC) = \sum (\text{base cations}) - \sum (\text{strong acid anions}) \quad \text{(Eq.1)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1806_76277641

or

$$ANC = \sum (Ca + K + Mg + Na) - (SO_4 + NO_3 + Cl) \quad \text{(Eq.2)}$$

The open site deposition ANC values at 1000-, 1200-, 1400-, and 1600-m elevation zones were found as 0.6, 1.31, 1.91, and 0.6, respectively. The ANC values of throughfall depositions were calculated as -3.36, 5.58, 1.95, and -2.41, respectively. Although the open site SO_4^{-2} deposition was high, base cations of the same depositions were also relatively high. Thus, the acid neutralization capacity of open site depositions remained high along all elevation zones. However, the acidifying potential of the throughfall depositions at 1000 and 1600 m was higher than at the other elevation zones.

Kimmins (1997) estimated that 45%, 35%, 66%, and 69% of the N, P, K, and Ca cycles, respectively, were biogeochemical cycles. Andesite, which constitutes the bedrock in the study sites, is mostly composed of plagioclase, hornblende, pyroxene, and biotite minerals. Plagioclase is one of the main sources of calcium in the soil. Augite and hornblende from pyroxenes also contain significant amounts of calcium. Biotite, also a component of andesite, contains a significant amount of potassium. Thus, a significant amount of calcium and potassium deposition may have local sources from the underlying bedrock. Kimmins (1997) also claimed that 31% of calcium and 12% of potassium have a geochemical cycle. Thus, some of the potassium and calcium deposited on the fir forests in the current study may have been transported from Central Anatolia via atmospheric movements. Stable isotope techniques can help to trace the source of the deposition. Demir et al. (2017) maintain that if the NO_3^- / SO_4^{-2} deposition ratio is less than "1", then the pollutant source may be local. Since in the current study this ratio is about 0.24, settlements and recreational activities in the region may have been a source of the pollutants. Bayramoğlu Karşı et al. (2017) stated that coal and wood fuels are among the most important causes of atmospheric potassium deposition. Moreover, agricultural practices may be one of the important sources of nitrogen deposition near forest areas (Waldner et al., 2015; Kosonen et al., 2019). Thus, intensive poultry farming, recreational activities, and the use of wood and coal as a fuel in settlements around the current study sites may contribute to the atmospheric pollution in the region.

The cumulative effects of increased deposition over decades may make the forest ecosystem vulnerable to diseases and prone to further disturbances. Due to their biological structure, most lichen and algae species are sensitive to acid-forming pollutants such as sulfur and nitrogen. Upon conducting a research in a coniferous forest in Finland, Salemaa et al. (2020) reported that an annual nitrogen deposition of more than 5 kg ha^{-1} significantly affected algae species. Moreover, there has been a significant loss in lichen diversity in European forests due to air pollution (Agnan et al., 2017). Studies conducted by Agnan et al. (2017) in the forests of France and Switzerland revealed that the lichen diversity index is strongly related to eutrophication. In a study conducted by Şahin (2017) in the Aladağ fir forests of the current study, 56 lichen taxa belonging to 33 genera were identified. He found that the population of *Bryoria*, *Lobaria, Ramalina,* and *Usnea,* as species sensitive to air pollution, decreased at 900–1100-m elevation zones, where heavy sulfate and nitrogen depositions were reported in the current study. Şahin (2017) also claimed that pollutant-sensitive species such as *Evernia* and *Ramalina* had not been encountered around the recreational areas. Therefore, even though the annual deposition concentration falls within certain limits, the accumulation of nitrogen and sulfur components over many years may cause sensitive species to be adversely affected.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76277641

The current study covered 1000–1600-m elevation zones. However, the ICP-Forests Level-II plots were established at 1600 m to monitor the regional fir forests. Therefore, the Level-II site located at only one elevation may not fully reflect the deposition on the fir forests of the region.

Conclusion and Suggestions

Sulfate, nitrogen, potassium, and calcium were the main ions deposited in the fir forests at the 1000–1600-m elevation zones in Aladağ. Even though only about 1/3 of the precipitation was intercepted, throughfall ion deposition was generally higher than the open site deposition, indicating that a significant amount of pollutant is captured by the canopy cover. This result implies the possibility of the filtering effects of forest canopy on pollutants deposited in openings around the forest. Therefore, instead of the forest openings, it would be more accurate to set up open site sampling plots above the canopy cover with the use of towers. The acid neutralization capacity of the deposition samples collected in the open site plots was high at all elevation zones. However, the acidifying capacity of the throughfall deposition at 1000 and 1600 m was higher than at the other elevation zones. Although the ion concentration values of the depositions remained within the ranges set by the ICP-Forests guidelines, the nitrogen and sulfur values of the current study were higher than those reported in many studies conducted in European forests. Different scenarios need to be developed addressing the problems that may arise from the long-term accumulation in the ecosystem, rather than focusing on annual concentration values. In addition, establishment of only one Level-II site at 1600 m for monitoring the condition might have been insufficient as a representation of fir forests. Therefore, either more Level-II monitoring plots should be established to represent different elevation zones or supporting studies should be conducted to increase the data validation.

Acknowledgements. This research was funded by the Duzce University BAP program through the project entitled “Bolu-Aladağ, Uludağ Göknarı (*Abies bormülleriana* Mattf.) Ormanlarında Atmosferik Çökelme [Atmospheric deposition in mature fir (*Abies bormülleriana* Mattf.) stands in Aladağ- Bolu]” and numbered as DÜBAP 02.02.213.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76277641

EFFECTS OF STAGE DROUGHT AND RE-WATERING ON PHOTOSYNTHESIS, ROOT SHOOT RATIO AND WATER USE EFFICIENCY OF SUMMER MAIZE (*Zea mays* L.)

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(Received 9th May 2020; accepted 29th Jul 2020)

Abstract. Field experiments were carried out to evaluate the effect of levels water stress and re-watering the growth characteristics and water use efficiency of summer maize (*Zea mays* L.) at different growth stages. The experiment included 3 water stress treatments at jointing-tasseling and flowering-filling stages, full water supply was set as a reference. The photosynthesis, dry matter accumulation, yield and water use efficiency were compared and analyzed under different drought and re-watering conditions. Results showed that the inhibition of photosynthesis not only occurred under of water stress, but also existed after re-watering. The degree of inhibition was related to both the degree of water stress and the growing stage. Meanwhile the degree of water stress and the growing stage were closely related to the stomatal restriction inhibiting the photosynthesis. The effects of moisture variation on dry matter distribution between root and cap were distinctive at different growing stages. The yield of maize did not significantly differ between the Treatment1, Treatment4 and control treatments, while the water utilization efficiency of Treatment1 and Treatment4 was higher that of the control check. Therefore, reasonable water stress not only ensures yield, saves irrigation water, but also effectively improve water use efficiency.
Keywords: *drought stress, rewater, biomass measurement, physiological measurement, water consumption*

Introduction

Henan province is the greatest agricultural province and the main grain-producing region of China. Affected by the climate of the Huang-Huai-Hai river basin, the region has complex hydrometeorological conditions and frequent drought disasters. Water deficit has become a bottleneck restricting the sustainable development of agriculture (Zhang et al., 2008, 2013) and affecting agricultural production and economic development seriously (Wang et al., 2014). Water deficit is one of the main factors affecting crop growth and yield (Kulkarni and Phalke, 2009; Nielsen et al., 2009; Peng et al., 2014). The effect of water on crop and the response of crop to water are very complex, there have been published research results about it at home and abroad. Studies on the effects of drought on summer maize have been a subject of scientific interest in recent years. Wu et al. (2015) revealed that effects of drought stress at different growth stages on the growth and yield of maize in Northwest Shandong through field experiments under rain-proof shed. Ji et al. (2012) concluded that the yield variation of maize in Northeast China was closely related to the growth stage of drought by field pond planting test. Guan et al. (2012) studied the effects of early flowering on growth, physiological characteristics and yield of potted maize and compensated growth of plants after re-watering. However,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76437653

the most of experiments were conducted under short-term drought stress at different growth stages, and there were regional differences, while the effects of short-term drought and long-term drought stress were significantly different. Barrel planting simulation experiments on maize response to persistent drought at different growth stages and even the whole growth period is still rare. Photosynthetic characteristics, root canopy and yield of maize are most directly related to drought, which a large number of studies have revealed the relationship between stomatal conductance, net photosynthetic rate and chlorophyll fluorescence of maize leaves and drought severity and growth period (Yao et al., 2012; Yu et al., 2015; Cai et al., 2017). Mangani et al. (2018) reported that drought decreased stomatal closure and photosynthesis. Other studies showed that drought significantly affected the root-shoot ratio of maize and was related to water stress, there were differences between root and shoot compensation effect in different growth period after re-watering (Li et al., 2016; Meng et al., 2016). Mi et al. (2017) through field experiments concluded that the degree of drought could quantitatively express the soil drought condition and there was a quantitative relationship between drought degree and maize yield reduction. Some studies have revealed the response of plant growth to drought and effect on yield (Kato et al., 2008; Daryanto et al., 2016, 2017; Wang et al., 2017). In addition, some studies have analyzed the growth and development, physiological processes and molecular genetic characteristics of maize in response to drought and used them to explain the effect of drought on yield (Deng et al., 2009; Zheng et al., 2010; Qi et al., 2010; Xu et al., 2017; Meng et al., 2018).

Maize is one of the most important crops in China and summer maize is a major food crop in North Plain China. As the main autumn grain, summer maize often suffered from drought during its growing period, which seriously affected the growth and final yield of crops and caused great agricultural economic losses. It is very important to study the effects of different degrees of drought after re-watering on maize growth and yield at different stages, and to determine the boundary thresholds of water demand time and quantity for drought recovery. It is the basic work of optimizing irrigation system and efficient utilization of water resources. Although there is a lot of research on maize under drought conditions, there is still no systematic and in-depth experimental study on the effects of re-watering on physiological characteristics and yield of maize after drought. Therefore, summer maize which the main economic crops in Huang-Huai-Hai Plain was taken as the research object, studied the effects of water deficit and re-watering at different growth stages on the growth and physiological characteristics of summer maize and analyzed the mechanism of photosynthetic characteristics, root cap and yield responses to drought of summer maize, which provided a certain technical basis for optimizing irrigation schedule and forecasting the influence of drought stress on the development trend of maize.

Materials and methods

Experimental design

The experiment was carried out in the barrel planting experimental area under the rainproof shed at Comprehensive Experimental Base of CHINESE ACADEMY OF AGRICULTURAL SCIENCES (35°18′N, 113°54′E, Height 81 m) from June to October, 2017. The average temperature of the area is 14 ℃, the frostless period is 210 d, the sunshine time is 2399 h and the precipitation is 580 mm. The summer maize variety was *Denghai 605* which was suitable for farming in Henan province, with good adaptability,

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DOI: http://dx.doi.org/10.15666/aeer/1806_76437653

good stable yield and high yield. They were sown on June 10, 2017, with two plants per barrel at three-leaf period. Water treatment at different growth stages is shown in *Table 1*. The water stress was set at four levels, such as full water supply, slight water stress, moderate water stress, severe water stress at jointing-tasseling stage and flowering-filling stage. The soil water content was controlled at 70%-80%, 60%-70%, 50%-60% and 40%-50% of field moisture capacity, respectively, expressed by CK, L, M and S. The experiment consisted of 7 treatments and each treatment was repeated 3 times. Soil moisture was controlled by daily weighing, and the daily amount of irrigation was the difference between the barrel weight of the adjacent two days, which is calculated in 60 cm deep soil layer and was measured accurately by measuring cylinder. The soil moisture content in the planned moisture layer (0-60 cm) was determined by drying method every 5 days, so that the irrigation could reach the upper limit of water control. The diameter and height of the test barrel were 40 cm and 60 cm, respectively. The compound fertilizer was applied at 10 g per barrel (2:1 ratio of N:P), and the soil bulk density of each barrel was controlled at 1.36 g/cm. All P and K fertilizers applied to summer maize were basal application.

Table 1. *Barrel planting test treatment*

Water treatment	Seedling stage	Jointing-tasseling stage	Flowering-filling stage	Milking stage
CK	CK	CK	CK	CK
T1	CK	L	CK	CK
T2	CK	M	CK	CK
T3	CK	S	CK	CK
T4	CK	CK	L	CK
T5	CK	CK	M	CK
T6	CK	CK	S	CK

Observation items

Determination of soil moisture content

The irrigation amount was measured and recorded every day, then calculated soil moisture by the weighting method. The soil moisture content in the planned moisture layer (0-60 cm) was measured by drying method every 5 days after the begin of drought treatment, and taken soil sample every 20 cm to determine the irrigation amount. The formula was as follows:

$$W = \gamma HA(W_s - W_o) \quad \text{(Eq.1)}$$

where: W is the irrigation amount (mL), γ is the soil bulk density (g/cm), H is the planned moisture layer (cm), A is the surface area of the barrel (cm^2), W_s (%) is the upper limit of design irrigation and W_0 (%) is the measured soil moisture content before irrigation.

Leaf photosynthesis parameters

Leaf photosynthetic rate P_n was measured by LI-6000 portable photosynthetic apparatus (LI-COR, USA) from 9:00 to 11:00, and stomatal conductance G_s was

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DOI: http://dx.doi.org/10.15666/aeer/1806_76437653

measured by AP4 plant porometer at jointing-tasseling stage and flowering-filling stage, respectively, weather is sunny and calm. The concentration of CO_2 was set at 400 μmol/mol, the flow rate was set at 500 μmol/($m^2 \cdot s^2$), and the light intensity was set at 1000 mmol/($m^2 \cdot s^2$) which was provided by the LED of the system-self. The third leaf from the top of maize was selected at jointing stage, which grew in the same direction, and the leaf position was consistent and fully expanded. Then three ear-leaves with uniform growth and light direction were selected at tasseling-filling stage. The photosynthetic rate was measured on the 0, 1 and 10 days after re-watering, and repeated three times for each treatment.

Root acquisition

During the milk stage of maize, firstly, the above-ground plants were cut off, second, the soil column that contain the root system was removed from the barrel to put into the prepared nylon net bag and soaked in the pool until the soil column becomes loose, then washed clean and dried finally. The dry weight of the root, canopy, ear, stem and leaf of the maize per plant were measured respectively and repeated three times for each treatment.

Biomass measurement

During the milk stage of maize, the above-ground biomass was classified according to the stem, leaf and ear organs. Then the samples were packed in a constant temperature drying oven and heated. The temperature of deactivation of enzymes in 100℃-105℃ for 0.5 hours, and then maintained at 70-80℃. After a week (The quality of the plant was constant), the dry matter of maize was weighed according to stem, leaf and ear.

Yield

After harvested artificially, threshed and dried, then grain number per spike and 100-grain weight were measured.

Water consumption

The water consumption of maize was calculated by water balance method. The maize experiment was conducted in barrel planting under rain-proof shed, so the rainfall, surface runoff and groundwater recharge could be neglected. Therefore, the formula of water consumption can be simplified to:

$$ET = \Delta W + I \quad \text{(Eq.2)}$$

where: ET is water consumption (mm), ΔW is 0~60 cm soil water storage change, I is irrigation amount (mm).

WUE is defined as:

$$WUE = \frac{Y}{ET} \quad \text{(Eq.3)}$$

where: WUE is water use efficiency (g/m^2·mm), Y is yield (g).

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Statistical analysis

Microsoft Excel and DPS 12.01 software were used for statistical analysis, and the least significant difference (LSD) method was used for the significance test with $P<0.05$ level and analysis of variance (ANOVA).

Result analysis

The effect of re-watering on stomatal conductance and net photosynthetic rate of maize at different growth stages after water stress

Fig. 1 and *Fig. 2* showed the change of stomatal conductance and net photosynthetic rate of maize under different water stress and re-watering later at two different growth stages. The stomatal conductance and net photosynthetic rate of maize leaves showed a gradual decline trend with the intensification of water stress. The more serious the water stress, the more obvious the decrease of stomatal conductance and net photosynthetic rate. Compared with CK, the stomatal conductance of T1 to T3 decreased by 26.6%, 40.1% and 64.8%, and the net photosynthetic rate decreased by 13.7%, 25.8%, 34.3%, the stomatal conductance of T4 to T6 decreased by 23.5%, 60.7% and 75.5%, and the net photosynthetic rate decreased by 4.1%, 17.4% and 62.4% respectively, the stomatal conductance and net photosynthetic rate with T1 to T6 showed significant difference with CK. After 1 day of re-watering the stomatal conductance and net photosynthetic rate of maize leaves were restored, but there were still significant differences between T1 to T6 from CK. Because the compensation effect usually needed a period of adjustment. After 10 days of re-watering, the stomatal conductance and net photosynthetic rate of maize began to recover significantly after different water stress treatments. There was no significant difference between T1, T2 and T3 in stomatal conductance and net photosynthetic rate, but there was still significant difference with CK. The stomatal conductance and net photosynthetic rate of T4 began to recover greatly, there was no significant difference with CK. But the stomatal conductance and net photosynthetic rate recovery of maize with T5 and T6 were limited, and there was a significant difference with CK. The stomatal conductance and net photosynthetic rate of maize at flowering-filling stage were significantly lower than jointing-tasseling stage after re-watering for 10 days under the same water stress treatments. Results indicated that the inhibition of photosynthesis not only occurred in the control process of water stress, but was still inhibited to some extent after re-watering, and the degree of inhibition was related to the degree of stress and growth stage.

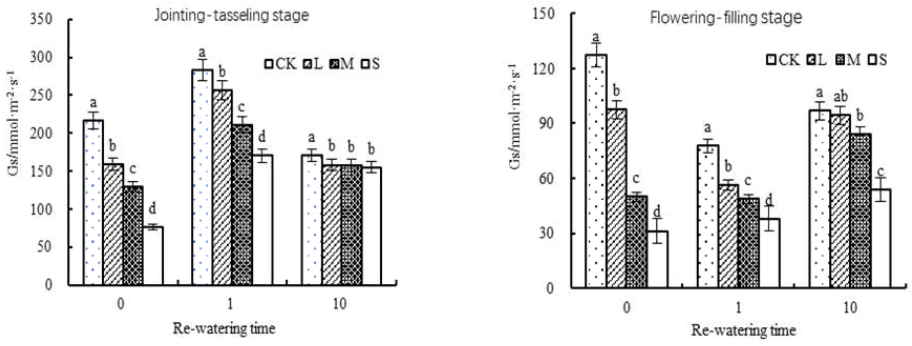

Figure 1. *Effects of re-watering on stomatal conductance of maize under water stress at different growth stages*

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DOI: http://dx.doi.org/10.15666/aeer/1806_76437653

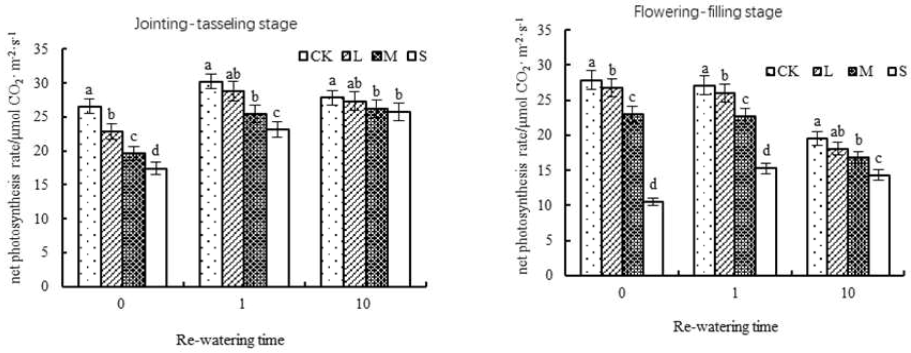

Figure 2. *Effects of re-watering on net photosynthetic rate of maize under water stress at different growth stage*

The effect of re-watering on dry matter distribution, root shoot ratio and yield of maize at different growth stages after water stress

The changed of dry matter distribution, root-shoot ratio and yield of maize under different water stress conditions were listed in *Table 2*. There was significant difference between the root dry weight and the crown dry weight per plant in T2, T3, T5 and T6 with CK, T1 and T4 was no significant difference, which indicated that water stress can inhibit the root and crown growth of maize. With the intensive of water stress, CK>T1>T2>T3, CK>T4>T5>T6, the root and the crown dry weight per plant of maize showed a decreasing trend. Water stress inhibited the growth and development of maize canopy and root system, the dry weight of canopy and root system were lower than CK in all treatments. However, the root-shoot ratio of T6>T5>T4>CK, T3>T2>T1>CK was found by compared the root-shoot ratio of each treatment. Results indicated that water stress inhibited the growth and development of root system more severe than the canopy, and T6>T3, T5>T2, T4>T1 showed the root-crown ratio of maize from flowering to filling stage was higher than that from jointing stage to heading stage under the same water stress. It showed that dry weight of stem and leaf per plant of maize was T6>T5>T4>CK, T3>T2>T1>CK in the *Table 2*, with the aggravation of water stress, dry weight of stem and leaf per plant increased gradually, the dry weight of per ear was CK>T1>T2>T3, CK>T4>T5>T6, the dry weight per ear showed a decreasing trend with the aggravation of water stress. Results indicated that with the intensification of water stress, the dry matter accumulation of each organs of maize under different water stress showed the same trend. The proportion of dry matter accumulation of stem and leaf per plant increased gradually, and the proportion of dry matter accumulation of spike per plant decreased gradually. Compared the grain number per ear and yield per plant, it showed that CK>T1>T2>T3, CK>T4>T5>T6, water stress from jointing stage to tasseling stage and flowering stage to filling stage can reduce the grain number per ear and yield per plant. They were negatively correlated, that was, the greater the degree of stress the grain number per ear and yield per plant of maize were smaller, meanwhile, T1>T4, T2>T5, T3>T6, under the same water stress the grain number per ear and yield per plant from jointing stage to tasseling stage was higher than flowering stage to filling stage. Compared the 100-grain weight of maize, T6>T5>T4>CK, T3>T2>T1>CK, water stress from jointing to tasseling stage and flowering to filling stage will increase the 100-grain weight of maize, moreover 100-grain weight is positively related to the degree

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of water stress, that was, the greater the degree of stress the greater the 100-grain weight of maize.

Table 2. *Dry matter, root shoot ratio and yield of maize at different growth stages under water stress and re-watering conditions*

Water treatment	Dry weight of root per plant (g)	Dry weight of Stem and leaf per plant (g)	Dry weight of shoot per plant (g)	Root-shoot ratio
CK	6.35^{a}	54.95^{b}	182^{a}	0.034^{b}
T1	6.05^{a}	81.72^{a}	167.61^{a}	0.036^{b}
T2	5.64^{b}	83.46^{a}	127.67^{b}	0.044^{a}
T3	5.46^{c}	85.79^{a}	119.14^{b}	0.046^{a}
T4	5.86^{a}	83.23^{a}	158.07^{a}	0.037^{b}
T5	5.24^{c}	84.44^{a}	117.78^{b}	0.045^{a}
T6	4.71^{d}	91.16^{a}	93.97^{c}	0.05^{a}
Water treatment	**Dry weight of spike per plant (g)**	**Grain number per spike**	**Hundred-grain weight (g)**	**Yield per plant (g)**
CK	127.05^{a}	364^{a}	28^{c}	57.95^{a}
T1	85.89^{a}	165^{b}	35.93^{bc}	56.02^{a}
T2	44.21^{b}	109^{bc}	40.76^{b}	43.33^{b}
T3	33.35^{b}	96^{c}	43.52^{b}	37.42^{b}
T4	74.84^{a}	147^{b}	33.8^{bc}	54.18^{a}
T5	33.34^{b}	99^{c}	42.28^{bc}	38.77^{b}
T6	2.81^{c}	14^{d}	48.88^{a}	2.07^{c}

Note: same letter in each column indicates no significant difference at 0.05 level, while difference letters mean significant difference at 0.05 level ($p<0.05$)

The effect of water stress on yield and water use efficiency of maize at different growth stages

The changed of maize yield and water use efficiency (WUE) under water stress at different growth stages were listed in *Table 3*. It showed that the water consumption and yield under T1 and T4 were lower than CK, but the WUE of T1 was the best, T4 was also higher than CK, which indicated that the suitable water (slight water stress) conditions were beneficial to the growth and development of summer maize and the distribution of dry matter in various organs. The water consumption of T1<T4, T2<T5, T3< T6, under same water stress the water consumption of maize at jointing-tasseling stage were lower than that at flowering-filling stage, but from the point of yield and WUE, T1>T4, T2>T5, T3>T6 which showed from analysis *Table 3*, the yield and WUE at jointing-tasseling stage were higher than that at flowering-filling stage. Results indicated that water condition was crucial of maize which can significantly reduce the yield and WUE at flowering-filling stage. The same as at jointing-tasseling stage water stress can also significantly reduce the yield, but the effect on WUE was not significant.

Therefore, when the WUE reached the maximum value, the yield did not reach the optimal value, it showed that the single-sided pursuit of the best maize yield, the WUE could not get the best, which will inevitably lead to waste of water resources.

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DOI: http://dx.doi.org/10.15666/aeer/1806_76437653

Table 3. *Yield and WUE of maize at different growth stages under water stress*

Water treatment	Yield (g/bbl)	Water consumption (mm)	WUE ($g/m^2 \cdot mm$)
CK	115.89[a]	297.33	1.38[b]
T1	112.04[a]	263.79	1.50[a]
T2	86.67[b]	252.47	1.21[b]
T3	74.84[c]	247.70	1.07[c]
T4	108.35[a]	270.56	1.42[b]
T5	77.54[c]	258.49	1.06[c]
T6	4.14[d]	251.95	0.06[d]

Note: same letter in each column indicates no significant difference at 0.05 level, while difference letters mean significant difference at 0.05 level ($p<0.05$)

Discussion

Under water stress, the photosynthetic ability of maize was inhibited, and the degree of inhibition increased with the aggravation of water stress. The compensation effect of maize after re-watering had an adjustment period. After 10 days, the differences in net photosynthetic rate and stomatal conductance between T1 to T5 and CK were significantly reduced, it may be that stomata were main factors affected photosynthetic characteristics under mild water stress, the stomatal resistance of leaves decreased and the limitation of CO_2 absorption was weakened for the stomatal, so photosynthesis of maize could recover quickly after re-watering (Yao et al., 2012). The main reasons for the significant difference of net photosynthetic rate and stomatal conductance between T6 and CK were the changes of hormone level, which metabolic disorders that destroy the structure and function of chloroplasts and cause permanent damage to mesophyll cells in leaves under severe water stress (Liu et al., 2012; Pan et al., 2015). The chlorophyll content decreased due to damage of leaf cell, therefore photosynthesis was inhibited even though stomatal resistance decreased after re-watering. The inhibitory action of water stress on Photosynthesis of maize was not only in the process of water stress but also in re-watering. The degree of inhibition varies with the degree of water stress and the growth stage of maize, and the degree of water stress and growth stage are closely related to the stomatal restriction inhibits photosynthesis of maize. Photosynthesis of maize was sensitive to water stress during flowering-filling stage. After 10 days of re-watering, the stomatal conductance and the net photosynthetic rate of T6 were significantly decreased by 44.5% and 26.7% compared with CK, respectively.

The results showed that water stress could significantly inhibit the root and crown growth of maize, and the treatment of moderate and severe water stress was significantly different from CK. The accumulation of dry matter in the stem and leaf organs of maize was significantly inhibited by water stress in the jointing - tasseling stage, mainly because the jointing-tasseling stage was the key period of maize vegetative growth (Xing et al., 2010), during this time, water stress affected the growth cell division of maize, resulting in short stalks and leaves. Water stress at flowering-filling stage had a great influence on yield, because it at flowering-filling stage seriously affected the differentiation of ear, reduced the activity of filament and pollen, and decreased the success rate of fertilization, thus leading to the reduction of seed number (Tian et al., 2013). Water stress increased the hundred-grain weight of maize which was similar to the result of the study that

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drought on the dry matter distribution of foxtail millet by Zhang et al. (2016). Water stress could reduce the proportion of ears distribution and increase the proportion of stems and leaves distribution, which was contrary to the results of spring wheat by Chen et al. (2017). Water stress inhibited the growth of maize root and crown, and the heavier the stress the larger the root-shoot ratio. From the perspective of different growth stages, water stress can increase the root-shoot ratio of maize, but water changed in different stages have different effected on the distribution ratio of dry matter between roots and crowns. The effected of water stress on increasing the root-shoot ratio during flowering-filling stage was higher than jointing-tasseling stage. In addition, the change of root-shoot ratio was consistent regardless of the degree of water stress.

The amount of irrigation water significantly affected the WUE of summer maize. After water stress and rewater with T1 and T4, the yield of summer maize per barrel was no different from CK but the WUE was higher than CK, increased by 8.7% and 2.8% respectively. Some compensation effects also appeared after re-watering with other water stress treatments but results were not obvious, both yield and WUE per barrel were lower than CK. Therefore, during the whole growth and development stage of summer maize, reasonable water stress should be carried out according to the water requirement characteristics of each growth period and the compensation law of re-watering after water stress, which not only gives full play to the compensation effect, but also guarantees the yield and reaches the goal of improving water use efficiency.

Conclusion

(1) The re-watering test after water stress indicated that the photosynthesis was inhibited not only during the control process of water stress, but also after re-watering. The degree of inhibition varies with the degree of water stress and the growth stage of maize, then the degree of water stress and growth stage were closely related to stomatal restriction inhibits photosynthesis. The stomatal conductance and net photosynthetic rate of re-watering of maize under severe water stress at flowering-filling stage were more difficult to recover than those at jointing-tasseling stage.

(2) In terms of different growth stages, water stress can increase the root-shoot ratio of maize, but the water change in different stages had different effect on the distribution ratio of dry matter between roots and crowns. The effect of water stress on root-shoot ratio was more obvious in the flowering-filling stage.

(3) The yield and WUE of maize decreased with the increase of water stress at jointing-tasseling stage and flowering-filling stage. The yield of maize under slight water stress showed no significant difference from CK and the WUE was higher than CK, the WUE of maize under T1 and T4 was improved by 8.7% and 2.8% compared with CK, respectively. Therefore, reasonable water stress (slight water stress) during jointing-tasseling and flowering-filling stages can guarantee yield, saves irrigation water and effectively improves WUE. The slight drought stress at jointing-tasseling stage and re-watering was the best.

(4) The experiment studied the relationship between water and yield, but it did not reveal the specific quantitative relationship of the influence of water deficit on yield loss at each growth stage of maize, and the specific quantitative relationship between yield reduction rate and water consumption should be further studied through repeated tests. Although some experimental results of summer maize drought re-watering were obtained

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by means of barrel cultivation, in the next, further verification and research should be carried out in combination with field experiment.

Acknowledgements. We are grateful to the research grants from the National Natural Science Foundation of China (51779093) and Supported by Program for Innovative Research Team (in science and Technology) in University of Henan Province (17IRTSTHN026), Supported by Innovation fund for doctoral students of North China University of Water Resources and Electric Power.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76437653

ABOVEGROUND BIOMASS ESTIMATES OF GRASSLAND IN THE NORTH TIBET USING MODIS REMOTE SENSING APPROACHES

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(Received 26th May 2020; accepted 17th Sep 2020)

Abstract. Grassland biomass is a key biophysical metrics to characterize grassland growth and a sensitive indicator of environmental change and ecological functioning. The quantification and timely information on aboveground biomass (AGB) of grassland are crucial for the sustainable use and management of grassland resources. In this study, AGB for main grassland types in the North Tibet was analyzed using field measurements and climatic controls of variations of aboveground biomass were explored, and the general estimate models for grassland AGB were further developed based on the relationships between MODIS vegetation indices (VIs) and the field measurements. The results indicated that alpine swamp meadow has the highest AGB of 356.8 g/m^2 on average, followed by temperate steppe (64.5 g/m^2) and alpine meadow (61.6 g/m^2), and AGB of alpine steppe is lowest at 48.9 g/m^2. Precipitation is main climate factor impacting variations of grassland AGB. The exponential relationships existed between grassland AGB and MODIS VIs and exponential models based on MODIS NDVI were found to be optimum for monitoring and estimating grassland AGB. Study also showed that AGB in the North Tibet spatially decreases from southeast to northwest, which is above 100 g/m^2 in the southeast and is below 20 g/m^2 in the northwest.
Keywords: *aboveground biomass, alpine grasslands, estimate model, satellite remote sensing, Tibetan Plateau*

Introduction

Grassland ecosystems are among the most important terrestrial ecosystems on the Earth, covering around one fourth of the Earth's terrestrial surface and providing essential ecosystem services, such as soil and water conservation, wind erosion prevention, sand fixation, wildlife habitat, air purification, and microclimate and global carbon cycle regulation (Dusseux et al., 2015; Han et al., 2018; Meng et al., 2018; Wang et al., 2019). Moreover, grassland ecosystems play a vital role in pasture animal husbandry development and environmental conservation, especially in areas where traditional animal husbandry practices (Zhang et al., 2018; Wang et al., 2019). Aboveground biomass (AGB) is an important component of grassland ecosystems and plays a critical role in the sustainable use of grassland resources and the global carbon cycle (Zhao et al., 2014; Eisfelder et al., 2017) and is a sensitive indicator of environmental change and ecological functioning, and largely influences biodiversity and environmental processes such as grassland degradation, hydrological cycle and soil erosion (Lu, 2006; Segoli et al., 2008; He et al., 2019). AGB also provides key information for understanding the responses of vegetation to climate change and resilience (Costanza et al., 1997; Houghton et al., 2000; Liang et al., 2016) and can be used to directly estimate grassland productivity (Lauenroth et al., 1986; Jobbagy et al., 2000; Meng et al., 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

The quantification of AGB is particularly important to monitor areas under degradation and desertification (Eisfelder et al., 2017) and to identify regions that are more vulnerable to changing climate. Monitoring and estimating grassland AGB in degraded and desertified areas can not only show the vegetation growth condition but also provide evidence that ecosystem managers and scientists can evaluate the effects of ecological restoration in these areas to realize sustainable development of grassland ecosystems (Tsalyuk et al., 2015; Yan et al., 2015; Yang et al., 2018).

Satellite remote sensing with various temporal and spatial resolutions has been widely used to estimate grassland biomass at large scales while traditional biomass harvesting is a common and reliable method of estimating site-specific AGB and is the only way to provide indispensable ground truth for satellite remote sensing verification (Shen et al., 2008; Anaya et al., 2009; Jia et al., 2016). How to combine these two methods more effectively has become main approaches to more accurately estimating AGB at regional to global scales (John et al., 2018; Wang et al., 2018; Liu et al., 2018).

Tibet, referred to as Tibet Autonomous Region (TAR) here, is main body of the Tibetan Plateau (TP) and one of the five major livestock production zones and pastoral regions in China (Ministry of Agriculture of China, 1996; Chu et al., 2007; Bai et al., 2008). Grassland is the most extensive land cover type in Tibet and plays a vital role in animal husbandry development and environmental conservation. Due to geographical location, complex terrain, and various environmental and climatic conditions, grasslands in Tibet are diverse and are available from subtropical to alpine desert grassland types. Of 18 types of grasslands in China, 17 types can be found in Tibet with the exception of savanna (Tibet Land Management Bureau and Tibet Animal Husbandry Bureau, 1994).

Grasslands in Tibet have been experiencing degradation and desertification since the 1980s under impacts of climate warming, human activities, fast growing grazing pressure and rodent damage (Harris, 2010; Wang et al., 2013; Chen et al., 2014). Grassland degradation is one of the most widespread and severe environmental issues on the TP (Wang et al., 2007; Liu et al., 2015), especially in the arid and semi-arid areas in which vegetation is susceptible to climate variations. It has currently become social concerns and restriction factors for the sustainable development of animal husbandry and the environmental conservation in TAR (Liu et al., 1999; Gao et al., 2006).

Many scientists and research institutes have conducted various studies on grassland degradation and desertification as well as productivity change in Tibet area based on the vegetation indices obtained from different remote sensing satellite platforms to retrieve degree of grassland degradation and biomass change. The firs grassland resources survey in TAR was carried out in the 1980s (Tibet Land Management Bureau and Tibet Animal Husbandry Bureau, 1994). Liu et al. (2017) investigated the spatial and temporal patterns of grassland aboveground biomass on the Qinghai-Tibet Plateau based on the relationship between MODIS NDVI and field-sampled AGB. The estimate models for aboveground biomass of alpine desert grassland in the northern TP were developed based on the relationships between SPOT-VGT NDVI and field-sampled data (Liu et al., 2015). The spatial patterns and environmental controls of aboveground biomass in alpine grasslands on the TP were investigated by integrating field-collected AGB data and concurrent enhanced vegetation index derived from MODIS data sets (Yang et al., 2009). Liang et al. (2016) evaluated various methods for estimating the aboveground biomass of alpine grassland vegetation in pastoral area of southern Qinghai Province, in combination with long-term climate and grassland monitoring data, and developed an operational multi-factor model based on latitude, longitude, and grass cover. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

artificial neural network and MODIS VIs were used to estimate grassland AGB in the Three-River Headwaters Region in the northeastern TP and it was found that the back-propagation artificial neural network model achieves better results than do the traditional multi-factor regression models (Yang et al., 2018).

Spatiotemporal dynamics of aboveground biomass (AGB) is a fundamental problem for the grassland management and environmental concerns on the TP. Advanced remote sensing is an effective approach for characterizing AGB, but the accuracy of the remote sensing-based inversion methods should be addressed and examined before putting into use. Compared to other pastoral regions in China, such as Inner Mongolia and Xinjiang provinces, few studies have focused on developing remote sensing-based approaches for estimating AGB in Tibet area and investigating climatic controls of AGB variations due to lack of continuous in-situ grassland observations. The spatial distribution and temporal variations of grassland biomass in Tibet also largely remain unknown. Particularly, there are no appropriate approaches available to more accurately estimating AGB of these inhomogeneous grasslands at larger spatial scales based on the remote sensing data, which can be used for monitoring grassland growth status and understanding degree of grassland degradation and desertification.

The study aims to present AGB magnitude of major grassland types in the North Tibet based on in-situ measurements first, and then to develop general models for AGB estimation using site-specific measurements and the corresponding MODIS vegetation indices, followed by analyzing main climatic controls of variations of AGB and validating models using independent ground truth data, and finally apply the proposed models to investigate the spatial distribution pattern of AGB in the study area. The study especially focuses on developing operational models suitable for monitoring grassland AGB for the TP area, which can be further used for understanding and providing timely information on grassland productivity, growth and degradation degree in the TP for the effective management and decision making to realize sustainable development of grassland resources.

Materials and methods

Study area

Study area is North Tibet, also named "Chang Tang" in Tibetan, which means Northern Plain, accounting for 42×10^4 km^2 or 35% of total TAR area. Average elevation of study area is over 4500 meters above sea level (masl) and belongs to temperate alpine climate zone. Grassland is predominant land cover type in the North Tibet and plays a vital role in pasture animal husbandry development and environmental conservation (Zhang et al., 2013). Shrub and sub-alpine meadow are primary grassland type in southeastern region due to temperate monsoon humid climate condition. In central region, main grassland is alpine meadow with dominant species of *kobresia pygmaea* adapted to temperate monsoon semi-humid climate. The northwestern region is largely covered by alpine steppe with dominant species of *stipa purpurea* under temperate monsoon semi-arid climate condition. In the far northwest, alpine dessert is main grassland type due to temperate monsoon arid climate condition (Nakchu Bureau of Animal Husbandry, 1992; Chu et al., 2007). The southern region of study area consisted of Lhasa River basin and is mainly covered by temperate steppe and mountain steppe. In the North Tibet, annual precipitation generally increases from northwest to southeast, whereas temperature decreases from south to north.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

Field measurements

The in-situ measurements of AGB were carried out two times a month in 2004 to obtain accurate and site-specific grassland biomass and to provide ground truth for validation of satellite-based AGB estimation methods. Therefore, 10 sampling sites were set up in relatively homogenous and spatially representative region with typical grassland types. AGB samples in these sites were collected two times within 3 days every 15th and 30th day of the month during the vegetation growing season from June to September. In addition, AGB and vegetation coverage at Lhundup sampling site measured by the Tibet Institute of Animal Husbandry Sciences (TIAHS) in 2004 during the growing season within the study area were also used in the study and there were 11 observation sites in total (*Table 1*).

Table 1. *AGB sampling sites and grassland/vegetation types*

Site	Longitude/º	Latitude/º	Altitude/m	Grassland type	Main vegetation type
Damshung A	91.1257	30.4975	4233	Alpine swamp meadow	*Kobresia littledalei*
Damshung B	91.0959	30.4948	4249	Alpine steppe	*Stipa purpurea*
Damshung C	90.9724	30.4127	4216	Alpine meadow	*Kobresia pygmaea Polygonum macrophyllum*
Damshung D	90.6275	30.2000	4590	Alpine meadow	*Kobresia pygmaea*
Damshung F	90.8933	30.3574	4236	Alpine swamp meadow	*Kobresia pygmaea*
Yangbajian	90.4720	30.0761	4300	Alpine steppe	*Stipa purpurea*
Riduo A	92.2927	29.6908	4418	Alpine meadow	*Kobresia pygmaea*
Riduo B	92.0968	29.7099	4150	Alpine swamp meadow	*Dasiphora parvifolia, Kobresia pygmaea*
Lamuxiang	91.5444	29.8043	3720	Temperate steppe	*Artemisia younghusbandii*
Lhasa	91.1452	29.6251	3693	Temperate steppe	*Artemisia younghusbandii, Pennisetum flaccidum*
Lhundup	91.2363	30.0919	4546	Alpine meadow	*Kobresia pygmaea*

In each sampling site, all aboveground biomass was clipped at ground surface in three quadrats of 0.5 m×0.5 m, and all residual litter and other non-plant materials were removed by hand from the harvested samples. Vegetation coverage, dominant species and digital photographs for each quadrat and vegetation type, latitude, longitude and elevation for each site were also recorded. All AGB samples collected were separated into green and residual materials and were oven-dried at 85°C for 24 hours until a constant dry biomass was obtained at the grassland laboratory of TIAHS. The average AGB value of three quadrats within one site was used to represent the AGB at one site. Biomass is expressed in dry weight per unit area since fresh weight varies according to environmental conditions (Barrachina et al., 2015). Dry weight of green grass material is green dry matter (GDM) content, also named green AGB, while dry weight of residual material is residual dry matter (RDM) content. AGB or total AGB is sum of GDM and RDM.

Methods and data

Vegetation indices derived from satellite remote sensing can reflect the photosynthetic activity of surface vegetation and have become the most effective approaches to biomass estimation at large spatial scales (Gaitán et al., 2013; Jin et al.,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

2014). A great number of vegetation indices have been proposed to characterize surface vegetation, biophysical process and terrestrial ecosystems (Gao et al., 2013b; Barrachina et al., 2015; Liang et al., 2016).

MODIS data are suitable for studying grassland vegetation at large scales due to their spatial and temporal advantages. MODIS vegetation indices(VIs) can better response vegetation growing status as compared to previous coarse spatial resolution satellites such as NOAA AVHRR and SPOT VEGETATION due to high signal-to-noise ratio, improved spectral signature and spatial characteristics, narrower bandwidth and frequent global earth imaging (Salomonson et al., 2004), and have been successfully used to characterize large-scale vegetation growth and dynamic change and have become the main remote sensing data source for monitoring grassland biomass and other biophysical parameters on the surface from regional to global scales.

Among various vegetation indices, NDVI (Normalized Difference Vegetation Index) and EVI (Enhanced Vegetation Index) are most frequently used for regression model development of biomass estimation. NDVI is designed to reflect vegetation activity by using information on chlorophyll radiation absorption in the red band and radiation scattering by mesophyll in the near-infrared band (Shen et al., 2014) and is expressed as follows:

$$NDVI = (NIR - R) / (NIR + R) \quad \text{(Eq.1)}$$

where "*NIR*" is the surface reflectance in the near-infrared wavelength (MODIS band 2), and "*R*" is the surface reflectance in the red wavelength (MODIS band 1).

However, the study shows that NDVI has several limitations, including saturation phenomenon in a multilayer closed canopy and sensitivity to both atmospheric aerosols and soil background (Heute, 1988; North, 2002). To overcome these limitations, EVI was developed as a new MODIS product to reduce saturation at high vegetation coverage and minimize impacts of soil background and atmospheric noise (Huete et al., 1994). EVI is expressed as follows:

$$EVI = G \times \left[\frac{(NIR - R)}{(NIR + C_1 \times R - C_2 \times B + L)} \right] \quad \text{(Eq.2)}$$

where "*B*" is the surface reflectance in the blue band (MODIS band 3); "C_1" is atmosphere resistance red correction coefficient (C_1=6.0); "C_2" is atmosphere resistance blue correction coefficient (C_2=7.5); "L" is canopy background brightness correction factor (L=1.0); "G" is gain factor (G=2.5).

To establish AGB estimate models, two cloud-free Terra/MODIS scenes on September 13 and 27 in 2004 received at Lhasa MODIS receiving station of Tibet Institute of Plateau Atmospheric and Environmental Sciences (TIPAES) were used in the study and radiometric and atmospheric corrections for two images were made accordingly. NDVI and EVI then were calculated based on the surface reflectances in MODIS band 1, band 2 and band 3 using *Eq.1* and *Eq.2*. In August, there were no cloud-free MODIS images available for the study area due to rainy season. Thus, MODIS product (MOD13Q1) downloaded from NASA's Land Processes Distributed Active Archive Center (LP DAAC) was used. MOD13Q1 is provided every 16 days at 250 m spatial resolution as a gridded level-3 product in the sinusoidal projection,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

including Terra/MODIS NDVI and EVI data, which are computed from atmospherically corrected bidirectional surface reflectances that have been masked for water, clouds, heavy aerosols, and cloud shadows.

The climate data included monthly precipitation and temperature obtained from Tibet Meteorological Information Center of Tibet Meteorological Bureau, which is consistent with field measurements. Based on the principle of meteorological station nearest to sampling sites, climate data from three meteorological stations are available for the study. For the sampling sites Riduo A and B the climate data from Medro Gongkar meteorological stations were used, for Damshung A, B, D and Yangbajian the data from Damshung meteorological station were used, and for sampling sites Lamuxiang and Lhasa the data from Lhasa meteorological station were used.

Results and discussions

AGB size of different grassland types

Vegetation growing seasons in the North Tibet last three months or less in general due to alpine climate and short monsoonal system, with starting in early June and ending in late August to early September, and its length decreases from southeast to northwest. Grassland vegetation tends to be mature from late August to early September and its production is generally at the highest stage in a year. However, constrained by alpine climate, soil and water conditions in this elevated plateau, grassland AGB is low compared to plain areas at same latitudes on the Earth. Averaged AGB of all grassland types is 96.9 g/m^2, among which average GDM and RDM is 77.7 and 19.2 g/m^2, respectively, with GDM accounting for 80.2% of AGB. In August and September, AGB in some sampling sites had no residual material appeared and only consisted of green plants (*Table 2*).

Table 2. *Field-sampled aboveground biomass of grassland (g/m^2)*

Grassland type	Items	Maximum	Minimum	Mean
Alpine steppe	GDM	45.1	26.2	39.0
	RDM	24.9	0.0	9.9
	AGB	58.2	41.6	48.9
Alpine meadow	GDM	70.1	18.9	44.4
	RDM	39.6	0.0	17.2
	AGB	91.8	37.1	61.6
Temperate steppe	GDM	77.1	23.7	52.1
	RDM	26.0	0.0	12.4
	AGB	87.2	49.7	64.5
Alpine swamp meadow	GDM	541.2	190.1	303.3
	RDM	81.3	22.7	53.6
	AGB	589.1	212.7	356.8
Average	GDM	541.2	18.9	77.7
	RDM	81.3	0.0	19.2
	AGB	589.1	37.1	96.9

There are great discrepancies in AGB size in different grassland types and sampling sites, ranging from 37.1 to 589.1 g/m^2, affected by grassland types, geographical locations, water and temperature conditions and so forth. Among these, AGB of alpine

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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swamp meadow in Damshung A located in lowland wetland near the Damshung County town is highest with 589.1 g/m^2, followed by Riduo B ranging from 212.7 to 327.0 g/m^2 as alpine swamp meadow also. Sampled AGB in the rest of sampling sites is lower than 100 g/m^2, with a minimum of 37.1 g/m^2 occurred in Riduo A as typical alpine meadow grassland. On average, AGB of alpine swamp meadow is highest with reaching up to 356.8 g/m^2, followed by temperate steppe (64.5 g/m^2) and alpine meadow (61.6 g/m^2), whereas AGB of alpine steppe is lowest at 48.9 g/m^2.

Likewise, there are considerable differences in GDM in four main grassland types as well, with a maximum of 541.2 g/m^2, a minimum of 18.9 g/m^2 and an average of 77.7 g/m^2. On average, GDM of alpine steppe is lowest with 39.0 g/m^2, followed by alpine meadow (44.4 g/m^2) and temperate steppe (52.1 g/m^2), while alpine swamp meadow also has the highest GDM of 303.3 g/m^2. Over the period from August to September the sampled value of RDM ranges 0-81.34 g/m^2. Alpine swamp meadow has the highest RDM of 81.3 g/m^2 and its lowest RDM reaches 22.7 g/m^2, which means residual dry plants always existed during the sampling period for alpine swamp meadow. In other three grassland types, the minimum of RDM is 0, which means there were no residual dry plants existed for some samples in August and September. Averaged RDM of alpine swamp meadow is highest at 53.6 g/m^2, followed by alpine meadow (17.2 g/m^2) and temperate steppe (12.4 g/m^2), while RDM is lowest in alpine steppe of 10.0 g/m^2. Averaged RDM of all grassland types is 19.2 g/m^2, accounting for 19.8% of total AGB.

The study on aboveground biomass in the North Tibet conducted by the TIAHS shows that AGB of alpine steppe and alpine meadow in August is 52.5 and 63.2 g/m^2, respectively (Ji et al., 2008). Yang et al. (2009) reported that average AGB of alpine meadow is 68.8 g/m^2 and average AGB of alpine steppe is 50.1 g/m^2 in the North Tibet (Gao et al., 2009). The first grassland resources survey in TAR conducted in the 1980s shows that general aboveground biomass of grasslands in Tibet ranges from 30 to 75 g/m^2 (Nakchu Bureau of Animal Husbandry, 1992). The field measurements in the TP during the growing season of 2013 shows that average AGB of alpine meadow was 70.7 g/m^2 and average AGB value of alpine steppe was 49.9 g/m^2 (Liu et al., 2017). Liang et al. (2016) reported that there were considerable spatial variations in the grassland AGB in the northeastern TP and average AGB in the peak period of grassland growth ranges from 32.9 to 365.3 g/m^2.

These results are consistent with main findings and grassland AGB magnitude in this study. The vegetation types, height, composition, and other properties are substantially different between grassland types, which could produce different AGB values even given the same grassland types (Gao et al., 2013b). Moreover, geographic location, topography, climate, soil, and grass types in the plateau also make great differences in grassland AGB size from region to region (Zheng et al., 2004; Chu, 2020).

Relationships between AGB and climate variables

The climate variables affect grassland vegetation growth, while hydrothermal condition among the variables is main factor influencing vegetation growth and biomass accumulation. In order to reveal the effects of climate variables on AGB in different grassland types and sample sites, the relationships between AGB and precipitation, temperature were explored using linear regression models. The models were developed using total aboveground biomass and fresh grass biomass (green AGB or GDM) as dependent variables and precipitation and temperature as independent variables,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

respectively. The result is shown in *Table 3*. It is clear that the linear correlations between aboveground biomass of grassland and precipitation are more prominent as a whole. Except for Lamuxiang and Lhasa sample sites due to higher spatial heterogeneity than other grassland types and sample site Damshung A as artificially fenced lowland alpine swamp meadow, there were significant correlations between AGB and precipitation (P<0.01) in all sample sites, whereas relationships with temperature were not statistically significant.

Table 3. *Linear correlations between aboveground biomass and climate variables*

Sample site	AGB		Green AGB(GDM)	
	Precipitation	Temperature	Precipitation	Temperature
Damshung A	0.34	0.15	0.85**	0.71*
Damshung B	0.88**	0.68	0.97**	0.82*
Damshung D	0.85**	0.64	0.97**	0.82*
Yangbajian	0.75*	0.66	0.97**	0.84**
Riduo A	0.80*	0.43	0.97**	0.72*
Riduo B	0.72*	0.59	0.94**	0.86**
Lamuxiang	0.37	0.00	0.97**	0.77*
Lhasa	0.14	0.15	0.94**	0.82*

Note: * represents P<0.01; ** represents P<0.001

Green AGB is dry weight of fresh grass biomass as biomass accumulation part produced through vegetation photosynthesis during the vegetation growing season. For all sample sites, the linear correlations between green AGB and precipitation were higher than that between total AGB and precipitation. Likewise, the linear relationships between green AGB and temperature were also obviously higher than that between total AGB and temperature. It is evident that precipitation is main climatic factor affecting grassland aboveground biomass in the study area and plays more important role than temperature for vegetation growth as a whole in this alpine monsoon climate zone. The correlation analysis above also indicates that the impact of climate factors (precipitation, temperature) on green biomass is more profound compared with total AGB. Biomass accumulation during the vegetation growing season is integratedly affected by precipitation and temperature in terms of climate perspective whereas precipitation contributes more to intra-annual variations of AGB than temperature and is main driving force of alpine grassland in the study area. These findings are consistent with the previous studies. The relationships between biomass and climate factors have been a key issue regarding the response of the grassland ecosystem to climate change (Gao et al., 2013a). In the Eurasian steppe region, precipitation is a principal climate factor impacting grassland ecosystem processes (Hsu et al., 2012; Gao et al., 2013a). Other studies have shown that climate is an important factor affecting aboveground biomass of temperate steppe in China, North America and South America, and its variations is mainly constrained by precipitation (Piao et al., 2009; Ma et al., 2010; Ni, 2011). In northern China the precipitation is a primary determinant for grassland ecosystem productivity, biomass accumulation, and inter-annual pattern of the AGB (Gao et al., 2013a). In the Tibetan Plateau, precipitation may serve as the primary driver of the onset and peak dates of greenness of alpine grasslands and it is reasonable to expect that precipitation plays a more important role in alpine grasslands under the pressure of rapid warming in the plateau (Wang et al., 2015).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

AGB estimate models

The essence of remote sensing-based biomass estimate method is to build empirical regression models based on the relationships between field-measured biomass and vegetation index derived from remotely sensed data to estimate biomass for larger spatial scales (Barrachina et al., 2015; Liang et al., 2016; Yuan et al., 2016; Liu et al., 2017; Eisfelder et al., 2017; Chu, 2020). In the study, biomass data collected in August and September were used to develop general AGB estimate models, while remaining data sampled in June and July were employed for model verification. The regression models were established by using sampled grassland AGB as a dependent variable and MODIS NDVI and EVI as independent variables, respectively. A linear and four commonly used nonlinear regression models (S model, logarithmic, power and exponential models) were tested and compared in order to select optimal AGB estimate models for monitoring grasslands in the North Tibet. The coefficient of determination (R^2) and F-test values are used to evaluate the performance of the regression models. Results show that the relationship between AGB and MODIS VIs can be best expressed by exponential models. Based on the higher R^2 and F-test values for regression modeling, NDVI-based exponential model estimates the best with the highest R^2 (R^2=0.63) and F-test values (F=49.21), as shown in *Table 4*. It means that NDVI-based exponential model explains the largest proportion (63%) of total variation in AGB. Other models based on the NDVI explain less than 53% of total variation in AGB. The fitting performance of EVI (R^2=0.50, F=28.87) is slightly lower than NDVI for estimating AGB, which presents that the saturation effect caused by high vegetation coverage and the soil background effect are very limited in the study area since moderate vegetation coverage is dominant in the northern TP. That is to say, EVI has no its advantage over the NDVI for the study area. Instead, the effectiveness of estimating AGB by NDVI is slightly better than that by EVI, showing that NDVI is more appropriate for monitoring and estimating grassland AGB in the North Tibet.

Table 4. *Regression models of aboveground biomass of grassland for the North Tibet*

Model	Vegetation index	Regression model	R^2	F
Linear regression $Y= b_0+b_1x$	NDVI	Y= 500.013x-107.258	0.52	31.01*
	EVI	Y= 908.641x-118.609	0.45	23.77*
Logarithmic $y=b_0+b_1Ln(x)$	NDVI	Y=258.805+165.992Ln(x)	0.36	16.09**
	EVI	Y=366.057+179.264 Ln(x)	0.32	13.78**
S model $y = e^{(b0 + b1 / x)}$	NDVI	$Y=e^{(4.998-0.254/ x)}$	0.26	10.19**
	EVI	$Y=e^{(5.170-0.189/ x)}$	0.26	10.34**
Exponential model $y = b_0e^{b1x}$	NDVI	$Y=19.421e^{(3.178x)}$	0.63	49.21*
	EVI	$Y=19.251e^{(5.507x)}$	0.50	28.87*
Power model $y = b_0(x)^{b1}$	NDVI	$Y=204.493x^{1.083}$	0.46	24.54*
	EVI	$Y=382.679x^{1.121}$	0.38	17.77*

Note: * represents P<0.001; ** represents P<0.01; and *** represents P<0.05

By using MODIS NDVI and EVI as independent variables respectively and field-sampled green AGB as a dependent variable, the estimate models for green AGB (GDM) were also developed. A linear and four nonlinear regression models above were tested and assessed for model development. Result shows that NDVI-based exponential model

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

performs the best for green AGB estimation as well with the highest R^2 and F-test values, as shown in *Table 5*, which means NDVI-based exponential model explains the highest proportion (69%) of total variation in AGB among models tested. Additionally, NDVI-based model (R^2=0.69, F=65.82) is better than that EVI-based model (R^2=0.59 and F=41.95). It is obvious that for monitoring and estimating green aboveground biomass of grassland in the North Tibet the fitting performance of NDVI is also better than EVI and it is an optimal vegetation index to estimate green biomass in the study area.

Table 5. *Regression models of green AGB (GDM) for the North Tibet*

Model	Vegetation index	Regression model	R^2	F
Linear regression $Y= b_0+b_1x$	NDVI	Y= 443.940x-103.588	0.51	29.75*
	EVI	Y=830.422x-119.282	0.47	25.48*
Logarithmic $y=b_0+b_1Ln(x)$	NDVI	Y=225.320+151.371Ln(x)	0.37	16.95*
	EVI	Y=328.219+166.867Ln(x)	0.35	15.41**
S model $y = e^{(b0 + b1 / x)}$	NDVI	$Y=e^{(5.050-0.376/x)}$	0.43	21.74*
	EVI	$Y= e^{(5.306-0.280/x)}$	0.43	22.11*
Exponential model $y = b_0e^{b1x}$	NDVI	$Y=10.929e^{(3.850x)}$	0.69	65.82*
	EVI	$Y=10.208e^{(6.916x)}$	0.59	41.95*
Power model $y = b_0(x)^{b1}$	NDVI	$Y=211.741x^{1.427}$	0.60	43.07*
	EVI	$Y=512.346x^{1.516}$	0.52	31.62*

Note: *: P<0.001; **: P<0.01; and ***: P<0.05

It can be seen from *Tables 4-5* that the exponential relationship between green AGB and NDVI (R^2=0.69) is better than that between total AGB and NDVI (R^2=0.63), which is attributed to high sensitivity of vegetation index to green vegetation compared to total vegetation cover in the presence of senescent vegetation (Butterfield et al., 2009; Hagen et al., 2012). It is clear that either using MODIS NDVI or EVI the performance of estimate models for green AGB is always better than that for total AGB, indicating that MODIS vegetation index is more applicable for green biomass estimation, which reflects unique spectral signature and response of green vegetation detected by satellite sensors (Zheng et al., 2004; John et al., 2018). Vegetation index is based on differences in reflectances between visible and near-infrared bands of satellite sensors, which presents that strong photosynthesis of green vegetation makes it has low reflectance and strong absorption in the red visible band and higher reflectance and weaker absorption in the near-infrared band (John et al., 2018; Wang et al., 2018). The most obvious differences in spectral responses for senescent and green vegetation presents that senescent vegetation has higher reflectance both in the red and near-infrared bands but has no strong absorption and low reflectance in the visible band like green vegetation due to chlorophyll absorption through photosynthesis. Furthermore, the reflectance of green vegetation in the near-infrared band is obviously higher than the reflectance of senescent or residual vegetation. Low chlorophyll content of senescent vegetation reduces the red-to-near infrared spectral contrast (Butterfield et al., 2009; Tsalyuk et al., 2015) and finally affects fitting performance of vegetation index for estimating grassland biomass.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

AGB estimate models for alpine meadow grassland in eastern TP were established using field-measured AGB and different remotely-sensed NDVI data and it was found that in the four types of grassland AGB estimation models (linear, logarithmic, power and exponential) the exponential model performs the best (Meng et al., 2018). Liu et al. (2015) presented that the relationship between grassland biomass and NDVI in the TP can be quantified by an exponential model with an acceptable coefficient of determination ($R^2 = 0.58$). Liang et al. (2016) reported that among the 12 VIs tested the MODIS NDVI exhibits the highest correlation with grassland AGB in the peak of grassland biomass in southern Qinghai Province in the northeastern TP and the NDVI-based exponential inversion model performs the best and is capable of accounting for 58% of the changes in the grassland AGB. In the North Tibet, the relationship between grassland AGB and vegetation indices showed a non-linear trend and the exponential function resulted in the best option for use in describing that relationship and around 90% of the changes in AGB can be explained by the changes estimated with the vegetation indices (Ji et al., 2008). In addition to relatively mature and widely used empirically-based grassland AGB retrieval methods based on the relationship between field AGB measurements and vegetation indices, He et al. (2019) proposed a novel physically-based grassland AGB retrieval method and AGB time series estimated by the method agreed reasonably well with the expected temporal dynamic trends of the grassland in Zoige Area in the eastern TP, and it has the potential for operational monitoring of grassland AGB at regional and even larger scales since its greatest advantage is fully physical nature and no field measurement is needed.

Model validation

To verify accuracy of biomass estimation models for the North Tibet, AGB estimated by NDVI-based exponential model is validated using field-sampled AGB during June and July of 2004. The result is shown in *Figure 1* and indicates that the linear correlation coefficient between field-sampled and NDVI-estimated AGB is 0.84, with statistically significant at a significance level ($p<0.001$). In addition, average AGB sampled and estimated is 62.9 g/m^2 and 70.1 g/m^2, respectively, which means averaged AGB estimated by the model is 7.2 g/m^2 higher than that sampled data. The overall trend as shown in *Figure 1* is that when field-measured AGB is lower than 50 g/m^2, NDVI will generally overestimate the actual AGB, while if it is higher than 90 g/m^2, NDVI-predicted AGB tends to underestimate the actual AGB, namely, a general trend of overestimation at lower AGB level and underestimation at the higher AGB level for MODIS VIs-estimated AGB, which is mainly attributed to great regional differences in grassland biomass, high spatial heterogeneity of grassland types, various influence factors from climate, environment and topography in the study area as well as model algorithm limitations. However, the linear correlation coefficient is 0.84 with RMSE (root mean square error) of 29.1 g/m^2 and MAE (mean absolute error) of 22.9 g/m^2. According to these statistic measures, it is concluded that estimating aboveground biomass of grassland in the North Tibet using MODIS NDVI is effective and reliable.

Spatial distribution of AGB

By using NDVI-based exponential model the spatial distribution of grassland AGB in the North Tibet was made and the final map is shown in *Figure 2*. It is clear that AGB presents decrease from southeast to northwest, corresponding well to precipitation gradient to a large extent in this region. AGB is over 100 g/m^2 in the southeastern

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DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

regions while it is below 20 g/m^2 in the northwestern corners. In the North Tibet, non-vegetation areas account for 4.24% of the total area, including lakes, perennial snow, glaciers, and alpine bare rock etc. Based on intervals of 10 g/m^2 apart, the areas where AGB ranges 30-40 g/m^2 cover the largest extent with 37.7% of the total area, mainly distributed in central North Tibet, followed by AGB between 20-30 g/m^2 with accounting for 27.9% of the entire areas and distributing in broad northern region close to Kunlun mountain range. The third is AGB of 40-50 g/m^2 with covering 9.1% of total area, and is distributed in the northern region of Nyainqentanglha Mountains. Other areas with 10 g/m^2 intervals cover less than 10%. AGB in the southeastern river valleys is above 100 g/m^2 due to more abundant rainfall and ground water, with accounting for 5.5% of total area and reaching over 200 g/m^2 in some lowland wetlands.

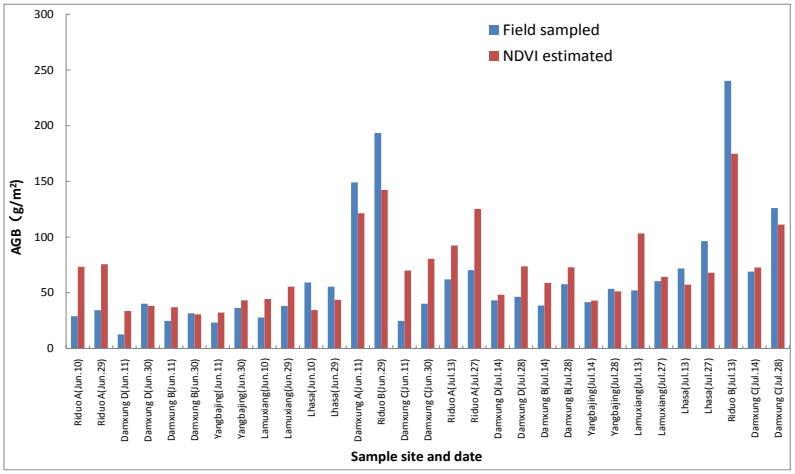

Figure 1. *Comparisons between field-sampled and MODIS NDVI-estimated AGB*

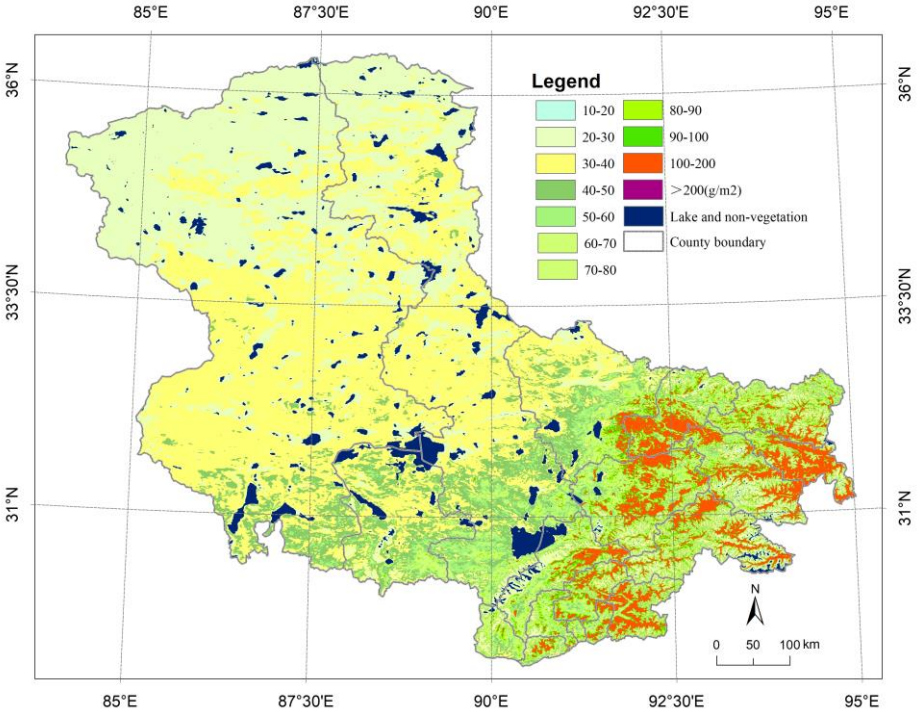

Figure 2. *Spatial distribution of aboveground biomass of grassland in the North Tibet*

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The spatial distribution of green AGB of grassland for the study area was also made using NDVI-based optimal model and the final result is shown in *Figure 3*. Its spatial distribution pattern is generally similar with total aboveground biomass, presenting gradually decrease from southeast to northwest. In the southeastern regions the green biomass is above 100 g/m^2 whereas it is lower than 20 g/m^2 in the northwestern regions. The green biomass ranges between 60 and 100 g/m^2 in most of southeastern regions while it ranges from 20 to 60 g/m^2 in central regions and is lower than 20 g/m^2 in the northwestern regions with covering 38.1% of total study area.

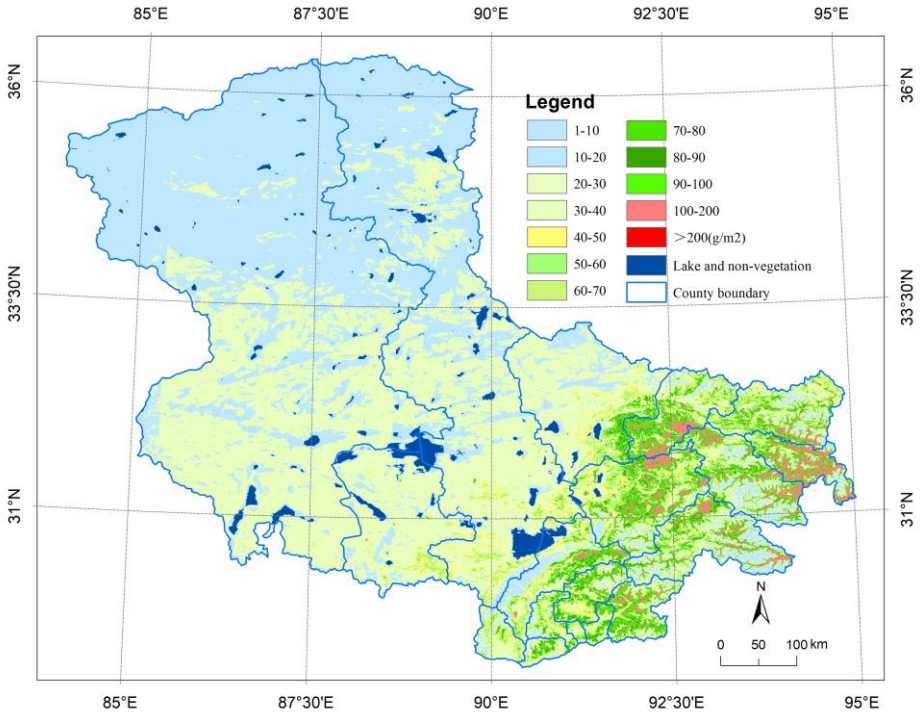

Figure 3. *Spatial distribution of green AGB of grassland in the North Tibet*

Conclusions

In this study, aboveground biomass of grassland for major grassland types in the North Tibet is investigated using field measurements of AGB first, and then empirical regression models for estimating grassland AGB are established based on the relationship between MODIS VIs and in-situ measurements and main climatic controls of AGB variations is further explored, and finally the spatial distribution of aboveground biomass in the North Tibet is mapped using optimum regression models developed. The study particularly focuses on combining field observations and remotely sensed data to develop methods for monitoring grassland biomass and generate a spatially explicit aboveground biomass map of grassland in the North Tibet.

(1) Affected by alpine climate, high attitude, soil fertility and moisture availability, grassland AGB in the North Tibet is low compared to same latitudes in the northern hemisphere. Average AGB of all grassland types between August and September is 96.9 g/m^2, of which green biomass is 77.7 g/m^2, accounting for 80.2% of the total AGB.

(2) AGB largely varies in different grassland types and sampling sites, ranging from 37.1 to 589.1 g/m^2. Based on the sample sites, AGB of alpine swamp meadow in

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DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

Damshung A is highest at 589.1 g/m^2, while a minimum of 37.1 g/m^2 occurs in Riduo A. Based on the grassland types, average AGB of alpine swamp meadow is highest at 356.8 g/m^2, followed by temperate steppe (64.5 g/m^2) and alpine meadow (61.6 g/m^2), whereas AGB of alpine steppe is least at 48.9 g/m^2. Biomass accumulation during the vegetation growing season is jointly affected by precipitation and temperature and in contrast the precipitation plays more important role than temperature and is main driving force for vegetation growth in this alpine region.

(3) Vegetation index derived from remotely sensed data is an important indicator to represent the vegetation characteristics of land surface and has been widely used for grassland biomass estimates. The study found that optimum regression models for estimating aboveground biomass in the North Tibet are MODIS NDVI-based exponential models. The spatial distribution maps of aboveground biomass of grassland made using optimum models show that both AGB and green AGB presents decrease from southeast to northwest, which is above 100 g/m^2 in the southeastern regions and decrease lower than 20 g/m^2 in the northwestern margin.

(4) Due to limited physical access to the TP and large spatial extent, remote sensing observations are becoming main data source and realistic approaches in monitoring this unique environment and have great potentials to further apply in various biophysical parameters retrieval. However, current accuracy of AGB estimates based on vegetation indices is still limited due to lack of sufficient field-sampled data and spatial heterogeneity and complexity of grassland types. Given that the accuracy of remote sensing-based AGB estimate models is largely dependent on the number and representativeness of the sampling sites. Therefore, the collection of a sufficient number of biomass sample sites in representative regions is a key for developing robust AGB estimate models and evaluating the estimate results in the future.

(5) Timely monitoring and estimating aboveground biomass are important for ensuring sustainable grassland ecosystem function and maintaining its environmental conservation values. Satellite remote sensing provides only feasible approach to monitoring and estimating aboveground biomass at large spatial scales. However, grassland AGB estimate models based on a single factor such as vegetation index have limited accuracy and high uncertainty, which would be further improved using unmanned aerial vehicle technology and finer spatial-resolution remote sensing data in the future.

Acknowledgments. The author would like to thank Prof. Kristy Tiampo and Prof. Richard Armstrong for their assistance during my visiting University of Colorado at Boulder. MODIS product (MOD13Q1) was downloaded from NASA's Land Processes Distributed Active Archive Center (LP DAAC). The climate data was provided by the Meteorological Information and Network Center of Tibet Meteorological Bureau. This research work was financially supported by the Second Tibetan Plateau Scientific Expedition and Research Program (2019QZKK010312), the Science & Technology Basic Resources Investigation Program of China (2017FY100501), the National Natural Science Foundation of China (41561017), and the Tibet Key Laboratory of Plateau Atmosphere and Environment Research through the Project of Scientific Investigation and Research for Main Land Surface Features in Tibet.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76557672

THE EFFECTS OF NANO PHOSPHATIC FERTILIZER APPLICATION ON THE PRODUCTIVITY OF SOME EGYPTIAN RICE VARIETIES (*ORYZA SATIVA* L.)

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(Received 31st May 2020; accepted 14th Sep 2020)

Abstract. A tow-season field investigation was conducted at The Experimental Farm of RRTC (Rice Research & Training Center), Sakha, Kafr El-Sheikh Governorate, Egypt, during 2018 and 2019 to examine the impact of nano phosphatic fertilizer utilization on the performance of four Egyptian rice varieties. The selected tested varieties were Sakha106, Sakha107, Giza177, and Giza179. The phosphorus fertilizer treatments included superphosphate (15% P_2O_5) as a soil application, nano-phosphorus as a foliar application, superphosphate as a foliar application and control (distilled water). Superphosphate was incorporated into the soil at the rate of 36 kg ha^{-1} for the first treatment. Nano-phosphorus (1%) and superphosphate (2%) were sprayed at 35 and 45 days after transplanting (DAT) for the second and third treatments. The application of phosphorus fertilizers increased the number of tillers per m^2, the number of branches per panicle, filled grains number per panicle, 1000-grain weight (g), grain yield (t ha^{-1}) and straw yield (t ha^{-1}) for al tested rice varieties. Superphosphate soil application and a foliar application of nano phosphorus to Giza 179 surpassed the other plural in terms of grain and straw yields in both seasons. Application of phosphorus as superphosphate or nano-phosphorus enhanced grain quality characteristics as well as nitrogen and phosphorus content in milled grains.
Keywords: *nano-phosphorus, foliar application, soil application, grain yield*

Introduction

Rice (Oryza sativa L.) is an important crop for Egyptian farmers as well as for Egyptian consumers. Rice farmers in the past used a huge quantity of synthetic fertilizers to treat their fields. The synthetic fertilizers have great benefits for enhancing rice yield. On the other hand, their cost is high and their effectiveness is low. They also pollute the environment. Heavy applications of mineral fertilizers affect the groundwater and also lead to eutrophication in aquatic ecosystems. In sustainable agricultural system, rice farmers have to look for new fertilizer technologies with high efficiency and lower pollution and cost. One of those technologies is Nanoparticles' fertilizers. The impact of Nanoparticles' fertilizers on agricultural generally and rice fields especially has been reported by several investigations.

Phosphorus (P) is critical in the metabolism of plants, playing a role in the transfer and storage of energy from photosynthesis and the metabolism of carbohydrates. It is also a structural component of the nucleic acids of genes and chromosomes and many coenzymes, phosphoproteins, and phospholipids. Application of P is recommended as a principal agronomical practice for increasing crop yield, given the fact that about 5.7 billion hectares globally require sufficient available P (Batjes, 1997). Phosphorus is quickly loaded inside grains of both indica and japonica rice genotypes between 6 and 15 days after flowering (Wang et al., 2016). With P absorption suppressed, some

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

varieties can maintain a stronger ability to gain greater plant biomass and grain yield with relatively lower tissue P content (Richardson and Simpson, 2011). The yield components of rice, such as panicle number, seed-setting rate, and grain weight significantly increased with P fertilization (Usman, 2013).

Nanotechnology is a very promising field of science and technology that has the potential to open up new applications in the field of agriculture and biotechnology (Siddiqui and Al-Whaibi, 2014). Ultrafine particles are between 1 and 100 nm in size. Nanoparticles can serve as magic bullets containing herbicides, chemicals, or genes which target plant parts to release the content (Jampílek and Kráľová, 2015). In agriculture, fertilizers are very important for plant growth and development, most of the applied fertilizer is rendered unavailable to the plants due to many factors such as leaching, degradation by photolysis, hydrolysis and degradation. Hence it is necessary to minimize nutrient losses in fertilization and to increase the crop yield through the exploitation of new applications with the help of nanotechnology and nanomaterials. Nanoparticles have unique physicochemical properties and the potential to boost plant metabolism (Giraldo et al., 2014).

Benzon et al. (2015) reported that the application of nanotechnology in agriculture is still in its budding stage. However, it has the potential to revolutionize agricultural systems, particularly where the issues on fertilizer applications are concerned. Nano fertilizer application promoted the growth, development, and antioxidant activity in rice and has the potential to improve crop production and plant nutrition.

Upadhyaya et al. (2017) reported that calcium phosphates are of great interest in medicine, biology, agriculture, and materials sciences. They evaluated the effect of calcium phosphates nanoparticles on biochemical changes in rice. Nanoparticles increased the growth rate and affect the physiology of the plant. Calcium phosphate nanoparticles may help in the formulation of new nano growth promoters and nano-fertilizers for agricultural use. Therefore, it could potentially help in the reduction of the quantity of fertilizer applied to crops and contributing to precision farming as it reduces fertilizer wastage and in turn environmental pollution due to agricultural malpractices. However, detail physiological and molecular understanding of its impact on rice crop plants is needed in the future to validate its prospective application in agriculture. This study aimed to examine the impact of nano phosphatic fertilizer utilization on the performance of four Egyptian rice varieties.

Materials and methods

At the experimental Farm of RRTC (Rice Research & Training Center), Sakha, Kafr El-Sheikh Governorate, Egypt, a two-year field experiment was conducted during 2018 and 2019 to study the behavior of four rice cultivars treated with different sources of phosphorus fertilization.

Representative soil samples were taken from the experimental site at the depth of 0-30 cm from the soil surface. Samples were air-dried, then ground to pass through a two mm sieve and well mixed. The procedure of soil analysis was conducted according to the methods of Black (1965) was used. The Results of chemical analysis in both seasons are shown in *Table 1*.

The selected tested varieties were Sakha106, Sakha107, Giza177, and Giza179 (*Table 2*). The treatments of phosphorus fertilizer were superphosphate (15% P_2O_5) as a soil application, nano-phosphorus as a foliar application, superphosphate as a foliar

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

application and control (distilled water). Superphosphate was incorporated into the soil at the rate of 36 kg P_2O_5 ha^{-1} for the first treatment. Nano-phosphorus (1%) and superphosphate (2%) were sprayed at 35 and 45 DAT for the second and third treatments.

Table 1. *Soil physical and chemical properties at the experimental site in 2017 and 2018 rice growing seasons*

Soil properties	2017	2018
Mechanical analysis:		
Clay %	55.04	54.24
Silt %	30.68	31.56
Sand %	14.28	14.20
Texture	Clay	Clay
Chemical analysis:		
Organic matter %	1.48	1.63
Total nitrogen, mg kg^{-1}	463.08	469.20
Available P, mg kg^{-1}	13.26	12.81
pH (1:2.5 soil suspension)	8.26	8.35
EC $dS.m^{-1}$ (soil paste)	1.87	2.14
Soluble cations, meq. L^{-1}:		
Ca + +	9.72	10.23
Mg + +	4.05	4.09
K +	1.83	1.87
Na +	15.13	15.53
Soluble anions, meq. L^{-1}:		
CO3 =	0	0
HCO3-	6.16	6.93
Cl-	8.51	8.65
SO4 =	16.05	16.14
Available micronutrients ppm		
Fe + +	6.22	5.92
Zn + +	0.72	0.95
Mn + +	3.57	3.07

Phosphorus Nanoparticles (P) was obtained from rock superphosphate. According to the manufacturing and observation on Transmission Electron Microscopy (TEM), the particle size distributions were ranged from 30 to 60 nm (*Fig. 1*). Nano phosphorus solution was prepared freshly by dispersing nanoparticles in de-ionized water through ultrasonication (300 W, 40 kHz) for 30 min.

Table 2. *Parentage, type and origin of the tested rice varieties*

Varieties	Parentage	Type	Origin
Sakha106	Giza 177/Hexi 30	Japonica	Egypt
Sakha 107	Giza177/BL 1	Japonica	Egypt
Giza 177	Giza 171/Yamji No.1//Pi No.4	Japonica	Egypt
Giza 179	Gz 1368-S-5-4/Gz 6296-12-1-2-1-1	Indica/Japonica	Egypt

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

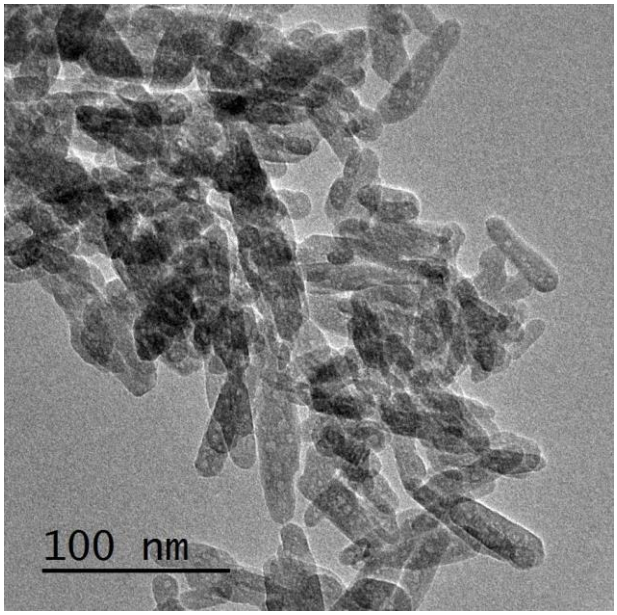

Figure 1. *Phosphorus nanoparticles under the transmission electron microscopy (TEM)*

Split-Plot Design with four replicates was used. Rice cultivars and phosphorus fertilization treatments were arranged as main plot and sub-plot, respectively. The plot size was 12 m^2. Rice seeds were sown in the nursery on the 2nd and 4th of May for 2017 and, respectively. Transplanted was done after 28 days of sowing nurseries with three to four seedlings per hill, spaced 20 X 20 cm within and between rows. All plots received optimum cultural practices. The recommended dosages of nitrogen and potassium were applied according to the recommendations of RRTC. Nitrogen was applied at the rate of 165 kg N ha^{-1} in the form of urea (46% N). Potassium was applied as potassium sulphate (50% K_2O) at the rate of 100 kg K_2O ha^{-1}.

The studied characteristics were; number of tillers m^{-2}, number of branches $panicle^{-1}$, unfilled grains number $panicle^{-1}$, filled grains number $panicle^{-1}$, 1000-grain weight (g), grain yield (t ha^{-1}) and straw yield (t ha^{-1}).

Grain quality characteristics: milling recovery was estimated according to Cruz and Khush (2000): 150 (g) cleaned rough rice at 14% moisture content was dehulled using an Experimental Huller Machine (Satake - Japan). The brown rice was separated and weighed then the hulling percentage was calculated. The brown rice was milled using MC GILL Rice Miller No.2. (S.K. Appliances – India). The total milled rice was weighed and milled rice percentage was calculated. Whole milled grains were separated from the total milled rice using a rice sizing device SKU: 61-220-50 (Seedburo – USA). The percentage of head rice was calculated.

Nitrogen and phosphorus contents in milled rice grain: samples were taken from the grain after milling (50 g of milled rice). All samples were placed in paper bags and oven-dry at 70 °C for 48 h. Grain samples were crushed to powder and digested according to Chapman and Pratt (1961). The nitrogen content of milled grains was determined by using the Microkieldahl method (Jackson, 1967). The phosphorus content of milled grain was determined using Spectronic 1201 Spectrophotometer (Milton Roy, USA) following the procedures of Watanabe and Olsen (1965).

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DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

The data was subjected to analysis of variance (Two-way ANOVA in randomized blocks), and the differences among treatment were compared by Duncan's Multiple Range Test ($P < 0.05$) according to Duncan (1955), using Costat Statistical Software.

Results and discussion

Numbers of tillers m^{-2}, branches $panicle^{-1}$, filled grains $panicle^{-1}$ and unfilled grains $panicle^{-1}$ were varied significantly among rice varieties and affected significantly by different phosphorus treatments in both seasons as shown in *Table 3*. Giza179 surpassed the other varieties in the above mention characteristics. The differentiation among the studied varieties in terms of those characteristics may be related to the genetic and physiological background of the tested varieties.

A foliar application of nano-phosphorus recorded the highest values of number of tillers per m^2, number of branches per panicle and number of filled grains per panicle. The highest numbers of unfilled grains per panicle were recorded when phosphorus was not applied without any significant differences with the foliar application of superphosphate in the first season only. Upadhyaya et al. (2017) found that rice growth increased with an increase in the concentration of calcium phosphate nanoparticles with respect to the control. Miranda-Villagómez et al. (2019) found a positive relationship between the number of rice tillers and the initial P concentration in the nutrient solutions due to phosphorus application as nanoparticles.

Table 3. *Tillers No. m^{-2}, branches No. $panicle^{-1}$, filled grains $panicle^{-1}$ and unfilled grains $panicle^{-1}$ of different rice varieties as affected by phosphorus treatment*

Treatments	Tillers No. m^{-2}		Branches No. $panicle^{-1}$		Filled grains $panicle^{-1}$		Unfilled grains $panicle^{-1}$	
	2017	2018	2017	2018	2017	2018	2017	2018
Rice variety:								
Sakha 106	523.3 c	501.7 c	9.57 c	9.18 c	118.04d	122.8 c	9.88 b	8.91 b
Sakha 107	525.1 c	496.6 c	10.00 b	9.64 b	125.2b	117.3 b	7.59 d	6.90 d
Giza 177	541.1 b	520.0 b	9.99 b	9.47 bc	122.5c	114.4 c	8.57 c	7.87 c
Giza 179	583.1 a	553.6 a	10.50 a	10.40 a	141.8a	129.8 a	14.08 a	13.25 a
F test	**	**	**	**	**	**	**	**
P treatment:								
Super P (soil)	584.2 b	550.8 b	10.37 b	9.91 b	134.6b	125.3 b	8.01 b	5.16 c
Nano-P (spray)	589.9 a	562.5 a	10.71 a	10.27a	141.5a	132.4 a	8.15 b	6.36 c
Super P (spray)	523.7 c	499.1 c	9.91 c	9.43 c	121.8d	112.6 c	11.6 a	11.85 b
Control	474.8 d	459.6 d	9.56 d	9.08 d	109.8d	104.01d	12.33 a	13.57 a
F test	**	**	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**	**	**

Values with the same letter in each column indicate no significant difference ($P < 0.05$)

The interaction between rice variety and phosphorus treatment effect was significant for numbers of tillers m^{-2}, branches $panicle^{-1}$, filled grains $panicle^{-1}$ and unfilled grains $panicle^{-1}$ (*Table 4*). A foliar application of nano-phosphorus to rice variety Giza179 recorded the highest numbers of tillers per m^2, branches per panicle and filled grains per

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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panicle in both seasons. There were no significant differences between the foliar application of superphosphate and nano-phosphorus to Giza179 rice variety in the first season only on number of tillers per unit area. Number of unfilled grains per panicle was higher when Giza179 did not receive any phosphorus fertilizers in both seasons which was at bar with those sprayed with superphosphate in the first season only.

Table 4. *Tillers No. $m^{-2,}$ branches No. $panicle^{-1}$, filled grains $panicle^{-1}$ and unfilled grains $panicle^{-1}$ as affected by the interaction between rice variety and phosphorus treatment*

Treatments	2017				2018			
	Sakha 106	**Sakha 107**	**Giza 177**	**Giza 179**	**Sakha 106**	**Sakha 107**	**Giza 177**	**Giza 179**
	Tillers No. m^{-2}							
Super P (soil)	568.6cd	559.5 d	577.1bc	631.8 a	535.4e	527.2 f	550.5d	590.2 b
Nano-P (spray)	578.2 b	560.5 d	584.5b	636.2 a	548.6d	535.01e	559.6 c	606.7 a
Super P (spray)	492.9 h	515.7 f	525.9e	560.3 d	481.9h	470.8 i	511.00g	532.5ef
Control	453.6 k	464.6 j	477.1 i	504.02g	440.8k	453.5 j	458.8 j	485.2 h
	Branches No. $panicle^{-1}$							
Super P (soil)	9.84 f	10.34 d	10.16de	11.13b	9.55 f	9.97de	9.52 f	10.62 b
Nano-P (spray)	10.25 d	10.55 c	10.33 d	11.72a	9.81 e	10.24c	10.07cd	10.97 a
Super P (spray)	9.12 h	9.93 f	10.02ef	10.58c	8.85 h	9.34 fg	9.28 g	10.24 c
Control	9.05 h	9.42 g	9.47 g	10.32d	8.53 i	9.03 h	9.00 h	9.78 e
	Filled grains $panicle^{-1}$							
Super P (soil)	124.2 f	134.8cd	129.5e	149.7b	117.6m	124.1cde	120.7ef	139.08b
Nano-P (spray)	126.2f	136.4c	136.5c	166.8a	122.8de	126.2cd	126.9 c	153.8 a
Super P (spray)	177.8g	119.3g	117.6g	132.0de	111.5 ij	113.9 hi	108.2jk	116.9gh
Control	103.8 i	110.3h	106.4 i	118.7 g	99.3	105.1	102.07	109.4
	Unfilled grains $panicle^{-1}$							
Super P (soil)	8.60 e	4.44 g	6.48 f	12.52b	5.86 f	3.11 h	3.57gh	8.09 e
Nano-P (spray)	10.72bcd	4.80 fg	5.65fg	11.44bc	5.79 f	4.36fgh	5..03fg	10.25 d
Super P (spray)	9.30 de	10.42cde	10.39cde	16.33 a	10.42 d	10.28 d	10.05d	16.67 b
Control	10.89bcd	10.68bcd	11.75 bc	16.02 a	13.58 c	9.87 d	12.82c	17.99 a

Different letters indicate statistical significance ($P < 0.05$) within the same characteristic and the same year

1000-grain weight, grain yield and straw yield of different rice varieties were affected significantly by phosphorus treatments as well as the interaction in both seasons (*Table 5*). Sakha 106 and Giza 177 recorded the highest values of 1000-grain weight in the first and second seasons, respectively. The highest values of grain and straw yields were obtained by Giza179 in both seasons. Those variations among the varieties might be due to the genetic potentiality.

Application of phosphorus fertilizers as a soil or a foliar application increased significantly the values of 1000-grain weight, grain yield and straw yield over control. The heaviest 1000-grain was observed with the application of either superphosphate as a soil application or nano phosphorus as a foliar application without any significant differences between both of them. Grain and straw yields reached to the maximum values with the application of nano phosphorus as a foliar application. Wang et al.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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(2011) indicated that application of nanomaterials could increase rice grain yield. Miranda-Villagómez et al. (2019) reported that application of P nanoparticles is particularly important in early growth stages of rice plant. It is mobile within the plant and promotes the development of roots, tiller creation, early flowering, and maturation.

Table 5. *1000-grain weight (g), grain yield (t ha^{-1}) and straw yield (t ha^{-1}) of different rice varieties as affected by phosphorus treatment*

Treatments	1000-grain weight (g)		Grain yield (t ha^{-1})		Straw yield (t ha^{-1})	
	2017	2018	2017	2018	2017	2018
Rice variety:						
Sakha 106	3.28 a	2.70 b	10.50 b	9.71 c	12.89 b	12.78 c
Sakha 107	3.14 b	2.72 b	10.14 d	9.41 d	12.39 c	12.63 d
Giza 177	3.14 b	2.88 a	10.23 c	9.99 b	12.81 b	13.02 b
Giza 179	3.03 c	2.70 b	11.28 a	10.82 a	14.11 a	14.23 a
F test	**	*	**	**	**	**
P treatment:						
Super P (soil)	3.30 a	2.86 a	10.97 ab	10.32 a	13.56 a	13.64 a
Nano-P (spray)	3.30 a	2.88 a	11.02 a	10.40 a	13.56 a	13.71 a
Super P (spray)	3.05 b	2.70 b	10.30 b	9.75 b	12.77 b	12.91 c
Control	2.91 c	2.57 c	9.86 d	9.46 c	12.31 c	12.40 c
F test	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**

Values with the same letter in each column indicate no significant difference ($P < 0.05$)

Concerning the interaction effects on 1000-grain weight, grain yield and straw yield, data in *Table 6* shows that application of phosphorus fertilizers increased 1000-grain weight for al tested rice varieties. The highest values of 1000-grain weight were recorded with the application of either superphosphate as a soil application or nano phosphorus as a foliar application to Sakha 106 in first season and to Sakha 106, Sakha 107 and Giza 177 in second season. Soil application superphosphate and a foliar application of nano phosphorus to Giza 179 surpassed the other combination in terms of grain and straw yields in both seasons.

Milling characteristics of tested rice varieties were affected significantly by phosphorus fertilizer treatments and the interaction (*Table 7*). Giza 179 recorded the highest percentages of hulling and milling grains. Sakha 106 recorded the highest percentage of head rice followed by Giza 177 in the first season. While Giza 177 rice variety produced the highest percentage of head rice in the second season. Milling characteristics were improved by the application of phosphorus fertilizers whatever the source and method. Application of either superphosphate as a soil application or nano phosphorus as a foliar application produced the highest percentages of hulling, milling and head rice in both seasons without any significant differences between both of them. The nano-fertilizers have a higher surface area it is mainly due to very less size of particles which provide more sites to facilitate the different metabolic processes in the plant system result production of more photosynthesis which improves the grain filling as well as milling characteristics (Qureshi et al., 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

Table 6. *1000-grain weight (g), grain yield (t ha^{-1}), straw yield (t ha^{-1}) and harvest index as affected by the interaction between rice variety and phosphorus treatment*

Treatments	2017				2018			
	Sakha 106	Sakha 107	Giza 177	Giza 179	Sakha 106	Sakha 107	Giza 177	Giza 179
	1000-grain weight (g)							
Super P (soil)	3.49 a	3.28 cd	3.26cde	3.17def	2.86a-d	2.83a-e	2.95 ab	2.80 b-f
Nano-P (spray)	3.41 ab	3.29bcd	3.33 bc	3.19def	2.92abc	2.82a-e	3.00 a	2.76 c-g
Super P (spray)	3.14 ef	3.02gh	3.08 fg	2.95 hi	2.68d-g	2.63 fg	2.83a-e	2.66 efg
Control	2.94 hi	2.96 gh	2.90 hi	2.83 i	2.36 h	2.60 g	2.72d-g	2.59 g
	Grain yield (t ha^{-1})							
Super P (soil)	11.06b	10.51d	10.53d	11.78a	10.05 f	9.74h	10.26d	11.24 a
Nano-P (spray)	11.02b	10.53d	10.67c	11.85a	10.17e	9.75h	10.38c	11.29a
Super P (spray)	10.24e	9.85 f	10.17e	10.95b	9.50 i	9.17 i	9.84 g	10.51 b
Control	9.66 g	9.67 g	9.56 g	10.53d	9.13 i	8.99k	9.47 i	10.25 d
	Straw yield (t ha^{-1})							
Super P (soil)	13.39c	12.82e	13.25cd	14.76 a	13.30 d	13.10 e	13.35 d	14.81 a
Nano-P (spray)	13.68b	12.4g	13.27cd	14.82 a	13.41cd	13.05ef	13.5 c	14.90 a
Super P (spray)	12.53fg	12.2h	12.68 ef	13.64 b	12.45 g	12.31 h	12.95 f	13.93 b
Control	11.95 i	12.02i	12.05 i	13.22 d	11.97 i	12.06 i	12.27 h	13.30 d

Different letters indicate statistical significance ($P < 0.05$) within the same characteristic and the same year

Table 7. *Hulling, milling and head rice % of different rice varieties as affected by phosphorus treatment*

Treatments	Hulling %		Milling %		Head rice %	
	2017	2018	2017	2018	2017	2018
Rice variety:						
Sakha 106	76.21 b	75.26 c	68.48 b	67.06 c	65.64 a	60.28b
Sakha 107	73.27 c	74.06d	65.14 d	66.42 c	60.39 c	59.32c
Giza 177	73.74 c	78.29b	66.92 c	69.40 b	64.17 b	63.36a
Giza 179	78.50 a	81.33a	71.44 a	72.68 a	59.82 d	60.32b
F test	**	**	**	**	**	**
P treatment:						
Super P (soil)	78.77 a	79.66 a	70.47 a	70.78 a	67.18 a	63.67b
Nano-P (spray)	78.92 a	80.10 a	70.80 a	71.19 a	66.73 a	64.60a
Super P (spray)	72.67 b	75.23b	65.92 b	67.43 b	59.57 b	58.27c
Control	71.38 c	73.95 c	64.78 c	66.17 c	56.55 c	56.73d
F test	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**

Values with the same letter in each column indicate no significant difference ($P < 0.05$)

Regarding the interaction effect, the application of phosphorus fertilizers enhanced them milling characteristics of all tested varieties (*Table 8*). The highest percentage of hulling was obtained when superphosphate as a soil application or nano phosphorus as a

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DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

foliar application was applied to Sakha 106 and Giza 179 in first season and to Giza 179 only in the second season. Application of superphosphate as a soil application or nano phosphorus as a foliar application to Giza 179 recorded the highest values of milling percentage in both seasons. Moreover, there were no significant differences between the effect of mentioned treatments on milling percentage and application of superphosphate as a soil application in first season only. The highest percentages of head rice were observed when superphosphate as a soil application or nano phosphorus as a foliar application was applied to Sakha 106 and Giza 177 in the first and second season respectively.

Table 8. *Hulling, milling and head rice % as affected by the interaction between rice variety and phosphorus treatment*

Treatments	2017				2018			
	Sakha 106	**Sakha 107**	**Giza 177**	**Giza 179**	**Sakha 106**	**Sakha 107**	**Giza 177**	**Giza 179**
	Hulling %							
Super P (soil)	81.41 a	76.68cd	76.08cd	80.92 a	77.80e	77.09e	80.42cd	83.35 a
Nano-P (spray)	79.63ab	76.78cd	77.84 bc	81.42a	79.50d	76.89e	81.70bc	82.34ab
Super P (spray)	73.33ef	70.44gh	72.04 fg	74.85de	73.06f	71.55g	77.11 e	79.20 d
Control	70.49gh	69.20 h	69.01 h	76.82 c	70.67g	70.74g	73.94 f	80.46cd
	Milling %							
Super P (soil)	72.74ab	67.43ef	68.72de	73.01ab	69.21 d	68.81 d	70.89bc	74.19 a
Nano-P (spray)	71.53bc	67.68 e	70.12cd	73.88 a	70.46 c	68.71 d	71.84 b	73.75 a
Super P (spray)	65.70 g	62.80 h	65.98fg	69.22 d	64.76fg	64.92 f	68.6 d	71.46 b
Control	63.97 h	62.63 h	62.87 h	69.65 d	63.83gh	63.23 h	66.29 e	71.33bc
	Head rice %							
Super P (soil)	72.05 a	64.95cde	64.82cde	66.88bc	64.07 bc	61.85 d	65.39ab	63.38 cd
Nano-P (spray)	70.62 a	62.85 e	67.61 b	65.83bcd	65.05 bc	61.94 d	66.93a	64.49 bc
Super P (spray)	60.28f	58.66 f	64.25de	55.08g	55.88 gh	57.67ef	61.91d	57.63efg
Control	59.61f	55.08 g	60.00 f	51.49h	56.11fgh	55.82h	59.20e	55.79h

Different letters indicate statistical significance ($P < 0.05$) within the same characteristic and the same year

The results of post-harvest milled grain analysis indicated that there were changes in nitrogen and phosphorus content percentages among the tested rice varieties as well as among phosphorus treatments (*Table 9*). Data showed that the highest values of nitrogen and phosphorus content percentages were obtained in Giza 179 rice variety. On the other hand application of phosphorus fertilizers as a soil or a foliar application enhanced the absorption of nitrogen and phosphorus by rice grains. Regarding the interaction effect, data in *Table 10* showed that the application of different phosphorus treatments increased the uptake of nitrogen and phosphorus by all rice varieties. These results confirm that nutrients uptake is controlled by source and method of phosphorus application which was observed also by Miranda-Villagómez et al. (2019). Rock phosphate if use as nano form it may increase the availability of phosphorus to the plant because the direct application of rock phosphate nanoparticles on the crop may prevent

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

fixation in the soil similarly there is no silicic acid, iron, and calcium for fixation of the phosphorus hence it increases phosphorus availability to the crop plants (Qureshi et al., 2019; Suriyaprabha et al., 2012). Wang et al., 2011 found that applying nanomaterials fertilizer can increase NPK absorption amount of rice plants and promoting transportation of those nutrients to grains. Dhansil et al., 2018 observed that the application of phosphorus nanoparticles showed a positive relationship between nitrogen and phosphorus uptake by pearl millet.

Table 9. *Nitrogen (%) and Phosphorus (%) of the tested rice varieties under phosphorus fertilizer treatments*

Treatment	Nitrogen (%)		Phosphorus (%)	
	2017	2018	2017	2018
Varieties:				
Sakha 106	1.15 b	1.19 b	0.400 b	0.455 b
Sakha 107	1.16 b	1.16 c	0.39 b	0.42 c
Giza 177	1.08 c	1.10 d	0.39 b	0.42 c
Giza 179	1.24 a	1.30 a	0.43 a	0.48 a
F test	**	**	**	**
P treatments:				
Super P (soil)	1.19 b	1.25 b	0.41 a	0.46 b
Nano-P (spray)	1.23 a	1.29 a	0.42 a	0.48 a
Super P (spray)	1.20 ab	1.20 c	0.41 a	0.47 ab
Control	1.02 c	1.02 d	0.37 b	0.40 c
F test	**	**	**	**
Interaction effect	**	**	**	**

Values with the same letter in each column indicate no significant difference ($P < 0.05$)

Table 10. *Nitrogen (%) and phosphorus (%) contents in milled rice grains as affected by the interaction between rice variety and phosphorus treatment*

Treatments	2017				2018			
	Sakha 106	Sakha 107	Giza 177	Giza 179	Sakha 106	Sakha 107	Giza 177	Giza 179
	Nitrogen (%)							
Super P (soil)	1.20cde	1.19 def	1.08 gh	1..28ab	1.27cd	1.22de	1.12 f	1..39 b
Nano-P (spray)	1.22bcd	1.26abc	1.12fgh	1.30 a	1.20 e	1.28 c	1.13 f	1.52 a
Super P (spray)	1.20 cd	1.13 efg	1.18 def	1.30 a	1.27 cd	1.13 f	1.12 f	1.28 c
Control	1.00 ij	1.05 hi	0.95 j	1.07 gh	1.02 g	1.02 g	1.04 g	1.02 g
	Phosphorus (%)							
Super P (soil)	0.41 bc	0.40bcd	0.36 ef	0.46 a	0.43def	0.47 bc	0.44cde	0.49 b
Nano-P (spray)	0.41 bc	0.41 bc	0.41bcd	0.43 b	0.49 b	0.48 b	0.40 fg	0.54 a
Super P (spray)	0.42 bc	0.38 de	0.39cde	0.46 a	0.50 ab	0.45 cd	0.43 def	0.49 b
Control	0.35 f	0.36 ef	0.42 b	0.36 ef	0.38 g	0.40 fg	0.41 ef	0.40 fg

Different letters indicate statistical significance ($P < 0.05$) within the same characteristic and the same year

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DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

Conclusion

The results demonstrated that the tested rice varieties displayed differential phosphorus response depending on the source and method of phosphorus fertilizer used. Phosphorus supplied from superphosphate as a soil application exhibited a higher response than supplied as foliar application. Nano-phosphorus application as a foliar promoted yield, quality and NP uptake by rice grain. Furthers studies are needed on the effect of nano-phosphorus on rice varieties behavior under Egyptian condition.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76737684

ROLE OF PLANT PROBIOTICS, SUCROSE AND SILICON IN THE PRODUCTION OF TOMATO (*SOLANUM LYCOPERSICUM* L.) SEEDLINGS UNDER HEAT STRESS IN A GREENHOUSE

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(Received 4th Jun 2020; accepted 20th Aug 2020)

Abstract. The rising temperature and global warming is a great challenge our planet facing nowadays. The production of vegetables in a greenhouse system under high temperatures could also be considered a vital threat for arid zones in particular. This study was carried out during two successive summer seasons under greenhouse conditions. The effect of different amendments including plant probiotics, silicon, sucrose and paclobutrazol were investigated on growth and quality of tomato seedlings under heat stress in a greenhouse. The main findings of this study were the followings: foliar applications of plant probiotics (100 mg l^{-1}) resulted in the tallest seedlings followed by sucrose (10%) and silicon (500 mg l^{-1}) treatments 28 and 35 days after sowing (DAS), whereas the shortest seedlings were obtained from the treatments of the control, silicon (100 mg l^{-1}) and paclobutrazol (100 mg l^{-1}). There were significant differences among all treatments for chlorophyll content, at both growth stages and after 28 and 35 DAS in both seasons in most cases. Tomato seedlings could overcome the high temperature stress under greenhouse conditions, with foliar application of plant probiotics, sucrose and silicon. Further studies are needed focusing on the gene expression and biochemical traits under previous treatment conditions.
Keywords: *high-temperature stress, seedling quality, chlorophyll, healthy seedlings, abiotic stress, vegetative growth, NPK contents, agro-chemical compounds, climate change*

Introduction

Tomato (*Solanum lycopersicum* L.) is considered a popular vegetable worldwide. This Solanaceous seedling has categorized as an important model plant in investigating the stress tolerance (Zhang et al., 2018; Alturki et al., 2020). So, several stress studies have been carried out on tomato seedlings such as heat stress (Lyu et al., 2018), water stress (Zhang et al., 2018), drought (Bian et al., 2019), salinity (Zhou et al., 2019), UV-B stress (Liu et al., 2020), chilling (Dong et al., 2020), and waterlogging (Elkelish et al., 2020). Many strategies could be adapted to overcome these stresses depending on the kind of stress, cultivated plants and its growth stage (Zhou et al., 2020). Many

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

researchers reported about these strategies in the case of tomato seedlings, which include application of spermidine (Sang et al., 2016), calcium, magnesium and potassium compounds (Sakhonwasee and Phingkasan, 2017; Nafees et al., 2019), ascorbic acid (Alayafi, 2020), melatonin and salicylic acid (Jahan et al., 2019a, b) as well as proline (Tonhati et al., 2020).

Egypt is ranked the fifth country worldwide in 2018 concerning tomato production (6,624,733 Mg), the area harvested was 161,702 ha and the yield recorded 40.96 Mg ha^{-1} (FAOSTAT, 2020). So, the high quality of tomato seedlings and its transplantation are common practice in successful production of tomatoes for fast sustainable establishment coupled with enhancement of earliness, uniform maturity and total yield as well as quality (FAO, 2017). Therefore, the production of healthy and vigorous tomato seedlings is the most important factor controlling this successful commercial production and the quality of tomato fruits (Gama et al., 2015; El-Shafeey et al., 2019). The produced seedlings also should be hardened to increase stress resistance by increasing content of carbohydrates, dry matter, and thickness of the wax layer as well as reducing plant size, water loss and slowing plant growth (Hossain et al., 2020). This target could be achieved by spraying the seedlings with some solutions such as paclobutrazol (Zhao et al., 2017; Zhang et al., 2019) and silicon (Zhang et al., 2018).

Climate change and global warming are serious problems threatening the agricultural production. The global temperature is projected to increase with a rate that may reach 1.5 and 3.5 °C by 2050 and 2100, respectively (Zhou et al., 2020). The high temperature or heat stress is considered one of the most devastating abiotic stresses, which becomes more serious due to global warming (Bilal et al., 2020). High temperature stress may disturb cellular homeostasis, affect physiological and biochemical reactions, as a consequence, it impedes plant growth, development and eventually reduces crop productivity (Fahad et al., 2017). Concerning the impact of high temperature on tomato, it is reported that the tomato growth may be impeded during the summer period threatening the yield (Zhou et al., 2020).

Silicon is considered the second abundant element in the earth's crust (28%) after oxygen (47%). There is no evidence that silicon is essential for growth of higher plants but this element has distinguished and beneficial roles as "*quasi-essential element*" in promoting the cultivated plants under different stresses (Zargar et al., 2019; Cao et al., 2020). Silicon can ameliorate the negative effects of stress (e.g., heat stress, drought and water stress) through improving the physiological and biochemical features of tomato (Zhang et al., 2018; Cao et al., 2020). Several studies have been published on the silicon and its roles in tomato production under different stresses (e.g., Li et al., 2015; Cao et al., 2017; Zhang et al., 2018; Cao et al., 2020), but no publications have been issued about the effects of heat stress.

Sucrose is an important carbohydrate in all plants, which has many functions as a storage compound, in regulating respiration and photosynthesis as well as in maintaining the osmotic pressure in plant cytosol (Xu et al., 2010). It is reported also that sucrose may act as a key signaling molecule in strawberry fruit ripening (Jia et al., 2013; Li et al., 2016; Luo et al., 2019, 2020). Sucrose also may play a part in the germination regulation and seedling development as a signaling molecule, although sucrose might readily convert into glucose and fructose (Xu et al., 2010). Sucrose has been applied in tomato production several years ago (Went and Carter, 1948) in order to inhibit the expansion of selected tomato fruits (Bussières et al., 2011), improve tomato seedlings quality (Javanmardi and Emami, 2013) and accelerate the postharvest of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

tomato fruit ripening (Li et al., 2016), but there have been no published studies about using sucrose in tomato seedlings under heat stress.

Probiotics could be defined based on the World Health Organization (WHO) as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (van der Geest et al., 2020). The term of plant probiotics has been used to characterize the "living plant-associated microorganisms", by which the growth of the host plant could be enhanced using adequate amount applied (Islam and Hossain, 2012). Plant probiotic microorganisms are beneficial microorganisms, which could reduce the problems of the environment and also known as bio-fertilizers, bio-controllers, bio-protectants, or bio-stimulants (Vandenberghe et al., 2017). Many studies used plant probiotics emphasizing their role in the agroecosystems (Kumar et al., 2017), such as to enhance plant growth and resistance of diseases (Jayakumar et al., 2019), but still further studies are needed on the growth of tomato seedling under heat stress (De Palma et al., 2017).

Therefore, the aim of this study was to improve the growth and quality of tomato seedlings under heat stress using plant probiotics as well as the individual application of sucrose, silicon and paclobutrazol.

Materials and methods

Plant materials and growth conditions

Two experiments were carried out during summer seasons of 2017 and 2018 (during July and August) under plastic greenhouse conditions in a private nursery in Qulin district, Kafr El-Sheikh Governorate, Egypt. The mean maximum and minimum air temperatures and relative humidity inside this greenhouse during the experiments were recorded daily as a mean to be 45/27 °C during day/night and 55 to 80%, respectively. Tomato seeds, super strain B variety were sown in seedling trays (209 cell). This variety is early maturing and produces oval/square fruits hybrid that could weigh up to 140 g and is vastly used for processing market all over the world, typically with dual purposes. It is resistant to *Verticillium* wilt, gray leaf spot and *Fusarium* wilt. These trays were filled with the commercial medium of the coco peat and vermiculite (1:1 volume), that was mixed with 300 g ammonium sulphate (20% N) + 150 g potassium sulphate (48% K_2O) + 400 g calcium super phosphate (15.5% P_2O_5) + 50 g micronutrients + 4 g calcium carbonate + 50 g fungicide. All previous components were mixed well during preparation the media mixture. The mixture must be well moisturized and filled well in the seedling trays. Tomato seedlings were watered daily and fertilized weekly with recommended nutrients solution. It is worth to mention that, the optimum temperature for tomato seedlings growth ranges from 18 to 30 °C, whereas the growing of seedling may stop when it increases over 35 °C.

Treatments and experimental design

Treatments were arranged in completely randomized design twice in both seasons in three random replicates, each replicate was one tray (209 cells) and actually occupied 153 cells per tray because the edge cells were not used for getting data. These treatments were arranged individually as follows:

1. Control (untreated).

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

2. Silicon (Si) at four concentrations (i.e., 100, 200, 300 and 500 mg l^{-1}) were sprayed on seedlings using diatomite (86-89% SiO_2). The diatomite as a source of silicon was bought from Al-Ahram Company.
3. Sucrose at three concentrations (i.e., 2.5, 5 and 10%) as a commercial product was added with foliar application.
4. Plant probiotics (100 mg l^{-1}) were imported from Hungary, as a commercial product "Okant-Agro". The ingredients of this product included water, Sustainable Community Development (SCD) microorganisms, bio sugar cane and rock flour.
5. Paclobutrazol (100 mg l^{-1}) was sprayed three times as a commercial product "New Coltar Super", which contains 25% paclobutrazol and produced by Starcheam Company, Egypt. Paclobutrazol (PBZ) is a plant growth retardant (as an antagonist of the plant hormone gibberellin) and tri-azole fungicide.

All previous treatments were sprayed on seedlings three times weekly after complete germination. Foliar application was carried out three times on the seedlings after 10, 17 and 24 days from sowing. The first true leaf emerged after 8 days from sowing and the germination of all seeds started after 4-5 days after cultivation. The measured parameters of vegetative growth were recorded after 28 and 35 days from sowing. The seedling parameters were included the height (cm), stem diameter (mm), leaf area (cm^2), fresh weight of roots and shoots (g), dry weight of roots and shoots (g).

Total chlorophyll and NPK contents in leaves

Total chlorophyll content was measured by the Soil Plant Analysis Development (SPAD) chlorophyll meter (Minolta, Co., Ltd, Japan), on fully expanded and apical leaves without destroying them. The leaves were dried at 70 °C until a constant weight, then ground and wet digested using sulfuric acid and hydrogen peroxide. The nitrogen, phosphorus and potassium contents in the digested plant leaves were determined using micro-Kjeldahl method, spectrophotometer and flame photometer, respectively (Sparks et al., 1996).

Statistical analyses

All the obtained data during both seasons of the study were tabulated and statistically analyzed using Duncan's Multiple Range Test for comparing between means of different treatments according to Snedecor and Cochran (1990). All statistical analyses were performed using analysis of variance technique by means of "M-STAT" computer software package and the means were compared by Duncan's multiple range test.

Results

The cultivation of tomato in greenhouse has become increasingly popular in Egypt particularly in the winter to avoid unfavorable weather and to protect cultivated seedlings, but in the summer a threat of heat stress may occur. The management of cultivating tomato seedlings under greenhouse or growth chamber has been reported in literatures. This management includes application of spermidine, ascorbic acid, melatonin, and recently proline as well as heat shock treatment of tomato seedlings (Yang et al., 2019). The proline may support the growth of tomato seedlings under

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

greenhouse conditions by lowering the hydrogen peroxide and malondialdehyde contents as well as alleviating the damage of high temperature and enhancing water use efficiency (Tonhati et al., 2020). In this study, the foliar application of plant probiotics, silicon, sucrose and paclobutrazol were investigated on tomato seedlings under greenhouse conditions during the highest temperatures in July and August in Egypt. The improvement of seedling growth and quality under such extreme conditions using the previous amendments was the main target of this study.

Seedling vegetative growth

The production of vegetables in particular tomato under large protected cultivation area has developed in recent years seeking for controlled intensive production. However, many serious problems have been established causing several stresses (e.g., heat, man-made- and secondary-soil salinization) in arid and semi-arid regions due to the intensive use of chemical fertilizers and unreasonable crop rotation (Li et al., 2015). The heat stress or rising temperature under greenhouse atmosphere has become a crucial problem that threatens the production of tomato and other vegetables under the protected cultivation zones particularly in the summer. Therefore, new approaches should be adopted to overcome the heat stress in particular under greenhouse production of tomato seedlings like silicon, sucrose, and plant probiotics as investigated in the current study.

The foliar applications of bio- (i.e., plant probiotics) and agro-chemical compounds (i.e., sucrose, silicon and paclobutrazol) on tomato seedlings after 28 and 35 days from sowing were impacted in general on the most studied treatments in both seasons (*Table 1*). Silicon is an important element, which already has been confirmed its mitigation role under biotic (e.g., pests and pathogens) and abiotic stresses (e.g., drought, salinity, etc.) such as salinity (Li et al., 2015; Muneer and Jeong, 2015), drought (Cao et al., 2017, 2020), osmotic stress (Ali et al., 2018), high-pH stress (Khan et al., 2019) and water stress (Zhang et al., 2018) as reported by many researchers, but the heat stress on tomato seedlings still needs more studies (Khan et al., 2020).

Different doses of silicon (100, 200, 300 and 500 mg l^{-1}) were foliar applied on tomato seedlings under greenhouse conditions. Regarding seedlings height, the tallest significant seedlings (6.23 and 13.0 cm) were recorded for the application of probiotics rate of 100 mg l^{-1} after 28 and 35 days from sowing, respectively in the first season, whereas the non-significant values were 6.1 and 13.3 cm in the second season. Comparing to the other Si doses, the silicon dose of 100 mg l^{-1} resulted in the shortest seedling, which obtained also from the control and paclobutrazol treatments (*Table 1*). The values of all studied vegetative parameters of seedlings (leaf area, stem diameter, fresh and dry weight of roots and shoots of seedlings) increased by increasing Si doses (*Tables 2* and *3*). The application rate 100 mg l^{-1} for both silicon and paclobutrazol showed significant differences with control treatment for almost vegetative growth parameters (*Table 1*).

Sucrose, as a common sugar, produced naturally in plants, consists of two mono-saccharides (i.e., fructose and glucose). It is also the main carbon form, which could be translocated in the higher plants. Sucrose also can regulate the genes, which may be involved in metabolism, photosynthesis, and developmental processes in plants (Xu et al., 2010). Sucrose may control the expression level of the "ApL3" gene that is encoded in tomato leaf and fruit as "Adenosine diphosphate (ADP) glucose focal phosphorylase (AGPase)" (Jia et al., 2016). The cultivated plants can use sugar "sucrose" as a source

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

for energy in the elongation of roots, initiation of lateral roots and as a compatible solute in roots for the osmotic adjustment (Xu et al., 2010).

Table 1. *The foliar application impacts of bio- and agro-chemical compounds on some vegetative growth parameters of tomato seedlings during both seasons (2017 and 2018)*

Treatments	Seedling height (cm)		Stem diameter (mm)		Leaf area (cm^2)	
	Sampling date after sowing (day)					
	28	35	28	35	28	35
	The first season					
Control	5.67 c	11.75 c	2.30 cde	2.59 efg	3.90 ef	7.17 cd
Silicon (100 mg l^{-1})	5.70 c	12.00 c	2.37 bcde	2.57 fg	4.23 cd	7.53 bc
Silicon (200 mg l^{-1})	5.77 bc	12.91 a	2.53 ab	2.56 g	4.40 bc	6.96 de
Silicon (300 mg l^{-1})	5.87 bc	12.75 ab	2.27 de	2.64 def	4.37 bc	7.46 bc
Silicon (500 mg l^{-1})	5.97 b	13.25 a	2.60 a	2.66 cde	4.80 a	7.60 ab
Sucrose (2.5%)	5.87 bc	12.26 bc	2.17 e	2.48 h	3.60 f	6.80 de
Sucrose (5%)	5.9 bc	12.99 a	2.30 cde	2.72 c	4.50 bc	7.70 ab
Sucrose (10%)	5.97 b	13.16 a	2.50 abc	2.88 b	4.80 a	7.97 a
Paclobutrazol, 100 mg l^{-1}	5.67 c	11.75 c	2.40 abcd	2.69 cd	4.07 de	6.77 e
Probiotics (100 mg l^{-1})	6.23 a	13.0 a	2.30 cde	2.96 a	4.60 ab	7.80 ab
	The second season					
Control	5.9	12.20 d	2.1 bc	2.95	3.50 c	7.1 bc
Silicon (100 mg l^{-1})	6.0	12.27 d	2.1 bc	3.06	3.75 abc	7.1 bc
Silicon (200 mg l^{-1})	5.9	12.40 cd	2.4 ab	3.00	3.80 abc	7.2 abc
Silicon (300 mg l^{-1})	6.1	12.50 cd	2.0 c	3.00	4.10 ab	7.2 abc
Silicon (500 mg l^{-1})	6.3	13.10 ab	2.6 a	3.20	4.30 a	7.3 abc
Sucrose (2.5%)	6.1	12.80 bc	2.0 c	3.00	3.50 c	7.1 bc
Sucrose (5%)	6.0	12.80 bc	2.3 abc	3.10	4.20 ab	7.2 abc
Sucrose (10%)	6.4	13.00 ab	2.4 ab	3.20	4.30 a	7.6 a
Paclobutrazol, 100 mg l^{-1}	6.0	11.70 e	2.1 bc	3.20	3.46 c	6.9 c
Probiotics (100 mg l^{-1})	6.1	13.30 a	2.0 c	3.10	3.70 bc	7.4 ab

Means in the same column followed by the same letter are not significantly different according to DMRT at 0.05

Different levels of sucrose (2.5, 5.0 and 10%) were foliar applied on tomato seedlings under greenhouse conditions. These levels of sucrose were investigated on different vegetative parameters of tomato seedlings (*Table 1*). The foliar application of 10% sucrose showed the highest values of some studied vegetative parameters of tomato seedlings at 28 and 35 days after sowing in both seasons (*Table 1*). Many studies have shown the positive effects of applied sucrose on growth of seedlings and fruit ripening of tomato and strawberry compared to untreated seedlings or plants (e.g., Jia et al., 2016; Li et al., 2016; Luo et al., 2019, 2020). These results are consistent with Javanmardi and Emami (2013), who found that application of sucrose solutions (25%) to tomato plants increased fresh and dry weights of shoots and roots. These results in partial agreed with the results from Hassankhah et al. (2014).

Plant probiotics can be defined as live bacteria capable to improve the crop yield, which may reduce or even eliminate chemical fertilizers. Many studies have shown that,

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

these bacteria can not improve the growth, but also the quality of foods. These probiotics are useful for human health because they can increase the food content of nutrients and many plant bioactive compounds (Woo and Pepe, 2018). The word "*probiotic*" is derived from the Greek, meaning *'for life'* and has had several different meanings. It induces plant stress resistance and mineralizes soil nutrients which results in enhancement of nutrient uptake by the plant (Menendez and Garcia-Fraile, 2017). Plant probiotics have significantly increased seedling emergence, vigor, plant weight, root system development compared with untreated rice plants (Khan et al., 2017). Also, probiotic bacteria have well-demonstrated mechanisms to support plant growth (Menendez and Garcia-Fraile, 2017). In the current study, the foliar application of plant probiotic had a positive effect on almost all vegetative growth aspects using rate of 100 mg l^{-1}. This impact might be attributed to the biological effect of probiotics as a biofertilizer, which enhances plant growth. Comparing with control, the values of vegetative growth in tomato seedlings have increased with the application of probiotics (*Table 1*).

Paclobutrazol (PBZ) is a member of the tri-azole group, which represents plant growth regulators. PBZ regulates excessive vegetative growth, establishes high density plantation, enhances flowering and induces early bearing (Gollagi et al., 2019). It may protect the cultivated plants from many environmental stresses such as salinity (Hu et al., 2017; Fan et al., 2020), high temperature (Baninasab and Ghobadi, 2011) and water stress (Mohan et al., 2020) as confirmed by many researchers (e.g., Soumya et al., 2017; Tesfahun, 2018). Paclobutrazol also has the ability to reduce gibberellin levels and affect the microbial population in soils as well as the activity of dehydrogenase enzyme (Gollagi et al., 2019). Concerning paclobutrazol and its impact on tomato seedlings, a few studies involved its inducing tolerance under water deficit stress (Pal et al., 2016) and use paclobutrazol in the production of tomato seedlings (Bene et al., 2014) but there are no investigations on heat stress and tomato seedlings in the presence of paclobutrazol.

In the current study, the foliar application of paclobutrazol had a negative effect on the vegetative growth aspects of tomato seedlings (*Table 1*). The reason might be due to paclobutrazol is delaying cell division and elongation in tissues of the seedlings. This result was in harmony with the results, which reported by Pal et al. (2016), who confirmed that PBZ application reduced the plant height, the seedling growth rate and consequently the seedlings size under deficit irrigation, whereas PBZ improved leaf number and the stem diameter. The paclobutrazol also has the ability to reduce shoot elongation, leaf expansion and stem diameter growth in many plant species like tomato because it is an active inhibitor of gibberellic acid biosynthesis, therefore it retards the division and elongation of cells, and consequently, growth in plant stem length (de Rezende et al., 2017).

Biomass production of seedlings

The biomass production of seedlings is the final desired harvest, where the high biomass production may support the seedlings to grow under the environmental stresses. It could be noticed that, the foliar application of plant probiotic (100 mg l^{-1}), silicon (500 mg l^{-1}) and sucrose (10%) separately have recorded the highest values on tomato seedlings in almost all vegetative growth parameters, whereas the lowest values belonged to the control and 100 mg l^{-1} of silicon as well as 2.5% sucrose (*Tables 1, 2* and *3*). For shoot and root fresh or dry weight, the highest values were recorded by seedlings treated by foliar application of sucrose (10%) and silicon application (500 mg l^{-1}) in both seasons and treatments 28 and 35 days after sowing. Sucrose treatment at 10% was produced the

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

highest plant fresh weight of seedlings as compared with the control treatment (more than 16%), which showed the lowest values in most cases (*Table 2*). Various results obtained including sucrose on tomato (Javanmardi and Emami, 2013), and sucrose on walnut (Hassankhah et al., 2014) were in harmony with our results. It is noticed also that, there is no publication concerning the role of sucrose, silicon, and plant probiotics on the growth and quality of tomato seedling under heat stress.

Table 2. *The foliar application of bio- and agro-chemical compounds on shoot and root fresh weights and the total fresh weight of tomato seedlings during both seasons (2017 and 2018)*

Treatments	Shoot fresh weight (g plant^{-1})		Root fresh weight (g plant^{-1})		Seedling fresh weight (g plant^{-1})	
	Sampling date after sowing (day)					
	28	35	28	35	28	35
	The first season					
Control	1.11 h	3.20 cd	0.30 j	0.80 j	1.42 i	4.04 h
Silicon (100 mg l^{-1})	1.25 e	3.00 e	0.32 h	1.00 h	1.60 f	4.01 i
Silicon (200 mg l^{-1})	1.29 d	3.20 cd	0.36 e	1.03 e	1.66 e	4.25 e
Silicon (300 mg l^{-1})	1.30 d	3.40 b	0.38 c	1.05 c	1.71 c	4.46 c
Silicon (500 mg l^{-1})	1.39 b	3.58 a	0.39 b	1.12 b	1.78 b	4.71 b
Sucrose (2.5%)	1.18 f	3.18 bc	0.36 f	1.01 g	1.55 g	4.10 g
Sucrose (5%)	1.31 d	3.32 g	0.37 d	1.04 d	1.69 d	4.37 d
Sucrose (10%)	1.45 a	3.70 a	0.40 a	1.14 a	1.85 a	4.85 a
Paclobutrazol, 100 mg l^{-1}	1.13 g	2.82 f	0.31 i	0.97 i	1.45 h	3.80 j
Probiotics (100 mg l^{-1})	1.35 c	3.13 de	0.34 g	1.02 f	1.70 cd	4.16 f
	The second season					
Control	1.12 e	3.00 d	0.26 e	1.20	1.38 e	4.29 e
Silicon (100 mg l^{-1})	1.22 d	3.03 d	0.26 e	1.20	1.49 d	4.32 e
Silicon (200 mg l^{-1})	1.27cd	3.15 c	0.30 d	1.20	1.57 cd	4.44 d
Silicon (300 mg l^{-1})	1.27cd	3.30 b	0.34 c	1.19	1.61 c	4.50 d
Silicon (500 mg l^{-1})	1.40 b	3.45 a	0.39 a	1.30	1.80 b	4.80 a
Sucrose (2.5%)	1.27cd	3.15 c	0.30 d	1.23	1.58 c	4.50 d
Sucrose (5%)	1.43 b	3.27 b	0.36 b	1.30	1.81 b	4.60 c
Sucrose (10%)	1.51 a	3.54 a	0.38 a	1.30	1.90 a	4.84 a
Paclobutrazol, 100 mg l^{-1}	1.22 d	2.85 e	0.26 e	1.30	1.49 d	4.11 f
Probiotics (100 mg l^{-1})	1.30 c	3.47 a	0.30 d	1.20	1.60 c	4.70 b

Means in the same column followed by the same letter are not significantly different according to DMRT at 0.05

Total chlorophyll content

Chlorophyll (Chl) is the essential plant organ for harvesting the light and transducing energy *via* photosynthesis. Photosynthesis is the main factor in the growth and development of cultivated plants. Under stress, the plants are required to protect the chlorophyll system from the degradation and to sustain Chl biosynthesis, which could be mediated by more than 17 enzymes (Li et al., 2015; Dong et al., 2020). The total

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

chlorophyll content in tomato seedlings was investigated using the foliar applications of studied bio- and agro-chemical compounds (*Table 4*). The foliar application of sucrose (10%), silicon (500 mg l^{-1}), and plant probiotics (100 mg l^{-1}) significantly increased chlorophyll content, whereas paclobutrazol (100 mg l^{-1}) decreased it or it was equal to the control values. The mean values of Chl after 28 days were recorded the highest, whereas the values were decreased after 35 days in both seasons. This may be due to 28 days being the optimum date for handling and transportation of seedlings and after that, the chlorophyll content was degraded by time in the nursery. These results are similar to those obtained by Javanmardi and Emami (2013) and Hassankhah et al. (2014), who stated that sucrose can ameliorate stress on chlorophyll of tomato and walnut.

Table 3. *The foliar application of bio- and agro-chemical compounds on shoot and root dry weights and the total dry weight of tomato seedlings during both seasons (2017 and 2018)*

Treatments	Shoot dry weight (g plant^{-1})		Root dry weight (g plant^{-1})		Seedling dry weight (g plant^{-1})	
	Sampling date after sowing (day)					
	28	35	28	35	28	35
	The first season					
Control	0.135 h	0.425 h	0.033 g	0.079 i	0.169 i	0.505 i
Silicon (100 mg l^{-1})	0.144 g	0.431 g	0.036 f	0.081 h	0.180 h	0.514 h
Silicon (200 mg l^{-1})	0.145 g	0.460 d	0.038 e	0.083 g	0.185 g	0.545 e
Silicon (300 mg l^{-1})	0.150 e	0.486 c	0.039 de	0.085 f	0.189 f	0.572 c
Silicon (500 mg l^{-1})	0.168 b	0.492 b	0.045 b	0.103 b	0.218 b	0.595 b
Sucrose (2.5%)	0.162 d	0.430 g	0.039 de	0.088 e	0.203 d	0.519 g
Sucrose (5%)	0.165 c	0.452 e	0.040 d	0.100 c	0.207 c	0.553 d
Sucrose (10%)	0.170 a	0.496 a	0.048 a	0.110 a	0.222 a	0.606 a
Paclobutrazol, 100 mg l^{-1}	0.148 f	0.402 i	0.043 c	0.092 d	0.192 e	0.475 j
Probiotics (100 mg l^{-1})	0.150 e	0.444 f	0.038 e	0.086 f	0.189 f	0.533 f
	The second season					
Control	0.14 cd	0.450 e	0.025 b	0.140 f	0.160 d	0.590 g
Silicon (100 mg l^{-1})	0.13 d	0.450 e	0.025 b	0.140 f	0.160 d	0.590 g
Silicon (200 mg l^{-1})	0.16 c	0.46 de	0.030 ab	0.144 d	0.190 bcd	0.603 f
Silicon (300 mg l^{-1})	0.17 b	0.490 b	0.037 b	0.148 c	0.217 abc	0.637 c
Silicon (500 mg l^{-1})	0.21 a	0.520 a	0.035 ab	0.154 b	0.223 ab	0.674 b
Sucrose (2.5%)	0.14 cd	0.450 e	0.029 ab	0.142 e	0.190 bcd	0.592 g
Sucrose (5%)	0.200 a	0.480 bc	0.033 ab	0.148 c	0.230 ab	0.628 d
Sucrose (10%)	0.210 a	0.530 a	0.039 a	0.158 a	0.250 a	0.688 a
Paclobutrazol, 100 mg l^{-1}	0.140 cd	0.470 cd	0.030 ab	0.154 b	0.180 cd	0.624 d
Probiotics (100 mg l^{-1})	0.150 c	0.470 cd	0.030 ab	0.142 e	0.180 cd	0.610 e

Means in the same column followed by the same letter are not significantly different according to DMRT at 0.05

Chemical composition of leaves

The NPK content in tomato seedling leaves represent one of the most important parameters of seedling quality due to the potentiality of NPK nutrients in several

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

metabolic processes. Several parameters could assess the quality of produced seedlings such as the growth rate, biomass accumulation, chlorophyll content, and NPK content. The chemical composition (i.e., NPK contents) of tomato seedlings was investigated using the foliar applications of studied bio- and agro-chemical compounds (*Table 4*). This chemical composition included the total chlorophyll content in seedlings and NPK contents in leaves as well. Leaf mineral content of tomato seedlings showed that application of both silicon (500 mg l^{-1}) and sucrose (10%) significantly increased potassium content (3.6 and 3.3%, respectively) compared to the paclobutrazol treatment, which recorded the lowest value (2.9%) in the first season. However, in the second season the application of Si (500 mg l^{-1}), sucrose (10%) and probiotics (100 mg l^{-1}) significantly increased leaf K-content comparing with paclobutrazol treatment, which resulted the lowest values (2.7%). The following treatments, which involved foliar applied silicon (500 mg l^{-1}), sucrose (10%) and probiotics (100 mg l^{-1}) had statistically the highest P content in leaves (0.19%) of tomato seedlings, while the reverse was true with those sprayed with water (control) or 100 mg l^{-1} silicon in both seasons (0.16%). The same trend was achieved in the case of N-content (1.9 and 2.0%) in seedling leaves for applied Si (500 mg l^{-1}), sucrose (10%) and probiotics (100 mg l^{-1}) in the first and second season (1.9 and 2.0%).

Table 4. *The foliar application of bio- and agro-chemical compounds on content of total chlorophyll, N, P and K in tomato seedlings during both seasons (2017 and 2018)*

Treatments	Total chlorophyll content (SPAD)		Nutrient content in seedling leaves		
	Sampling after sowing (day) (%)				
	28	35	N	P	K
	The first season				
Control	4.90 de	2.97 c	1.5 e	0.160 e	3.2 c
Silicon (100 mg l^{-1})	4.57 ef	3.37 ab	1.7 cd	0.160 e	3.2 c
Silicon (200 mg l^{-1})	4.43 f	2.93 c	1.8 bc	0.165 de	3.1 cd
Silicon (300 mg l^{-1})	4.63 ef	3.00 bc	1.8 bc	0.175 bc	3.2 c
Silicon (500 mg l^{-1})	5.27 bcd	3.43 a	1.9 ab	0.190 a	3.5 ab
Sucrose (2.5%)	4.70 ef	3.37 ab	1.7 cd	0.170 cd	3.0 de
Sucrose (5%)	5.63 ab	3.57 a	1.9 ab	0.180 b	3.4 b
Sucrose (10%)	5.77 a	3.73 a	2.0 a	0.190 a	3.6 a
Paclobutrazol (100 mg l^{-1})	5.23 cd	3.37 ab	1.6 de	0.180 b	2.9 e
Probiotics (100 mg l^{-1})	5.50 abc	2.87 c	1.9 ab	0.190 a	3.4 b
	The second season				
Control	5.8 ab	3.6	1.4 c	0.160 e	3.1 abc
Silicon (100 mg l^{-1})	5.8 ab	3.8	1.6 bc	0.160 e	3.0 abc
Silicon (200 mg l^{-1})	5.8 ab	3.8	1.7 abc	0.165 de	3.0 abc
Silicon (300 mg l^{-1})	6.0 ab	3.6	1.7 abc	0.168 cde	2.9 bc
Silicon (500 mg l^{-1})	6.0 ab	3.95	1.9 ab	0.175 bcd	3.3 ab
Sucrose (2.5%)	5.8 ab	3.6	1.7 abc	0.180 abc	2.9 bc
Sucrose (5%)	5.9 ab	3.9	1.8 ab	0.180 abc	3.1 abc
Sucrose (10%)	6.1 ab	4.0	1.9 ab	0.189 a	3.3 a
Paclobutrazol, 100 mg l^{-1}	5.6 b	3.6	1.8 ab	0.185 ab	2.7 c
Probiotics (100 mg l^{-1})	6.2 a	3.7	2.0 a	0.185 ab	3.3 a

Means in the same column followed by the same letter are not significantly different according to DMRT at 0.05

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

The regulation of plant growth with synthetic plant growth regulators has become a common agricultural practice. Such available synthetic plant growth regulators, paclobutrazols are potent at low concentrations to inhibit shoot growth and promising in reducing vigor of many species especially under high temperature conditions. Paclobutrazol is a very potent growth retardant that inhibits cell elongation and seems to inhibit gibberellin biosynthesis (Chen et al., 2020). So, paclobutrazol was investigated in this study to get healthy and strong seedlings.

Discussion

The agricultural production faces a serious threat represented by the climate changes and global warming particularly the raising atmospheric temperature. These high temperatures create heat stress, which may decrease the growth and productivity of many cultivated crops due to extreme damage in plant cells, death in a short time and then the loss in crop yield (Alayafi, 2020). This problem may be accelerated due to the "*greenhouse effect*". The production of tomato seedlings under greenhouse system may face this problem in particular in arid and semi-arid regions in the summer (mainly July and August), where the temperature is often over 40 C during these months. The optimal growth temperature of tomato ranges from 25 to 30 C during daytime (Zhou et al., 2020). Therefore, the production of tomato seedlings under greenhouse conditions in Egypt needs to manage the projected high temperature stress. This management was carried out in this current study using some biological (plant probiotics) and agro-chemical compounds (different doses of silicon and sucrose) comparing with paclobutrazol and control.

Foliar application of silicon (up to 500 mg l^{-1}) had positive effects on vegetative growth aspects that might be attributed to the effect of silicon in improving the tolerance of the seedlings to stress, increasing photosynthetic activity, water metabolism, chlorophyll content, antioxidant activities, protecting enzymes and enhancing uptake of necessary nutrients. The exogenous Si also may alleviate the chlorophyll degradation under stress. The heat stress could mainly affect biochemical reactions in particular restraining the photosynthetic process in plants (Zhou et al., 2017). The foliar application of high level of Si (500 mg l^{-1}) increased vegetative growth parameters (e.g., seedling height and leaf area) due to the beneficial role of Si under stress (Zhang et al., 2017). Silicon also protects the photosynthetic system and chlorophyll from degradation under stress. The photosynthetic damage may include the morphology of stomata and chloroplast, thylakoid electron transport and the fixation of carbon (Dong et al., 2020).

Many vegetables could be produced through the transplanting from seeds germination like tomato, pepper and eggplant. These seedlings should be healthy to get a higher yield. The transplanting process may increase the length of crop season, decrease the risk of adverse environmental conditions and reduce the cost of vegetable production (Javanmardi and Emami, 2013). The foliar application of sucrose up to 10% resulted in the best values of vegetative growth, biomass accumulation and chlorophyll content as well as NPK content in seedling leaves compared to control in the current study. The sucrose represents an important source for feeding the cultivated seedlings under heat stress because this source may provide seedlings with an additional carbohydrate, which is required for metabolic processes. A pivotal role of sucrose metabolism could be distinguished under biotic and abiotic stress besides its potential in mediation a lot of developmental processes and ripening of tomato fruits (Li et al., 2016). Heat stress is

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projected to cause many problems for tomato seedlings on the morphological, physiological (e.g., chlorophyll fluorescence and gas exchange) and biochemical level (e.g., the content of pigments and carbohydrates) as reported by Zhou et al. (2020).

Due to the inappropriate use of agro-chemicals, which has led to intensive degradation in soil and water resources besides environmental pollution, plant probiotics are considered as a crucial approach in the agriculture nowadays (Sohrabi et al., 2020). So, the using of plant probiotics has gained much more attention during the last decades of sustainable agriculture. Plant probiotics could support the cultivated plants through their potential of promoting plant growth, siderophore production, phytohormone biosynthesis, biostimulators, and phosphate solubilization (Menendez and Garcia-Fraile, 2017). These previous approaches may support cultivated plants under stress. The activity of plant probiotics depends on the environmental conditions particularly temperature, it increases with temperature till a limit. In the current study, the foliar application of probiotics recorded high values in vegetative growth, biomass production of tomato seedlings as well as chlorophyll content beside the NPK contents. These high values were nearly like those, which were recorded by applied sucrose (10%) and silicon (500 mg l-1). The mechanism may be resulted from solubilizing minerals in soil, which subsequently enhanced growth of plants. In other words, the mechanism may include the action of probiotics as bio-control, bio-fertilization and bio-stimulation, which enhances the plant growth and induces seedlings resistance to abiotic stress. Different managements which tomato seedlings could adapt under heat stress are summarized through a comparison between the current study and some published articles under different growth conditions (*Table 5*).

Table 5. *A comparison between the current study and some published articles concerning heat stress on tomato seedlings and growth conditions*

Seedling age at heat stress treating	Growth conditions and cultivated cv.	Most important findings of the study	Ref.
At the 3rd true leaf, 38/28 °C day/night for 7 days, spaying 1 mM spermidine	Growth chamber and "cv. Puhong 968"	Spraying spermidine may alleviate the damage of heat stress through enhancing oxidative stress of non- and enzymatic antioxidants	Sang et al. (2016)
At the 3rd week old of seedlings, 39/29 °C for 14 days	Growth chamber and "cv. Luktho"	Foliar application of the $CaCl_2$, $CaNO_3$, $MgCl_2$, or KNO_3 solution mitigated heat stress effect by reducing the ROS	Sakhonwasee and Phingkasan (2017)
Seedlings transferred into pots, grown for 25 days at 45/32 °C (day/night)	Growth chamber, NUN 5024 var., India, seeds primed in $Mg(NO_3)_2$	Priming seeds in $Mg(NO_3)_2$ (from 5 to 10 mM) may improve germination in normal (25 °C) and ameliorate high temperature stress (40 °C)	Nafees et al. (2019)
At the 4th true leaf, seedlings exposed to 42 °C for 24 h	Growth chamber, cv. Hezuo 903, China, at 28/19 °C (day/night), melatonin (100 μM) sprayed for 7 days	Melatonin may control over-accumulation of hydrogen peroxide and superoxide, then lower the lipid peroxidation content	Jahan et al. (2019a)
At the 5th true leaf, seedlings exposed to 42 °C for 36 h	Growth chamber, cv. Hezuo 903, China, at 28/19 °C (day/night), salicylic acid (1 mM) sprayed for 7 days	Salicylic acid may increase proline content, the activity of photosynthesis and antioxidant enzyme functions	Jahan et al. (2019b)

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DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

After transfer into hydroponics, 40 °C for 8 h/day for 7 days, applied 0.5 mM AsA	Plants were grown in peat moss for 35 days, then transferred into hydroponic system	Ascorbic acid (AsA) may be considered a key signaling molecule that enhances the thermo-tolerance of tomato seedlings	Alayafi (2020)
At 18th day-old seedlings, sprayed 3 times and sampling after 4 and 5 weeks	In greenhouse, super strain B variety, under heat stress 40/25 °C (day/night) for 35 days	Foliar application of sucrose, silicon and plant probiotics improved the growth and quality of seedlings	Current study

Conclusion

The planet suffers from the climate changes and global warming, which lead to increase in the atmospheric temperature especially under the greenhouse conditions. Under the conditions of Egypt, the production of vegetables under greenhouse systems particularly tomato seedlings during the highest temperatures (mainly July and August) is a great challenge due to the heat stress. So, this production should be managed through the application of proper amendments including the agro-chemical (e.g., Si, Se, Ca, Mg), organic (e.g., sucrose, spermidine, ascorbic acid, melatonin and proline) and biological candidates (e.g., plant probiotics). Therefore, this current study was carried out to improve the growth and production of tomato seedlings under greenhouse conditions during the highest temperatures in Egypt (i.e., July and August). This investigation demonstrated that the growth and quality of tomato seedlings under greenhouse conditions could protect the chlorophyll system against heat stress through foliar application of silicon (500 mg l^{-1}), sucrose (10%), probiotics (100 mg l^{-1}) and paclobutrazol (100 mg l^{-1}) as well. At the same time, the quality of produced tomato seedlings also could be enhanced by the application of previous treatments to include the content of seedlings of NPK. These NPK could guarantee many metabolic processes in leaves of tomato seedlings such as proteins synthesis (N), the cellular division and formation of energetic structures (P) and the control of stomata closure, transportation of sugars, also it increases the tolerance to stress (K). It could be concluded also that, the foliar application of Si, sucrose, probiotics and paclobutrazol are effective on the production of tomato seedlings under heat stress. Further studies are needed to emphasize this role in separate and combined or multiple stresses.

Acknowledgements. The Authors thank the staff members of Physiology and Breeding of Horticultural Crops Laboratory, Dept. of Horticulture, Fac. of Agric., Kafrelsheikh University, Kafr El-Sheikh, Egypt for supporting this study as well as their help in completing this work.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_76857701

PHYTOREMEDIATION POTENTIAL OF LEMONGRASS (*CYMBOPOGON FLEXUOSUS* STAPF.) GROWN ON TANNERY SLUDGE CONTAMINATED SOIL

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(Received 7th Jun 2020; accepted 17th Sep 2020)

Abstract. Phytoremediation is one of the safest and sustainable approaches to reclaim metal contaminated soil. The present study assessed the phytoremediation potential of lemongrass (*Cymbopogon flexuosus* Stapf.) by growing it on soil mixed with different concentrations of tannery sludge i.e., 5, 10 and 15% regarded as 5% TS + S, 10%TS + S, and 15% TS + S whereas the soil without tannery sludge was taken as control (0%TS + S). After 35 days of growth, the plants were harvested to record various morpho-physiological attributes and accumulation of various heavy metals in above and below ground plant parts. Results revealed that soil mixed with TS reduced the morphological attributes i.e., the fresh weight, dry weight, number of roots, number of leaves, number of tillers, and shoot length up to 37.50, 26.86, 23.46, 56.25, 16.13 and 19.31%. Moreover, tannery sludge application enhanced the free radicals i.e., 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and regulated the antioxidant activities. Overall metal uptake was higher in the root than shoot whereas the amount of heavy metals uptake by plants was the highest at 15% concentration. The trend of metal uptake was recorded as: Cr > Cd > Cu > Mg > Fe > Zn > Ni > Pb.
Keywords: *antioxidants, chlorophyll contents, diphenyl picrylhydrazyl, lemongrass, metals*

Introduction

Environmental pollution is a major challenge of current century and poses serious threats to environment, agro-ecosystems and food safety (Ali and Khan, 2017; Hashem et al., 2017). Heavy metals are comparatively more dangerous than other pollutants because of its long persistence and ubiquitous nature (Lou et al., 2015; Ashraf et al., 2015). If level of these heavy metals exceeded by given threshold limits, it will impose devastating effects on living beings and their biological functions (Peng et al., 2009). Categorically, pollution is divided in to several types e.g., air, water, noise and soil pollution (Azizullah et al., 2011; Henschel et al., 2012). Consequently, soil pollution has only recently attracted considerable attention (Ashraf et al., 2017). Soil quality is directly related to food safety, human health, and sustainable economic and social development (Teng et al., 2014; Ashraf et al., 2018). Soil contamination with various heavy metals is one of the main threats for agricultural production systems (Ashraf and Tang, 2017; Beesley et al., 2011; Ali et al., 2013).

Leather industry is largely responsible for environmental pollution, due to the use of various hazardous chemicals in leather processing. For example, the skin of sheep, buffaloes, cows and camels are treated by these industries and almost 130 types of different potent chemicals are used for every different kind of material to be processed (Bareen and Tahira, 2011). Upon entrance, heavy metals interfere with plant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77037715

metabolism and thus caused morpho-physiological and biochemical changes in plants (Morkunas et al., 2018). In general, over production of reactive oxygen species (ROS), superoxide free radicals, hydroxyl free and non-free radical species such as oxygen and hydrogen peroxide are associated with highest metal uptake. Production of some cytotoxic compounds like methylglyoxal also disturbs the balance between pro-oxidant and anti-oxidant homeostasis within the plant cells thus causing oxidative stress (Oliveira et al., 2012; Thanikaivelan et al., 2005). Overall, heavy metal contamination reduced growth and productivity of crop plants by altering morpho-physiological functions and crop growth. Therefore, developments of various methodologies/ techniques are required to remediate the heavy metal contaminated soils. Bioremediation is also an eco-friendly approach to cleanse the metal polluted soils where hyperaccumulator plants are grown on contaminated lands either alone or with combination of suitable microbes (Edward, 2013).

Lemongrass (*Cymopogon flexuosus* L.) is an economically important herb and widely cultivated over the globe. It is used for versatile purposes e.g., leaves are used as green tea, stem in soups, poultry items and seafood (Jasha and Chase, 2014). Its leaves and inflorescence also produce an essential oil that is used in aromatherapy, perfumes and medicines (Verma et al., 2014). Lemongrass also exhibits various anti-cancerous (Kumar et al., 2008), antioxidant (Anand et al., 2011), antimicrobial, insecticidal (Rajeswara et al., 2015) and anti-inflammatory (Figueirinha et al., 2010) properties. Polyphenols, is a natural antioxidant, extracted from lemongrass, has been reported from preventing many diseases like cancers, cardiovascular, and degenerative diseases (Ogura et al., 2008). Hence, medicinal properties of Lemongrass are well recognized and widely acknowledged world-wide; however, its phyto-remediation potential in heavy metal contaminated soil was rarely investigated and/or explored. The present study was therefore conducted to evaluate the uptake and accumulation behavior of different heavy metals and/or phytoremediation potential of Lemongrass in tannery sludge contaminated soil.

Materials and methods

Experimental details

The samples were collected from sludge dumping site of Kasur Tannery Waste Management Agency (KTWMA) Kasur, Pakistan (31° 05’ 16.32” N, 74° 28’ 36.20” E; *Fig. 1*). Samples of tannery sludge were collected and stored in plastic drums. The garden soil samples were collected in polythene bags from the lawns of University of Education, Lahore. The respective samples of soil were sieved through a sieve mesh size of 2 cm^2 for further use. The experiment was set-up in the wire-house of Department of Botany, University of Education, Township Lahore, Pakistan. The treatments were comprised of four different concentrations of tannery sludge mixed with soil i.e., 0% Soil (Garden soil as a control), 5% TS + S (5 g of tannery sludge in 95 g of soil), 10% TS + S (10 g of tannery sludge in 90 g of soil) and 15% TS + S (15 g of tannery sludge in 85 g of soil) with three replicate. The experiment was setup in the month of November, 2018. The fresh seedlings (15 cm) of lemongrass were transplanted (one plant/pot) and were irrigated regularly with tap water (*Fig. 2*). After 35 days of growth, the plants were harvested to record the fresh and dry weight, number of roots, leaves and tiller per plant, and shoot length whilst the leaf chlorophyll contents were measured by SPAD meter (SPAD-502 Plus, Japan).

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DOI: http://dx.doi.org/10.15666/aeer/1806_77037715

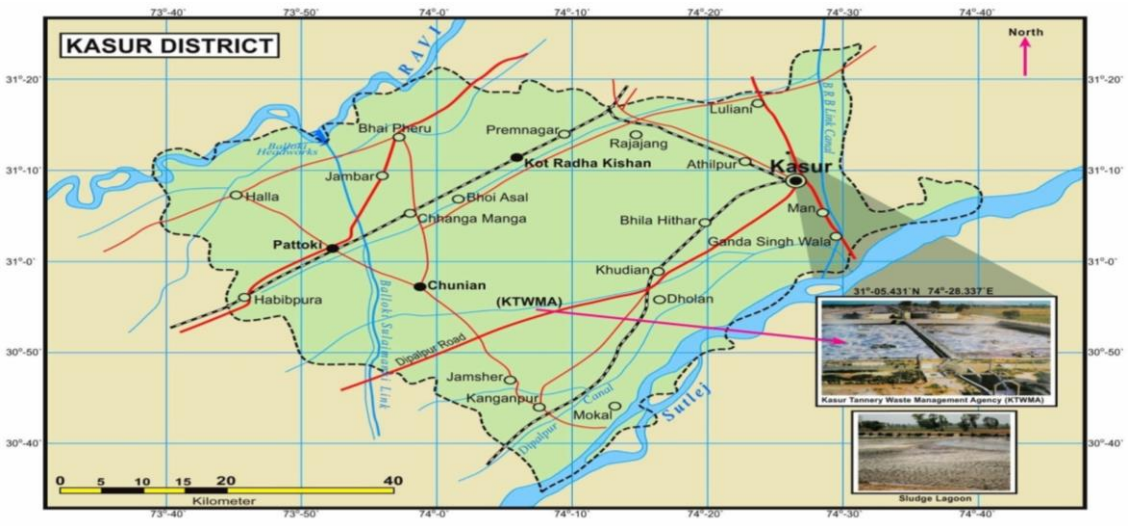

Figure 1. *The map of the Kasur District showing location of the sampling site*

Figure 2. *Plants of lemongrass growing on different concentrations of tannery sludge amended soil i.e. 0, 5, 10, and 15% after 35 days of exposure. Scale bar = 20 cm*

Pre-sowing physico-chemical analysis of soil amended with different concentrations of sludge

The pH and electrical conductivity (EC) of soil and sludge were determined by using Multiparameter (Model HI 9835, HANNA), carbonates and biocarbonates, chlorides were determined in different concentrations of soil and sludge by titration methods (Saeed, 1980). The organic matter content was estimated by using Muffle Furnace (Ney Vulcan, D-550, Dentsply Ceramco, USA; Ball, 1964). The bulk density of sludge was estimated by the given formula (*Eq. 1*):

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$$Bulk\ density\ [\ (cm^3)^{-1}] = \frac{Mass\ of\ the\ dried\ sludge\ (g)}{Volume\ of\ the\ container} \quad \text{(Eq.1)}$$

Acid digestion of plants, sludge and soil samples for the estimation of heavy metals

The soil and sludge samples were digested by using HNO_3 and $HClO_4$ (1:4) and then 4 ml of HNO_3: H_2O (1:1) were added and heated on hot plate at 85 °C for 30 min to reduce the volume to half (Greenberg et al., 1998). The digested samples were then filtered and final volume was made up to 100 ml with distilled water. The solution was then filtered and used for the estimation of metals by using Atomic Absorption Spectrophotometer (GBC Savant AA, Australia).

Antioxidant analysis based on the scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical

Preparation 0.002% ascorbic acid solution for standard curve

Ascorbic acid (2 mg) was dissolved in 25 ml of distilled water with constant stirring for 5-10 min. Then the final volume of solution was raised up to 1000 ml by adding distilled water. The prepared solution was saved in a dark bottle in the refrigerator (Brand Williams et al., 1995).

Standard curve of ascorbic acid

For sample preparation, 3.9 ml of DPPH (2, 2-diphenyl-1-picrylhydrazyl) with 0.1 ml (100 µM) of solution prepared from every dilution (2-20 µg ml^{-1}) of ascorbic acid, was taken in different test tubes. Prepared sample of DPPH was taken as control as well as blank. Initial readings of all the samples were taken on Spectrophotometer (T80 + UV/VIS Spectrophotometer, PG, UK) at 517 nm, which was then kept in dark for 30 min to let the reaction take place. Then final reading was taken to determine the leftover DPPH. Scavenging activity of DPPH was expressed as optical density (OD).

Preparation of plant leaves extract

Fresh leaves samples (15 g) were taken from plants growing on different concentrations (0-15%) of Tannery sludge amended soil and air-dried in dark at the room temperature. Then the plant sample was grinded to fine powder for the sake of extract preparation. The powdered leaves were soaked in 100 ml of 100% methanol and kept on orbital shaker for 24 h. After that the sample was filtered with Whatman filter paper no. 1 and it was evaporated by rotary evaporator to obtain the final plant extract which was then diluted for further experiment.

DPPH assay

The capability of *Cymbopogon* to scavenging DPPH free radical was determined by using DPPH assay (Clark et al., 2013). DPPH {(0.0024 g (6×10^{-5} M)} was dissolved into 100 ml of 100% methanol for the preparation of stock solution of DPPH. Scavenging activity of % of extract was measured by using following formula (*Eq. 2*):

$$\%\ Radical\ Scavenged = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \quad \text{(Eq.2)}$$

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Statistical analysis

The least significance difference of calculated means for selected parameters for the data was statistically analyzed by using SPSS software 20.0.0. The differences amongst means were compared according to least significant difference (LSD) test at 5% probability level. Duncan's multiple range tests were also done to compare means.

Results

Pre-sowing of plant and post-harvest analysis of soil amended with different concentrations of tannery sludge

All physico-chemical properties of soil varied with the different concentrations of sludge application. For instance, carbonates were absent in all concentrations of sludge whereas, bicarbonates were 21, 45, 49, 56 mg L^{-1}, in 0, 5, 10 and 15% concentrations of tannery sludge, respectively. Moreover, 15% (TS + S) has the highest pH, EC, bicarbonates, chlorides, bulk density and organic matter % than rest of the concentrations of tannery sludge amended soil. The amount of heavy metals was enhanced with increasing concentration of tannery sludge. The order of heavy metals at various concentrations of tannery sludge were remained as Cr > Cd > Cu > Zn > Mg > Ni > Fe > Pb.

The TS application altered the physico-chemical characteristics of soil after 35 days of experiment. The pH values were reduced i.e., at 5% TS (8.11-8.01), 10% TS (8.20-8.12) and 15%TS (8.70-8.37), respectively. Similarly, chloride content was reduced from 28.45 to 18.5 mg L^{-1} and bicarbonates from 56 to 49 mg L^{-1} was calculated in 15% TS. Heavy metal content was also reduced after 35 days of harvesting. The percentage reduction was found to be maximum for Cr as compared to all other TS concentrations. Similarly, all other metals reduced gradually in all concentrations of tannery sludge amended soil. The percentage reduction for highest concentration of tannery sludge i.e., 15% TS was found to be for Cd (23%), Cr (35%), Cu (16%), Fe (36%), Mg (49%), Ni 32%), Pb (31%) and Zn (49%) as compared to the control respectively (*Table 1*).

Morphological attributes and chlorophyll contents

The tannery sludge substantially reduced the morphological characters and chlorophyll contents in lemongrass. After 35 days of plants growth, the fresh/dry weight was: 128.33, 34.67 g and chlorophyll contents were 52.76 in control plants while the no. of leaves, roots and tillers was 75, 93, and 36, respectively. The shoot length was also higher (74.33 cm) as compared to the various TS concentrations. All the above-mentioned parameters were reduced significantly in plants growing at 5, 10 and 15% concentration of tannery sludge in soil. The fresh/dry weight and chlorophyll content was 93.33 and 27.33 g 37.70 respectively at 15% concentration of sludge. Similarly, the no. of leaves/roots/tillers was 48, 75, and 31, respectively. Dose-dependent reduction in seedling length was also observed up to 62.3 cm at 15% concentration of sludge. Moreover, the chlorophyll contents were also decreased by 8.11, 25.92, and 39.95% at 5, 10 and 15% (TS + S) as compared with control. The growth variation of lemongrass before and after 35-days of experiment is presented in *Table 2*.

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Table 1. *Pre-sowing and post-harvest physico-chemical analysis of soil amended with different concentrations tannery sludge*

Parameters	Pre-sowing analysis	Post-harvest analysis	Pre-sowing analysis	Post-harvest analysis	Pre-sowing analysis	Post-harvest analysis	Pre-sowing analysis	Post-harvest analysis
	0% (Soil only)		5% (TS + S)		10% (TS + S)		15% (TS + S)	
pH	8.01±0.01^{c}	7.90±0.005^{cd}	8.11±0.1^{bc}	8.01±0.008^{c}	8.20±0.01^{bc}	8.12±0.002^{bc}	8.70±0.02^{a}	8.37±0.007^{b}
ECe (dS cm^{-1})	1.30±0.09^{cd}	1.10±0.001^{d}	1.50±0.08^{c}	1.22±0.006^{cd}	2.92±0.01^{b}	2.22±0.001^{bc}	3.74±0.01^{a}	2.89±0.001^{b}
Bicarbonates (mg L^{-1})	21.00±0.05^{cd}	14.00±0.04^{d}	45.00±0.08^{ab}	31.00±0.01^{c}	49.00±0.01^{ab}	37.00±0.08^{c}	56.00±0.04^{a}	49.00±0.12^{ab}
Carbonates (mg L^{-1})	ND	ND	ND	ND	ND	ND	ND	ND
Chlorides (mg L^{-1})	3.50±0.03^{d}	2.60±0.06^{e}	7.1±0.01^{c}	6.50±0.02^{cd}	17.75±0.01^{b}	13.80±0.07^{bc}	24.85±0.00^{a}	18.50±0.04^{b}
Bulk density [g(cm^{3})$^{-1}$]	1.21±0.01^{a}	0.77±0.10^{d}	1.15±0.06^{ab}	0.33±0.01^{de}	1.09±0.02^{bc}	0.10±0.05	1.02±0.01^{c}	0.03±0.08^{e}
Organic matter content (%)	5.60 + 0.10^{c}	3.22±0.11^{d}	12.00±0.06^{ab}	8.00±0.04^{b}	16.00±1.2^{ab}	11.56±0.06^{ab}	19.00±0.80^{a}	5.33±0.01^{c}
Cd (mg kg^{-1})	ND	ND	3199±1.34^{d}	2518±1.27^{e}	5557±1.29^{b}	4635±098^{c}	6588±166^{a}	5011±0.99^{bc}
Cr (mg kg^{-1})	ND	ND	4268±1.28^{d}	2420±1.21^{e}	6135±1.40^{b}	4701±1.66^{d}	8627±1.27^{a}	5582±1.43^{c}
Cu (mg kg^{-1})	ND	ND	2298±1.34^{d}	1892±1.58^{e}	3798±1.65^{c}	3168±1.55^{cd}	6340±1.32^{a}	5298±1.29^{b}
Fe (mg kg^{-1})	ND	ND	1223±1.30^{d}	996±1.67^{e}	1527±1.68^{b}	1161±1.20^{de}	1967±1.23^{a}	1345±1.16^{c}
Mg (mg kg^{-1})	ND	ND	1359±1.70^{c}	1019±1.19^{cd}	2243±1.2^{b}	1561±1.01^{c}	4002±1.19^{a}	2027±1.23^{b}
Ni (mg kg^{-1})	ND	ND	1700±1.10^{d}	1558±1.41^{de}	2563±1.58^{c}	2219±1.29^{cd}	3339±1.20^{a}	3046±1.55^{ab}
Pb (mg kg^{-1})	ND	ND	344±1.21^{c}	240±1.07^{d}	426±1.45^{bc}	204±1.67^{d}	821±1.21^{a}	561±1.50^{b}
Zn (mg kg^{-1})	ND	ND	2230±1.31^{c}	1053±1.29^{e}	3060±1.35^{b}	1801±1.70^{d}	4408±1.21^{a}	2231±1.44^{c}

Values are means ± SE of 9 replicates. Means values followed by a same letter in the rows are not significantly different at P < 0.05 by Duncan's multiple range test. ND = not detected; TS = tannery sludge

Table 2. *Morphological attributes and chlorophyll contents of lemongrass grown in different concentrations of tannery sludge (TS) amended soil*

Parameters	Treatments				
	0% (Soil only)	5% (TS + S)	10% (TS + S)	15% (TS + S)	LSD (P < 0.05)
Fresh weight (g)	128.33±6.18^{a}	121.63±5.3^{b}	107.67±7.4^{c}	93.33±9.28^{d}	12.02
Dry weight (g)	34.67±2.86^{a}	33.00±1.63^{b}	30.33±3.85^{c}	27.33±5.79^{d}	7.56
Number of roots	93.00±2.44^{a}	86.67±4.49^{b}	80.00±1.63^{c}	75.33±3.29^{d}	9.33
Number of leaves	75.00±3.29^{a}	73.00±8.17^{b}	60.00±6.23^{c}	48.00±2.49^{d}	11.20
Number of tillers	36.00±1.69^{a}	34.00±2.86^{b}	32.00±5.79^{c}	31.00±4.49^{bc}	5.6
Shoot length (cm)	74.33±3.29^{a}	72.67±5.79^{b}	66.67±1.69^{c}	62.30±2.49^{d}	8.56
Chlorophyll contents	52.76±1.47^{a}	48.80±1.97^{b}	41.90±1.22^{c}	37.70±3.12^{d}	13.28

Values are the means ± standard deviation of 9 replicates. Means values followed by a same letter in the rows are not significantly different at P < 0.05 by Duncan's multiple range test. LSD: least significant difference at P < 0.05

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Heavy metal accumulation in root and shoot

The concentration of heavy metals in root and shoot plants was increased in a dose dependent manner of TS and remained the highest at 15% TS + S as compared to other TS treatments. The overall trend of metal uptake at 15% TS was recorded in order of: Cr > Cd > Cu > Mg > Fe > Zn > Ni > Pb both in root and shoot. At 15% TS treatments, the contents of Cd, Cr, Cu, Fe, Ni, Mg, Pb, Zn were increased up to 87.68, 91.21, 92.54, 64.16, 61.19, 87.65, 57.43, and 43.69% in root and 79.58, 83.01, 64.23, 71.43, 74.06, 81.33, 41.73, and 68.05% in shoot as compared with control. Comparatively, the metal contents were found higher in roots than shoots (*Fig. 3*).

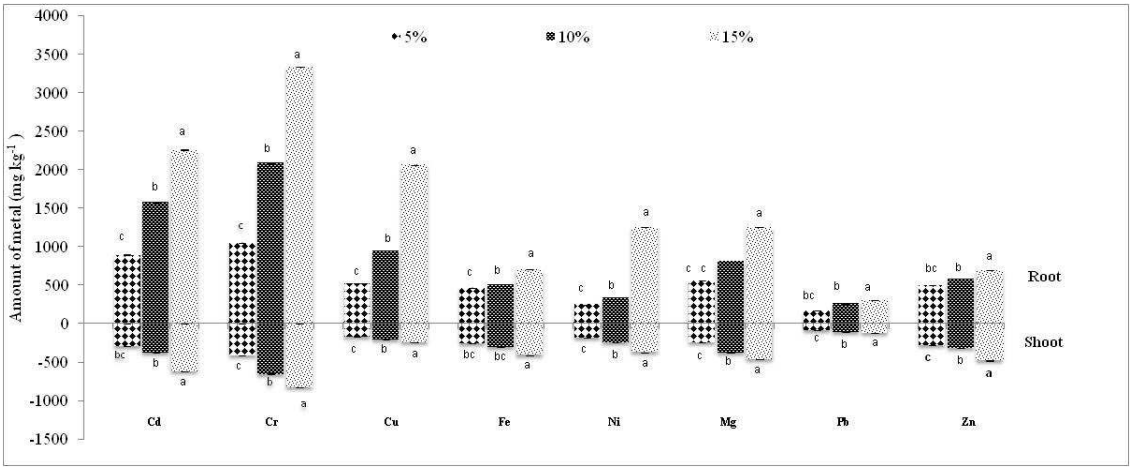

Figure 3. *Concentration of various heavy metals in root and shoot of 35 days old lemongrass plants under different levels of TS; Values are the means ± standard deviation (n = 9); Means with similar letters are not significantly different at P < 0.05 according to Duncans multiple range test*

Antioxidant activity analysis based on the scavenging of DPPH radical

The inhibition (%) and absorbance of 35 days-old-plants indicates that with increasing concentration of stock solution, absorbance of light goes on increasing from 0.076 to 0.12 in stock solutions of 2-20 µg ml^{-1} at 517 nm wavelengths of light for control plants. Antioxidant activity decreases with increasing concentration of stock solutions. Range of percentage inhibition was 79.3 to 56.6% in control and 83.85 to 59.9% in 2-20 µg ml^{-1} concentration of stock solution for 15% tannery sludge concentrations (*Figs. 4* and *5*).

Discussion

Heavy metal pollution has become a global environmental issue and posing serious threat for safe food production (Hu et al., 2017). In present study, the amount of heavy metals present in the TS was found in higher concentrations whereas these metals were found in the order of Cr > Cd > Cu > Zn > Mg > Ni > Fe > Pb. Different physico-chemical properties of soil with the different concentrations of tannery sludge application denoted that 15% TS + S has the highest values for the all pollution parameters than other concentrations of tannery sludge amended soil. The amount of heavy metals was enhanced with increasing concentration of tannery sludge application.

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The concentration of all heavy metals increased with increasing concentrations of tannery sludge in soil in the order of 0% < 5% < 10% < 15%. The pH is increased from 8.01-8.70 (*Table 1*). Previously, an increase in pH was reported to increase the adsorption of metals by competition between protons and metal cations and by the increase in the solubility of organic matter (Werkenthin et al., 2014). The increased in the pH was might be due to the use of alkalis and acid in tanning process (Kesarwani et al., 2015). The increased values of soil ECe are corroborates with Carmo et al. (2016) who reported that the ECe evaluates the concentration of soluble salts present in the soil. Higher ECe in the soil implies higher concentrations of heavy metals in the soil are present in soluble form. The highest amount of TDS was recorded in 100% concentrations of soil was confirmed by previous studies of Sabur et al. (2013). All other physico-chemical parameters increased by increasing the percentage of sludge in the soil.

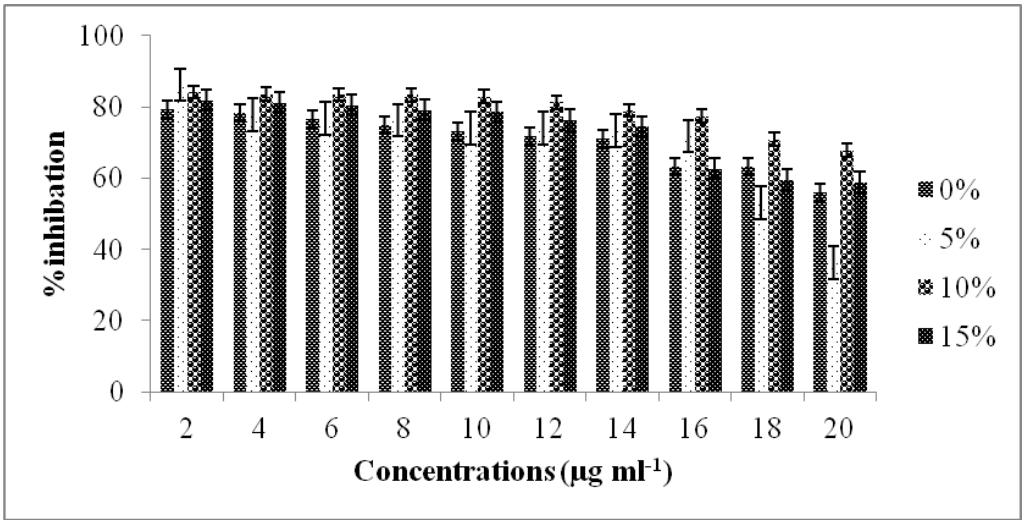

Figure 4. *Variation in percentage inhibition of various concentrations of 35 days old lemongrass plants extract growing in different concentrations of tannery sludge amended soil*

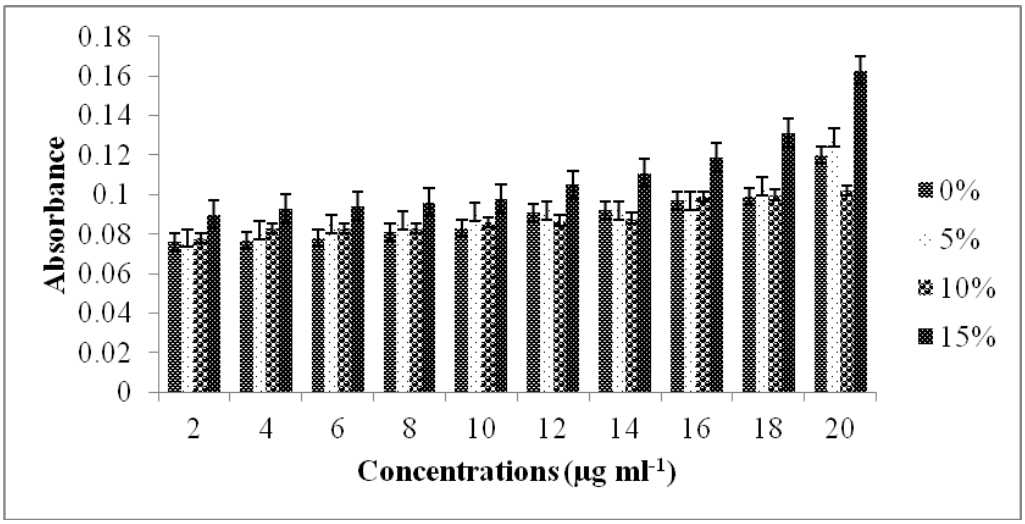

Figure 5. *Variations in absorbance of various concentrations of extract of 35 days old lemongrass plants growing in different concentrations of tannery sludge amended soil i.e., 0, 5, 10, and 15%*

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The morphological traits such as the fresh weight, dry weight, number of roots, number of leaves, number of tillers, and shoot length as well as the chlorophyll contents were reduced in TS treatments as compared with control (*Table 2; Fig. 2*). The reductions in morphological attributes and chlorophyll contents possibly due to increased amounts of heavy metals e.g., Cd, Cr, Pb, Cu and Zn present in sludge that might cause stress conditions to the growing lemongrass plants. Recently, Shah et al. (2017) reported that maximum growth of plants showed highly alkaline soil whereas Patel and Patra (2017) reported substantial reductions in dry weight as well as other morpho-physiological features of plants grown in metal contaminated soil. Though, a plant that was not such amendments was found with normal growth. Interestingly, the growth rate was higher during first week of experiment due to presence of some essential metals in TS amended soil. Excessive accumulation of heavy metals in plants disrupts various physiological processes such as stomatal conductance, photosynthesis, transpiration and enzymatic activities were badly affected (Farooqi et al., 2009). Significant reductions were observed in the morphological growth of maize as well as dry biomass accumulation when grown under marble effluent amended soil (Farid et al., 2020).

The amount of heavy metals was estimated in the roots and shoots of lemongrass growing in different concentration of TS after 35 days showed the following trend of metal uptake i.e., Cr > Cd > Cu > Ni > Fe > Zn > Mg > Pb. It was observed that metal uptake was higher in roots as compared to shoots whereas uptake of Cr by root and shoot was higher at 15% concentration as compared to all the other concentrations i.e., 0, 5 and 10%. The minimum amount of Pb was observed in 0 and 5% treatments in the roots and in shoots respectively (*Fig. 3*). Previously, Andaleeb et al. (2008) reported that the uptake rate of Cr was comparatively higher as compared to other metals present in the rhizosphere. Roots are the first vegetative part that accumulates its maximum metallic ions and thus highly affected by the concentration of heavy metals in the growth medium (Emamverdian et al., 2015). Amount of metals accumulated in roots were comparatively higher than shoots at all TS levels. Thamayanthi et al. (2013) also observed higher accumulation of metallic ions into the roots of *Tagetes erecta* followed by aerial parts respectively. Excessive accumulation of heavy metals in the different plant parts especially in the roots was due to the immobilization of metallic ions in the vacuole of plant root cells (Oliveira, 2012; Nematshahi et al., 2012). Accumulation of heavy metals ultimately reduces the dry matter production with limited development of stems and leaves during early growth of plants. Previous findings of Maksymiec (2007) confirmed that lemongrass can accumulate lower amounts of heavy metals whereas excessive amounts could be lethal for lemongrass plants. So, lemongrass could be grown successfully in low to mild concentration of metal contaminated soil in accordance to its phytoextraction capabilities.

The activities of various antioxidants i.e., SOD, POX, CAT etc. remained higher in plants grown under stress environment. Stress condition generally enhanced the anti-oxidant activities to neutralize the over-production of ROS (Li et al., 2017). In present study, the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was checked. An antioxidant activity was found to be increased (*Figs. 4* and *5*). by increasing concentrations of TS treatment that confirms the findings of Balakrishnan et al. (2014) who stated that antioxidant activity increases with increasing concentrations of sludge. Overall, lemongrass could be grown in metal contaminated soils as a phytoremediation approach.

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DOI: http://dx.doi.org/10.15666/aeer/1806_77037715

Conclusions

Tannery sludge (TS) application reduced the morphological growth as well as chlorophyll contents of lemongrass and such effects were more prominent at 15% TS treatment. Moreover, the metal accumulation concentrations varied with different TS treatments. The anti-oxidant activities were increased under TS treatments than control whereas the overall trend of metal uptake was recorded as: Cr > Cd > Cu > Ni > Mg > Fe > Zn > Ni > Pb both in root and shoot under all TS treatments. This study indicates that lemongrass has phytoremediation potential due to its accumulation of metals and induction of antioxidant enzyme activity and thus can be used for heavy metal extraction of Tannery sludge. Future investigations are needed to enhance the metal up take efficiency of lemongrass by using plant growth promoters to cleanse the metal contaminated soils.

Acknowledgments. Thanks are due to University of the Punjab for the financial assistance in the form of PURC research project to SAK.

Conflict of interests. It is declared that there is no conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1806_77037715

FIRST ASSESSMENT OF POLLUTION IMPACT AT ESSAOUIRA COAST (MOROCCO) USING BIOTIC AND ABIOTIC PARAMETERS AND THE RED ALGAE *ELLISOLANDIA ELONGATA* AS POTENTIAL BIOINDICATOR OF ORGANIC POLLUTION

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(Received 30th Jun 2020; accepted 17th Sep 2020)

Abstract. Environmental pollutants might significantly affect the ecological integrity of coastal waters. Biological indicators like seaweeds have been used globally to assess water pollution. In the present work, the seaweed *Ellisolandia elongata* was used to evaluate the pollution status in coastal waters around Essaouira city. Three sites were chosen: One as reference station (S1) and two polluted ones (S2 and S3). Seaweed biodiversity, physiologic parameters of *E. elongata,* as well as, abiotic parameters were studied. Results showed that at the polluted stations, seaweed biodiversity was significantly lower than in S1. However, the concentrations of Total Suspended Solids, Electrical Conductivity, Biological Oxygen Demand, Chemical Oxygen Demand, Ammonium, and Orthophosphates at S2 were significantly higher than at S1. Metal content of *E. elongata* remained below detection limit at all stations except for Zn and Cu at S2 considered the most polluted station. With respect to the physiologic parameters, Proline, Glycine Betaine and Polyphenol contents at S2 were above, whereas, Chlorophyll a content and axis length were below those determined at the reference station. From the results it can be concluded that seaweed *E. elongata* could be a good indicator to determine organic pollution in marine ecosystems.
Keywords: *seaweed, organic pollution, physicochemical parameters, Ellisolandia elongata physiology, biomonitoring*

Introduction

Boarding to the Atlantic Ocean and the Mediterranean Sea, the Moroccan coastlines are exposed to various types of anthropogenic stress. This environment is constantly exposed to human influence through domestic and industrial waste waters, which often introduce high quantities of pollutants without pre-treatment. In addition, the habitats undergo physical destruction which produces a significant impact on the coastal

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

environment and lead to significant ecological deterioration (Islam and Tanaka, 2004; Moore et al., 2004; Gu et al., 2007; Amin et al., 2009; Er-Raioui et al., 2012; Hong et al., 2020), causing serious changes in marine organisms, including macrofauna, seagrasses, algae and others (Sivadas et al., 2010; Sabri et al., 2017; Boundir et al., 2019). In fact, algae are the basis of many marine food webs. Their composition fluctuates depending on several conditions, such as temperature, light, salinity, pH, and nutrients (Huertas et al., 2011). According to literature, it is known that the amount of macronutrient of coastal areas are the main factors which control the structural composition of algae communities (McGlathery et al., 2007; Leterme et al., 2014; Nassar et al., 2015). Celis-Plá et al. (2014) demonstrated that Nitrate, ammonium and orthophosphate are essential macronutrients for algae growth. However, these nutrients can have a serious negative impact on algal communities if they reach high concentrations near urban areas (Guerra-García and Koonjul, 2005; Nassar et al., 2015). In addition, urbanization and industrialization which increases due to population expansion is often coupled to a release of heavy metals into coastal waters which are considered among the most serious contaminants of aquatic ecosystems due to their high potential to enter and accumulate in food chains (Erdoğrul and Erbilir, 2007).

However, only limited published information on organic and inorganic pollutants is available for the Moroccan coast, and in particular for its Atlantic section (Kaimoussi et al., 2002; Mouradi et al., 2014; Rezzoum et al., 2016). At certain concentrations, these compounds could have an impact on seaweeds, causing disappearance of some species or declining or shifting biodiversity (DÍez et al., 1999; Nassar et al., 2015; El-adl et al., 2017; Sabri et al., 2017). Many authors used seaweeds to evaluate the degree of pollution, as they are considered to be excellent indicators of environmental change (Harley et al., 2006; De Faveri et al., 2010; Benkdad et al., 2011; Reis et al., 2014; Shams El-Din et al., 2014; García-Seoane et al., 2018). When seaweeds are exposed to stress (e.g. heavy metals or pathogens) they may respond in characteristic ways, such as decrease of axis length or chlorophyll content. Also overproduction of specific metabolic products, such as proline, glycine betaine (GB) or total phenolic compounds (TPC) may compensate the cellular imbalances caused by environmental stress (Häder et al., 1997; Coelho et al., 2000; Di Martino et al., 2003; Koivikko et al., 2005; Liang et al., 2013). Thus, these metabolites allow certain seaweeds to acclimatize to some degree and to ensure survival (Fatma et al., 2007; Lamalakshmi et al., 2017). Previous studies already reported that algal size and the content of molecules like chlorophyll, TPC, GB and proline may be used as bioindicators to monitor air pollution (Anbazhagan et al., 1988; Agbaire, 2016; Khairallah et al., 2018; Mukherjee et al., 2019), salt stress (Ali et al., 1999; Carillo et al., 2008; Amirjani, 2010; Fariduddin et al., 2013), drought stress (Si et al., 2015; Mirshad and Puthur, 2016; Mao et al., 2019) and freezing stress (Nomura et al., 1995), as well as inorganic (Alia and Saradhi, 1991; Amna et al., 2015; Varun et al., 2015; Saif and Khan, 2018; Boundir et al., 2019) and organic pollution (Abdel and Abdel, 2015; Bibi et al., 2019).

A precondition to use seaweeds as a bioindicator is that they are present in the areas under study. This is the case for the Corallinaceae which exist in almost every habitat type, even in polluted areas, within the photic (Adey and Macintyre, 1973; Steneck, 1986; DÍez et al., 1999). Likewise, *Ellisolandia elongata* which occurs along the Atlantic intertidal zone of Essaouira and its surroundings was chosen in the present study to investigate its applicability as a bioindicator. For doing so, the species and numbers of macroalgae present were determined at two polluted sites and compared to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

those at an unpolluted site during different seasons in 2017/2018. In addition, variations in the content of selected metabolic substances and heavy metals of the tissue of *Ellisolandia elongata* samples were determined and compared together with the seasonal differences of the physicochemical parameters of the waters at the study sites.

Materials and methods

Study area

The study area is located along the Atlantic Coast west of Central Morocco in the Essaouira Province, which is administered under the Marrakech-Safi Region government (Morocco). It extends longitudinally among 9°67' and 9°77' West and latitudinally among 31°51' and 31°63' North (*Fig. 1*).

The study area has a diverse and exceptional climate, shaped by its geographical location between the Sahara Desert and the Atlantic Ocean. The aridity increases from west to east. The western narrow coastal fringe around Essaouira is reached by cold currents coming from the Canary Islands, which generate a microclimate with a relatively homogeneous average temperature of about 16.7 °C throughout the year. As a consequence, the difference between average temperatures in the hottest and the coldest month is relatively small (Mwambo, 2007; Bazairi et al., 2010). Agriculture, fishing, craft, mining, trade, tourism, energy production and some other industrial and recreational activities are the most dominant economic sectors in Essaouira city.

Three stations, located on rocky substrates along the Essaouira coast, were chosen for this study (*Fig. 1*).

- Moulay Bouzerktoune Station (S1), located approximately 30 km away from the city (31°63'N-9°67'W), is considered as a reference station, affected by anthropogenic activities only during ephemeral tourism activities at the end of spring and during summer.
- Bab Doukkala Station (S2), the industrial district of Essaouira, located in the city, north of the port (31°51'N-9°76'W), allows us to assess the impact of the discharges of Essaouira city on marine macroalgae. Indeed, this urban coast receives significant domestic and some industrial wastewater, as well as limited solid discharges that are mainly composed of household waste.
- Port Station (S3), also located within the city (31°51'N-9°77'W), receives some industrial discharges from the port, as well as domestic discharges from the city districts.

Sampling

In order to study the phytobenthos biodiversity, seaweed samples were collected seasonally (once per season) during 2017 to 2018 in autumn, winter, spring and summer at low tide (0 to 1.5 m) along the Essaouira coast at the three stations (S1, S2, S3), following the quadrat method (Ar Gall and Connan, 2004). 30 quadrats were provided for each bathymetric level because of the irregularity of algal distribution in the foreshore. Samples were transferred to the laboratory and stored at 4 °C in a cooler. The species of the seaweeds were identified *in situ* and at the laboratory using a magnifying glass and a binocular microscope.

A large quantity of the Corallinaceae *Ellisolandia elongata* (J. Ellis and Solander) K. R. Hind and G.W. Saunders with basionym *Corallina elongata* (Guiry and Guiry, 2020)

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was collected in order to study its physiology and metal uptake. The algal material sampled was carefully washed with seawater, in order to omit epiphytes, sediments and associated fauna, and was cleaned thereafter with distilled water to remove excess salt. The algal material thus prepared was lyophilized, ground to powder in a dust-free atmosphere and using metal-free equipment to avoid contamination and finally stored at -20 °C for toxicological and physiological analysis.

In addition, water was sampled in glass bottles (500 ml) and transported to the lab in a cooler on ice (4 °C) to reduce adsorption, precipitation, contamination or evaporation (Rodier, 2009).

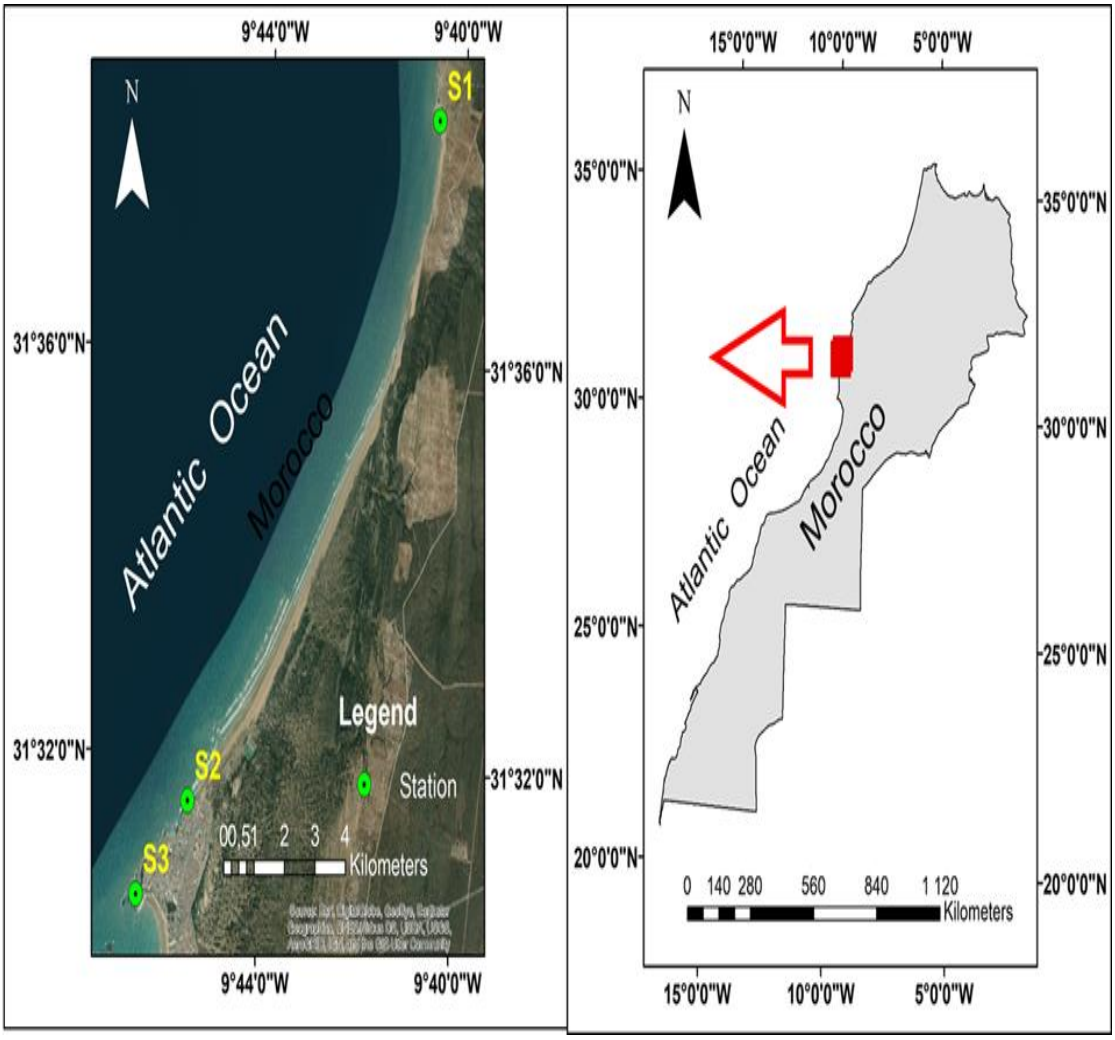

Figure 1. *Geographical position of the study sites along the coast of Essaouira. (S1) My Bouzerktoune: 31°63'N-9°67'W – (S2) Bab Doukkala: 31°51'N-9°76'W – (S3) The port: 31°51'N-9°77'W*

Physicochemical parameters

The water temperature, electrical conductivity (EC) and pH were determined on site with a multimeter instrument (Orion 4 Star), while dissolved oxygen (DO) was measured by a digital pocket oxymeter (HANNA-HI9829). All parameters were always measured *in situ* during collection of the samples at all sites and in all seasons.

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Concentrations of Total Suspended Solids (TSS) in water were determined by filtration of 200 ml of water sample through glass filters (0.45 µm mesh), which was subsequently dried and weighed. Biological Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), and concentrations of Nitrogenous and Phosphorus compounds were measured according to AFNOR norms (Association Française de NORmalisation) reported in *Table 1*. The oxidizable matter concentrations were calculated using *Equation 1:*

$$\text{Oxidizable matter} = \frac{2 \times BOD_5 + COD}{3} \quad \text{(Eq.1)}$$

Table 1. *AFNOR norms of the studied parameters*

Parameters	AFNOR norms
BOD_5	NF T90-103
COD	NF T90-101
Total nitrogen	NF T90-061
Nitrate	NF T90-012
Ammonium	NF T90-015-2
Total phosphorus	NF T90-023
Orthophosphate	NF T90-022

Physiological parameters

Chlorophyll a

Chlorophyll a (Chla), was determined according to the method of Jeffrey and Humphrey (1975), based upon extraction of 10 g of fresh algae in 10 ml of acetone 90%. The tubes were incubated in the dark at 4 °C for 24 h and subsequently centrifuged at 19,000 g for 30 min. Absorbance of the resulting supernatant was measured at 662 and 644 nm, using a spectrophotometer (Boeco Germany S20).

Proline

The algal proline content was quantified following the method of Monneveux and Nemmar (1986). 100 mg of dried tissue was homogenized in 2 ml of methanol (40%) and then placed in a water bath at 85°C for 1 h. After cooling the samples were centrifuged at 4000 g for 10 min at 4 °C. 1 ml of supernatant was added to a mixture composed of 1 ml of acetic acid, 25 mg of ninhydrin and 1 ml of a solution composed of distilled water, glacial acetic acid and ortho-phosphoric acid of density 1.7 (120, 300, 80: v/v/v). The mixture was heated again for 30 min at 100 °C in a water bath and subsequently allowed to cool at room temperature, and then added to 5 ml of toluene. The upper phase was collected and dehydrated with a pinch of anhydrous Na_2SO_4. The absorbance was read at 520 nm.

Glycine betaine (GB)

The GB essay was performed according to the protocol of Grieve and Grattan (1983). 0.5 g DW of seaweed was mechanically shaken with 20 ml of deionized water at 25 °C for 48 h. After filtration, 0.5 ml of extract was mixed with 0.5 ml of 2N sulfuric

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acid. Then 0.2 ml of KI-I_2 reagent (containing 15.7 g iodine and 20 g KI in 100 ml) was added and shaken in ice cold water for 1 h. The tubes were stored at 0-4 °C for 16 h and then centrifuged at 10,000 rpm for 15 min at 0 °C. The supernatant was carefully drawn off and the pellet was dissolved in 9 ml of 1,2-dichloroethane (chilled at -10 °C). The absorbance was measured after 2 h at 365 nm.

Total phenolic compounds (TPC)

TPC were measured according to Silvia Taga et al. (1984). A quantity of 0.5 g of dried algae was extracted in 60:40 acidified methanol/water (0.3% HC1). Supernatant at 100 µl was mixed with 2 ml of Na_2CO_3 (2%). After 2 min 100 µl of Folin-Ciocalteu's phenol reagent (50%) were added and the mix was allowed to stand at room temperature for 30 min. The absorbance was then read at 750 nm within 2 h. The results were expressed as gallic acid equivalents (GAE), based upon measurement of gallic acid standards ranging in concentration from 10 mg mL^{-1} to 200 mg mL^{-1}.

Morphometry

The main axis length of seaweeds was determined using a non-destructive method by Murray et al. (2002), which does not require detaching the seaweed. The lengths of individual axes were measured *in situ* with a ruler.

Heavy metals

The method of Topcuoğlu et al. (2003) was adopted to quantify trace metals after digestion with hydrogen peroxide, sulfuric and nitric acids. If necessary, samples thus obtained were diluted depending on their metal concentrations to allow for analysis with an Atomic Absorption Spectrophotometer (AA-6300 SHIMADZU).

Statistical analysis

Morphometric measurements were conducted on 30 algal samples, while *in situ* measurements and laboratory analysis were established in triplicate and mean values and standard deviations were calculated. For statistical data analysis, Kruskal-Wallis and Wilcoxon tests were applied to analyze differences among the three sampling stations. Linear correlations between parameters were analyzed with the Pearson coefficient. The regression coefficient was calculated between concentrations of different compounds in wastewater, and Principal Component Analysis was used as a classification tool. The SPSS Statistics version 23 package (IBM, USA) was used for data analysis. All statistical hypothesis testing was based upon use of a probability value p of 0.05.

Results

Algal diversity

A total of 309 taxa was recorded at the three sampling stations located along the coastline of Essaouira city. The maximum of specific richness was found at S1 with 221 species, followed by S3 with 140 species and S2 with only 111 species (*Fig. 2*). Generally, most species belonged to the Rhodophyceae (68%), followed by the Chlorophyceae (17%) and the Phaephyceae (16%). The green algal species at S2 and

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S3 contributed 29% and 21%, respectively, while this percentage was lower at S1 (13%). On the other hand, the red and brown algae contributed 73% and 14%, respectively, at reference station S1 and where thus more dominant at this station than at the polluted stations S2 and S3. The dominant species at S1 were *Hypnea musciformis*, *Gelidium corneum*, *Carpodesmia tamariscifolia* and *Bifurcaria bifurcata*. The brown seaweeds were associated with epiphytic species (e.g. *Aglaothamnion tripinnatum*, *Polysiphonia atlantica, P. elongata, Lomentaria articulata, Plocamium raphelisianum, P. cartilagineum*). The S2 and S3 stations exhibited mostly common species, which were generally dominated by the Chlorophyceae (*Ulva* and *Codium* genera), by some epiphytic species (e.g. *Derbesia lamourouxii, D. tenuissima, cladophora polifera, Chaetomorpha aerea*) and by the red alga *Caulacanthus ustulatus*.

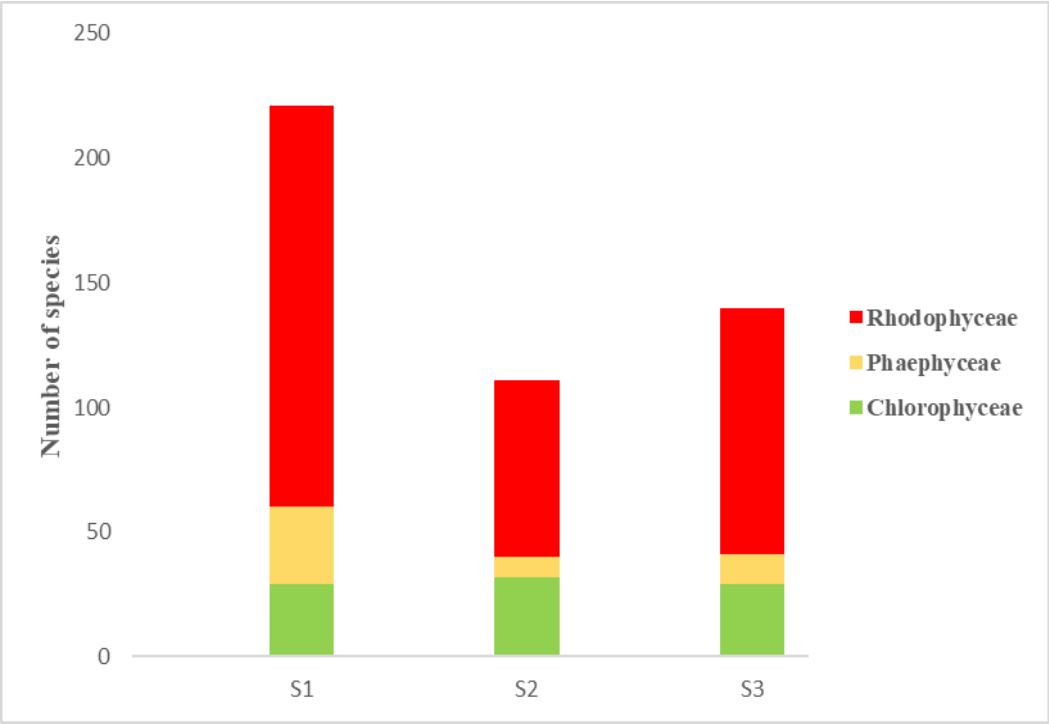

Figure 2. *Algal diversity in the studied areas*

Physicochemical parameters

The results of the physicochemical parameters of seawater measured *in situ* during the study period are presented in *Table 2*.

Temperature values vary between 15.5 °C during winter and 23.46 °C during summer all recorded at S3. For this parameter, statistical analyses did not show clearly a significant difference between the study sites, while the other parameters showed a significant difference between the control station and the polluted ones, especially between S1 and S2 ($p < 0.05$). pH and DO contents ranged from 7.2 to 8.7 and from 5.6 to 8 mg L^{-1}, respectively, and were significantly higher at S1 in comparison to the other stations (S2 and S3). In contrast, the highest values of EC were recorded at the two polluted stations. Their maxima reached 45500 µs cm^{-1} at S3 and 47000 µs cm^{-1} at S2, but only 39000 µs cm^{-1} at S1.

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Table 2. *Seasonal variation of temperature (°C), pH, electrical conductivity (µs cm^{-1}), and dissolved oxygen (mg L^{-1}) in the seawater at the three studied areas*

Season	Station	T	pH	EC	DO
Autumn	S1	18.6 ± 0.00^{AB}	7.9 ± 0.00^{A}	32500 ± 0.00^{A}	7.64 ± 0.00^{A}
	S2	18.8 ± 0.00^{A}	7.2 ± 0.00^{B}	45000 ± 0.00^{B}	6.50 ± 0.00^{B}
	S3	18.2 ± 0.00^{B}	7.6 ± 0.00^{AB}	40100 ± 0.00^{AB}	6.85 ± 0.00^{AB}
Winter	S1	16.0 ± 0.00^{AB}	8.7 ± 0.00^{A}	32000 ± 0.00^{A}	7.50 ± 0.00^{A}
	S2	17.2 ± 0.00^{A}	7.5 ± 0.00^{B}	43100 ± 0.00^{B}	6.20 ± 0.00^{B}
	S3	15.5 ± 0.00^{B}	8.4 ± 0.00^{AB}	34000 ± 0.00^{AB}	7.21 ± 0.00^{AB}
Spring	S1	17.9 ± 0.00^{A}	8.6 ± 0.00^{A}	39000 ± 0.00^{A}	7.85 ± 0.00^{A}
	S2	18.8 ± 0.00^{B}	8.1 ± 0.00^{B}	44000 ± 0.00^{B}	5.60 ± 0.00^{B}
	S3	18.2 ± 0.00^{AB}	8.5 ± 0.00^{AB}	41000 ± 0.00^{AB}	7.10 ± 0.00^{AB}
Summer	S1	20.0 ± 0.00^{A}	8.5 ± 0.00^{A}	32300 ± 0.00^{A}	8.00 ± 0.00^{A}
	S2	22.6 ± 0.00^{AB}	7.9 ± 0.00^{B}	47000 ± 0.00^{B}	6.80 ± 0.00^{B}
	S3	23.46 ± 0.00^{B}	8 ± 0.00^{AB}	45500 ± 0.00^{AB}	7.5 ± 0.00^{AB}

Different upper-case letters in the same column indicate differences between the studied stations for the four seasons ($p < 0.05$)

The spatial variation of TSS, BOD_5, COD, and OM in sea water are given in *Table 3*. The statistical analysis of these data revealed a significant difference between the control and the polluted stations, especially between S1 and S2 ($p < 0.05$).

Table 3. *Seasonal variation of total suspended solids, biological oxygen demand, chemical oxygen demand, and oxidizable matter (mg L^{-1}) in the seawater at the three studied areas*

Season	Station	TSS	BOD	COD	OM
Autumn	S1	42.00 ± 1.00^{A}	05.66 ± 0.57^{A}	13.00 ± 1.73^{A}	08.11 ± 0.83^{A}
	S2	55.66 ± 1.15^{B}	45.00 ± 5.00^{B}	83.00 ± 4.35^{B}	57.67 ± 4.58^{B}
	S3	53.33 ± 0.57^{AB}	32.66 ± 2.51^{AB}	59.00 ± 1.00^{AB}	41.44 ± 2.01^{AB}
Winter	S1	31.57 ± 0.51^{A}	03.66 ± 1.15^{A}	8.33 ± 2.08^{A}	05.22 ± 0.19^{A}
	S2	127.33 ± 0.57^{B}	31.33 ± 1.53^{B}	75.67 ± 3.51^{B}	46.11 ± 1.95^{B}
	S3	71.16 ± 0.29^{AB}	25.33 ± 2.52^{AB}	41.00 ± 1.73^{AB}	30.56 ± 1.71^{AB}
Spring	S1	26.41 ± 0.37^{A}	3.66 ± 0.58^{A}	34.00 ± 1.00^{A}	13.78 ± 0.69^{A}
	S2	86.26 ± 0.12^{B}	37.00 ± 1.73^{B}	70.67 ± 1.53^{B}	48.22 ± 0.84^{B}
	S3	66.66 ± 0.01^{AB}	30.33 ± 0.75^{AB}	36.33 ± 2.08^{AB}	32.33 ± 1.00^{AB}
Summer	S1	52.21 ± 0.03^{A}	6.33 ± 0.58^{A}	16.33 ± 1.53^{A}	09.67 ± 0.88^{A}
	S2	40.00 ± 0.01^{AB}	56.67 ± 1.53^{B}	94.67 ± 4.16^{B}	69.33 ± 2.31^{B}
	S3	33.77 ± 0.22^{B}	41.33 ± 1.53^{AB}	69.00 ± 2.00^{AB}	50.56 ± 1.68^{AB}

Different upper-case letters in the same column indicate differences between the studied stations for the four seasons ($p < 0.05$)

The TSS values varied between 26.41 and 127.33 mg L^{-1}. Particularly high levels were recorded at S2, especially during winter and spring, reaching 127.33 and 86.26 mg L^{-1}, respectively. Medium concentrations were recorded at S3 that varied between 33.77 and 71.16 mg L^{-1}, while the lowest concentrations were observed at S1

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(*Table 3)*. The highest BOD values were recorded at S2 (56.67 mg L^{-1}) and S3 (41.33 mg L^{-1}) both recorded during summer, while considerably lower values were recorded at S1 especially during winter (3.66 mg L^{-1}). Likewise, maximum COD values were recorded during summer at S2 (94.67 mg L^{-1}), and the lowest values were found at S1 (*Table 3*). The same was true for OM which exhibited a minimum at S1 of 5.22 mg L^{-1} and a maximum at S2 (69.33 mg L^{-1}) recorder during winter and summer, respectively (*Table 3*).

Concentrations of nutrients (TN, NH_4-N, NO_3-N, TP and PO_4-P) exhibited significant differences, Also, between control station S1 and the other polluted station S2 ($p < 0.05$) (*Table 4*).

Total nitrogen concentrations (TN) ranged from 6 mg L^{-1} at S1 to 12.97 mg L^{-1} at S2. The minimum value of ammonium was also recorded at S1 (0.12 mg L^{-1}) and the maximum at S2 (0.53 mg L^{-1}). However, elevated nitrate concentrations were found at S1 (Maximum: 4.41 mg L^{-1}) in comparison to the other stations (*Table 4*).

For phosphorus compounds, the lowest concentrations of PO_4-P were found at S1 in season autumn (0.02 mg L^{-1}), whereas the highest concentration was measured at S2 in spring (0.21 mg L^{-1}) (*Table 4*).

Table 4. *Seasonal variation of TN, NH_4-N, NO_3-N, TP and PO_4-P (mg L^{-1}) in the seawater at the three studied areas*

Season	Station	TN	NH_4-N	NO_3-N	TP	PO_4-P
Autumn	S1	6.25 ± 0.09^{A}	0.32 ± 0.01^{A}	3.53 ± 0.01^{A}	0.39 ± 0.01^{A}	0.02 ± 0.01^{A}
	S2	9.27 ± 0.04^{B}	0.53 ± 0.01^{B}	2.20 ± 0.01^{B}	0.52 ± 0.01^{B}	0.06 ± 0.01^{B}
	S3	8.06 ± 0.08^{AB}	0.50 ± 0^{AB}	2.69 ± 0.01^{AB}	0.42 ± 0.01^{AB}	0.03 ± 0.01^{AB}
Winter	S1	6.00 ± 0.04^{A}	0.06 ± 0^{A}	4.41 ± 0.02^{A}	0.38 ± 0.01^{A}	0.06 ± 0.01^{A}
	S2	12.97 ± 0.03^{B}	0.23 ± 0^{B}	2.85 ± 0.02^{B}	0.60 ± 0.01^{B}	0.12 ± 0.01^{B}
	S3	7.91 ± 0.05^{AB}	0.18 ± 0^{AB}	3.52 ± 0.01^{AB}	0.50 ± 0.01^{AB}	0.07 ± 0.01^{AB}
Spring	S1	8.77 ± 0.03^{A}	0.14 ± 0^{A}	2.09 ± 0.02^{A}	1.92 ± 0.07^{A}	0.13 ± 0.01^{A}
	S2	10.87 ± 0.13^{B}	0.23 ± 0^{B}	1.33 ± 0.02^{B}	7.20 ± 0.05^{B}	0.21 ± 0.01^{B}
	S3	8.96 ± 0.07^{AB}	0.17 ± 0^{AB}	1.40 ± 0.01^{AB}	2.90 ± 0.05^{AB}	0.14 ± 0.01^{AB}
Summer	S1	9.64 ± 0.10^{A}	0.12 ± 0^{A}	1.79 ± 0.01^{A}	5.20 ± 0.03^{A}	0.04 ± 0.01^{A}
	S2	11.98 ± 0.24^{B}	0.22 ± 0^{B}	1.69 ± 0.01^{B}	7.38 ± 0.02^{B}	0.06 ± 0.01^{B}
	S3	10.3 ± 0.14^{AB}	0.18 ± 0.01^{AB}	1.70 ± 0.01^{AB}	5.73 ± 0.05^{AB}	0.05 ± 0.01^{AB}

Different upper-case letters in the same column indicate differences between the studied stations for the four seasons ($p < 0.05$)

Heavy metals

Pb, Cr, Ni and Cd contents of *E. elongata* were always below the detection limit at the different sites during the four seasons. In contrast, Cu and Zn were detected and their concentrations differed significantly between the studied stations throughout the study period ($p < 0.05$) (*Table 5*). The highest values were always recorded at S2, with up to 2.64 µg g^{-1} DW and 0.99 µg g^{-1} DW of Zn and Cu, respectively.

Physiological parameters

Table 6 shows the physiological parameters of the seaweed *E. elongata*. The statistical analysis detected significant differences for all the parameters between the

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DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

control station S1 and the station S2 ($p < 0.05$), except for TPC and axis length during winter.

Table 5. *Seasonal variation of zinc and copper (µg g^{-1} dry weight) in E. elongata at the three studied areas*

Season	Station	Zn	Cu
Autumn	S1	1.13 ± 0.003^{A}	0.04 ± 0.002^{A}
	S2	2.02 ± 0.00^{B}	0.12 ± 0.001^{B}
	S3	1.41 ± 0.001^{AB}	0.07 ± 0.00^{AB}
Winter	S1	1.31 ± 0.002^{A}	0.07 ± 0.002^{A}
	S2	2.64 ± 0.003^{B}	0.14 ± 0.003^{B}
	S3	1.50 ± 0^{AB}	0.13 ± 0.002^{AB}
Spring	S1	1.94 ± 0.002^{AB}	0.09 ± 0.001^{A}
	S2	2.03 ± 0.001^{A}	0.17 ± 0.001^{B}
	S3	1.13 ± 0.001^{B}	0.17 ± 0.002^{AB}
Summer	S1	0.96 ± 0.001^{A}	0.09 ± 0.00^{A}
	S2	1.79 ± 0.170^{B}	0.99 ± 0.001^{B}
	S3	1.31 ± 0.001^{AB}	0.13 ± 0.003^{AB}

Different upper-case letters in the same column indicate differences between the studied stations for the four seasons ($p < 0.05$)

Samples from station S1 exhibited both the highest content of chlorophyll a (Maximum: 433.68 µg g^{-1} FW during winter) and the longest thalli (maximum: 5.77 cm during summer), respectively. Lower values were found at S2, with 86.67 µg g^{-1} FW during spring and 1.53 cm during summer, respectively. In contrast, the highest values of proline and GB were recorded at S2 (54.72 mg g^{-1} DW and 869.39 µg g^{-1} DW, respectively), while their minimum was always recorded at S1 (*Table 6*). With the exception of spring TPC also always reached its minimum at S1 (absolute minimum: 477.17 µg g^{-1} DW). Seasonal maxima were always observed at S2 or S3 (absolute maxima: 2116.58 and 2794.83 µg g^{-1} DW, respectively).

Table 6. *Seasonal variation of Chla (µg g-1 fresh weight), proline (mg g-1 dry weight), GB and TPC (µg g-1 dry weight) and axis length (cm) of E. elongata at the three studied areas*

Season	Station	Chla	GB	Proline	TPC	Axis length
Autumn	S1	315.85 ± 0.44^{A}	291.16 ± 10.27^{A}	21.35 ± 0.47^{A}	477.17 ± 15.62^{A}	5.60 ± 0.26^{A}
	S2	182.83 ± 0.25^{B}	704.76 ± 06.23^{B}	54.72 ± 0.99^{B}	657.81 ± 21.29^{A}	3.60 ± 0.10^{B}
	S3	216.53 ± 0.42^{AB}	518.37 ± 08.16^{AB}	32.10 ± 0.72^{AB}	671.44 ± 15.62^{A}	4.57 ± 0.35^{AB}
Winter	S1	433.68 ± 0.80^{A}	303.40 ± 13.12^{A}	06.67 ± 0.13^{A}	1960.53 ± 35.91^{A}	5.47 ± 0.50^{A}
	S2	180.94 ± 0.19^{B}	855.78 ± 08.50^{B}	17.07 ± 0.26^{B}	2116.58 ± 46.86^{AB}	4.43 ± 1.04^{A}
	S3	232.92 ± 0.41^{AB}	629.93 ± 06.23^{AB}	10.09 ± 0.27^{AB}	2794.83 ± 56.31^{B}	4.03 ± 0.15^{A}
Spring	S1	254.04 ± 0.36^{A}	521.09 ± 06.23^{A}	14.08 ± 1.61^{A}	961.15 ± 27.05^{AB}	5.00 ± 0.50^{A}
	S2	86.67 ± 0.56^{B}	869.39 ± 08.16^{B}	39.77 ± 1.61^{B}	1428.09 ± 50.44^{A}	2.57 ± 0.12^{B}
	S3	189.20 ± 0.31^{AB}	606.80 ± 08.50^{AB}	23.94 ± 0.61^{AB}	576.01 ± 56.31^{B}	3.50 ± 0.50^{AB}
Summer	S1	328.75 ± 1.26^{A}	533.33 ± 10.27^{A}	12.79 ± 1.38^{A}	562.38 ± 30.68^{A}	5.77 ± 0.38^{A}
	S2	121.37 ± 0.40^{B}	858.50 ± 04.71^{B}	39.18 ± 1.38^{B}	749.83 ± 51.46^{AB}	1.53 ± 0.38^{B}
	S3	157.74 ± 0.56^{AB}	801.36 ± 04.71^{AB}	21.99 ± 0.69^{AB}	940.70 ± 30.68^{B}	2.60 ± 0.26^{AB}

Different upper-case letters in the same column indicate differences between the studied stations for the four seasons at the level ($p < 0.05$)

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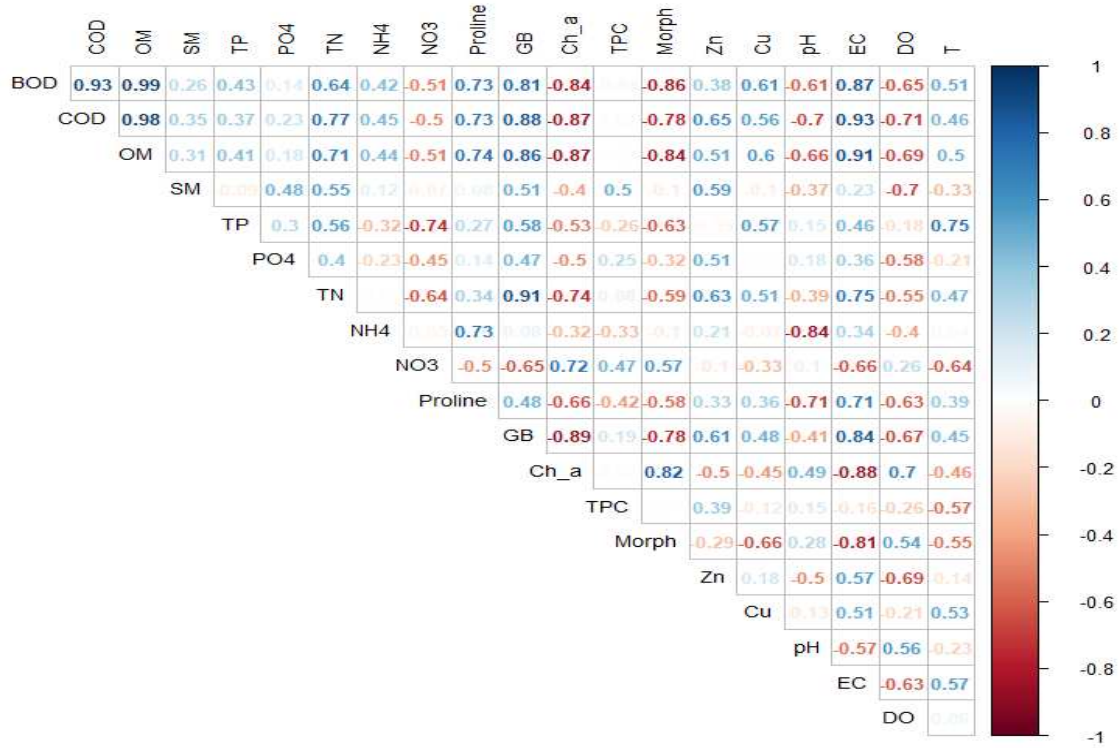
Statistical analysis

As shown on the Pearson correlation matrix (*Table 7*), parameters that reflect organic pollution (BOD, COD and OM) exhibit strong positive correlations with TN, GB and proline ($r > 0.7$), but negative correlations with Chla and morphometry ($r > -0.7$). However, the organic parameters show mainly weak relationships with the remaining parameters (e.g., TPC, PO_4).

Table 7. *Pearson correlation matrix between the parameters studied*



Principal Component Analysis (PCA) of the physiological parameters (Morphometry, Chlorophyll a, Proline, and Glycine betaine (GB) combined with the physico-chemical parameters (T, pH, EC, TSS, DO, BOD, COD, and OM) and heavy metal concentrations (Cu and Zn) allowed the identification of two principal components that together explain more than 75.72% of the overall variability of samples (*Table 8)*. Our PCA analysis allows to identify the most representative variables that distinguish the studied stations (*Fig. 3*). It demonstrates that BOD, COD, OM, Proline, GB, and EC are correlated positively with the PC1 (0.941; 0.975; 0.974; 0.775; 0.892; 0.934, respectively). In contrast, the algal axis length, Chla and DO are negatively correlated with the PC1 (0.834; 0.891; 0.779, respectively). TSS and Cu together correlate with PC2, while T and Zn together correlate along PC1. However, both heavy metals did not correlate particularly well with the algal physiological parameters (*Table 8*).

Discussion

Our study aimed for the first time to use physiological parameters of *Ellisolandia elongata* in combination with macroalgal biodiversity and physico-chemical

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

parameters for the assessment of coastal pollution. Three stations were investigated for this purpose. S1 is the reference site, while S2 and S3 received domestic and industrial wastewaters, containing organic and inorganic pollutants. The impact of pollution was partly reflected by the algal biodiversity existing in the studied area. Likewise, we found at the reference station S1 which suffers only small anthropogenic impacts the highest biodiversity of seaweeds. Genera such as *Cystoseira*, *Bifurcaria* and *Saccorhiza* were present at this site, which typically indicate small anthropogenic impacts or non-affected areas (Munda, 1980; Riadi, 1998; DíEz et al., 1999; Arévalo et al., 2007; Ballesteros et al., 2007; Orfanidis et al., 2011; Biskup et al., 2014; Rubal et al., 2014; Celis-Plá et al., 2016; Boundir et al., 2019). In contrast, a dramatic decrease of diversity was observed in sites S2 and S3, located in the urban area close to places where domestic and some industrial waste waters are discharged. Several authors have proven the disappearance of mostly perennial seaweeds including the associated or epiphytic species due to pollution impact (Munda, 1982; Schramm, 1999; Sava et al., 2011). Our finding is in agreement with these earlier reports from other areas and it also generally supports the work of Chouikh et al. (2019) that was based on the assessment of polychaete distribution and conducted in the same study area. Indeed, many epiphytic algae inventoried at S1 (e.g. *Aglaothamnion tripinnatum, Polysiphonia atlantica, P. elongata, Lomentaria articulata, Plocamium raphelisianum, P. cartilagineum*) were not found at S2 and S3. In contrast, it has been noticed that species known to indicate pollution, like *Caulacanthus ustulatus*, *Codium tomentosum*, and other species belonging to the *Cladophora* and *Ulva* genera, were dominant at the last two stations. These findings are in accordance with literature (Jupp, 1976; DÍez et al., 1999; Godeh et al., 2010; Orfanidis et al., 2011). In order to support our conclusions an established indicator of pollution was calculated, which is the mean ratio of red algal and green algal species diversity in a given area (the so-called corrected R/C index). It is based on the fact that the number of Rhodophyceae is generally higher in less polluted areas, whereas Chlorophyceae are more diverse in polluted habitats, pollution is coupled to a decrease of the ratio (Sfriso et al., 2006; Orfanidis et al., 2011). At S1, the R/C ratio was always above 2 (on average 3.84), indicating a high percentage of Rhodophyceae, while at the stations S2 and S3 it never exceeded 0.91 and 1.37, respectively. Moreover, a stronger dominance of opportunistic species was observed at S2 (78%) and S3 (66%), as compared to S1 (42%). Again, similar observations at differently impacted sites have been reported from other coastal areas (Sfriso et al., 2009).

Nitrate, ammonium and orthophosphate are essential macronutrients for algal growth and stress management (Celis-Plá et al., 2014). However, excess concentrations can have a negative impact, as they can generate additional stress and thus inhibit growth (Guerra-García and Koonjul, 2005; Nassar et al., 2015). The macronutrients of coastal areas are often a main factor that drives changes in the structural composition of algal communities (McGlathery et al., 2007; Leterme et al., 2014; Nassar et al., 2015). Such nutrients are present in natural environments, but their concentration near urban areas is usually elevated (Nixon, 1995; Scavia and Bricker, 2006). In the present study, the physiology of *E. elongata* did not correlate significantly with most of the inorganic parameters. This indicates that the state of this seaweed is not really affected by the nutrient concentration at the study site varied between 6 and 12 and 0.3 and 7 for TN and TP mg L^{-1}, respectively, which has also been observed by some other authors (Schaffelke, 1999; Boundir et al., 2019).

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DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

Table 8. *Loading vectors of physiological parameters and heavy metal concentrations in E. elongata and physicochemical parameters in the study areas along principal component axes 1 and 2*

Parameter	Component	
	1	2
BOD	0.941	0.136
COD	0.975	-0.011
OM	0.974	0.071
TSS	0.383	-0.724
Proline	0.775	0.135
GB	0.892	-0.091
Chla	-0.891	0.031
Morphometry	-0.834	-0.295
Zn	0.526	0.554
Cu	0.607	-0.540
pH	-0.658	0.229
EC	0.934	0.091
DO	-0.779	0.472
T	0.473	0.676
Variance (%)	41.85	33.86
Cumulative (%)	41.85	75.72

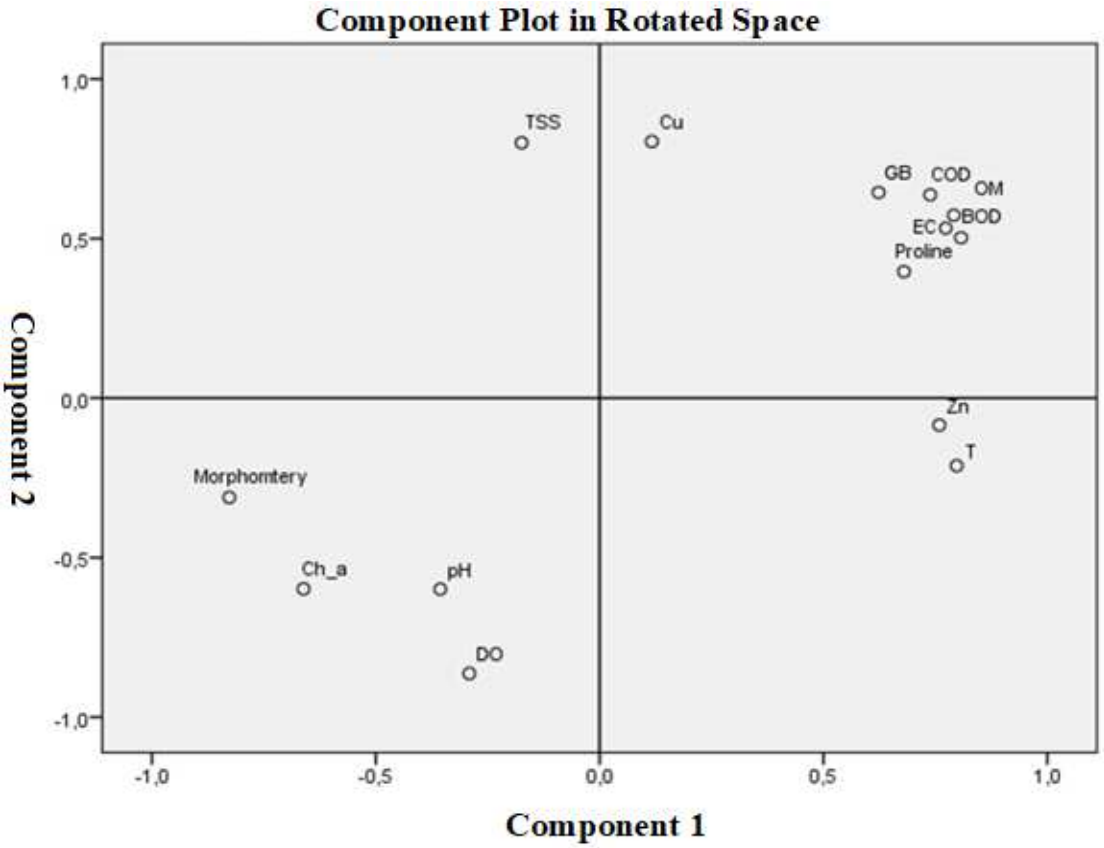

Figure 3. *Component plot in rotated space for the parameters studied. TSS: total suspended solids; T: temperature; EC: electrical conductivity; DO: dissolved oxygen; BOD: biological oxygen demand; COD: chemical oxygen demand; OM: oxidizable matter; Ch a: chlorophyll a; GB: glycine betaine; Zn: zinc; Cu: copper*

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DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

A significant increase of tissue concentrations of GB and proline in *E. elongata* and a decrease of Chla content, DO and algal axis length was observed together with an increase in concentrations of EC, BOD, COD and OM at the polluted stations S2 and S3. These results of our PCA analysis are in accordance with our expectation of an impact of organic pollution on *E. elongata's* physiology. Overall, algal length, chla and DO values were higher in S1 than in S2 and S3, which shows that *E. elongata* was affected by pollution in the two last stations.

Previous studies have demonstrated that pollution stress causes a decrease of chlorophyll contents, reducing algal photosynthetic activities, and correspondingly a reduction of production of dissolved oxygen, which can be decreased if pollution comes along with increased input of organic matter and subsequent degradation processes (Sawyer and McCarty, 1978). Additionally, some authors have shown that the pollution affects also the algal size (Doblin and Clayton, 1995; Coelho et al., 2000; Morin, 2006). However, toxic heavy metals (Cd, Pb and Cr) were not detected in *E. elongata*. Moreover, the Zn and Cu concentrations observed are much lower than those reported by other authors in calcareous red seaweeds (Malea et al., 1994; Abdallah et al., 2006; Dadolahi-sohrab et al., 2011; Khaled et al., 2014) and cannot be expected to be toxic. Working in our study area, some authors already demonstrated that brown algae such as *Carpodesmia tamariscifolia* (as *Cystoseira tamariscifolia*) and *Saccorhiza polyschides* are significantly more contaminated with heavy metals at sites S2 and S3 than at site S1 (Cherifi et al., 2018; Boundir et al., 2019). However, certain studies have already shown that calcareous Rhodophyceae have a reduced capacity for biosorption of metals as compared to other algal groups (Jordanova et al., 1999; Benguedda-Rahal, 2012). Bioaccumulation of heavy metals may be affected by many factors, such as pH, the binding capacity of various sediment components and the specific affinity of species for heavy metals. This binding capacity is largely dependent on the nature and chemical composition of cell wall components. The latter might be a main reason for the findings at the study area, given that brown algae generally exhibit a greater ability to accumulate metals than red and green algae (Leonardi and Vasquez, 1999; Davis et al., 2000; Kaimoussi et al., 2002; Abdallah et al., 2006; Benkdad et al., 2011; Mouradi et al., 2014).

While the inorganic pollution parameters had no impact on the physiological parameters of *E. elongata* at the study sites there was a clear correlation with the parameters indicating organic pollution like BOD_5, COD, OM. The highest concentrations of BOD_5, COD, and OM were always recorded in the polluted areas S2 and S3 which were elevated by a factor 5-10 compared to the unpolluted site S1. That organic matter is an indicator for pollution by discharge of wastewater was shown before (Sawyer and McCarty, 1978; Broecker and Peng, 1983; El-Sonbati et al., 2012; Er-Raioui et al., 2012; El-zeiny et al., 2016). This does not explain, why and how the organic matter affected the seaweed. There are two possible explanations: the organic matter load included substances which were toxic or the organic matter enhanced heterotrophic degradation processes which affected the primary production. The latter can be ruled out because although > 50% of the COD was biologically degradable the parameters like temperature, pH, DO and conductivity did not differ significantly at all study sites and during all season. It is thus highly possible that toxic organic pollutants entered the coastal areas with the discharge of waste water. Because this kind of pollution would have otherwise remained undetected, our approach to use physiological responses of *E. elongata* as indicators of variable kinds of pollution was verified. This is

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in accordance with a growing interest in using seaweeds for the biomonitoring of different kinds of pollution (Pereira et al., 2009; García-Seoane et al., 2018, 2019; Roleda et al., 2019). A number of researchers identified that stressful conditions of seaweeds induce the decrease of chlorophyll contents, algal size and also proline and GB accumulations (*Table 9*). Emphasis should be put on the development of methods which allow to indentify the type of pollution too.

Table 9. *Main factors causing algal stress*

Stress factors	Algal species	References
Solar radiation	*Ellisolandia elongata*	Häder et al. (1997)
Thermal stress	*Ellisolandia elongata*	Nannini et al. (2015)
Acidification effect	*Lithophyllum incrustans*	Noisette et al. (2013)
Light and desiccation stress	*Ulva linza*	Guan et al. (2016)
Heavy metals	*Cystoseira tamariscifolia*	Boundir et al. (2019)
Organic pollution	*Ellisolandia elongata*	Current study

Conclusion

The present work evaluated the pollution degree in the Essaouira city coastal area (Morocco) by using, for the first time, the calcareous seaweed *Ellisolandia elongata* (Rhodophyta) as a bioindicator. Our results show that organic pollution is the main factor that affects the macroalgae diversity at the Essaouira coast. A dramatic decrease of seaweed biodiversity has been noticed in the Bab Doukkala (S2) and Port (S3) stations where urban wastewater is discharged without any treatment, causing pollution. Several species reflecting these polluted conditions have been identified in these two stations and not in the reference station of My Bouzerktoune (S1). The pollution impact is more evident at S2 than at S3. Physico-chemical parameters and inorganic pollution did not show a real impact on the algal biodiversity and the physiological state of *E. elongata*. Thus, this red alga could be used as a tool to monitor organic pollution in Morocco and might be applicable as a bioindicator also in other areas. Further studies should be undertaken on other macroalgae and on other stressful conditions in order to support these results.

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DOI: http://dx.doi.org/10.15666/aeer/1806_77177738

EFFECT OF SHADING ON THE WATER USE EFFICIENCY OF WINTER WHEAT (*TRITICUM AESTIVUM* L.) IN SEMI-ARID AND SEMI-HUMID REGIONS OF CHINA

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(Received 7th Jul 2020; accepted 17th Sep 2020)

Abstract. Solar radiation has a substantial influence on winter wheat (Triticum aestivum L.) growth and water consumption. To elucidate the effect of solar radiation on the yield and water consumption of winter wheat, two-season pot experiments were conducted at Xi'an University of Technology in China from 2015 to 2017. Four shading treatments and a non-shaded control (CK) treatment were applied using black shading net, including 80% (L80), 60% (L60), 40% (L40), and 20% (L20) of the CK. With the increase in the shading degree, the time of winter wheat growing stage was prolonged, but the dry matter accumulation and water consumption decreased. The maximum of the water consumption and water consumption intensity for the winter wheat occurred at the filling stage, and the water-saving rate was higher than the shading rate from heading to the mature period under the L80 treatment. The water-use efficiency (WUE) under the L80 treatment and CK did not differ significantly. The harvest index for the L80 treatment was higher than that of the CK. To obtain high WUE, wheat must not receive less than 80% of solar radiation.
Keywords: *solar radiation, pot experiment, shading net, evapotranspiration, harvest index*

Introduction

Solar radiation is the energy source of all things and the most important factor that affects photosynthesis (Kalyanasundaram and Graetzel, 2010). Plant leaf photosynthesis and transpiration are both driven by solar energy. When the radiation reaches the saturation point of the crop, the photosynthetic rate no longer increases with higher radiation, but the transpiration rate does. A total of 98-99% of the water absorbed by the roots is dispersed into the air in the form of water vapor, and only approximately 1% of the water is absorbed for the growth of the plants. Crop water use efficiency is essentially the ratio of the photosynthates to water consumption. Based on the influence of the light intensity on photosynthesis and transpiration, the WUE must change when the solar radiation changes.

During the day, the radiation periodicity changes, and the daily evapotranspiration (ET) of the crops also shows obvious diurnal regularity. From dawn to sunrise, the ET is at the lowest level during the day. After sunrise, with the increase in light intensity, the physiological activities of the crops become more active, and the ET gradually increases before gradually decreasing with the decrease in the radiation intensity (Yu and Wang, 2010). These laws all indicate that solar radiation intensity has an effect on the ET, and a moderate reduction of the radiation intensity is accompanied by a decrease in water consumption. Most of the arid and semiarid areas in China are in the northwest with abundant radiation resources and large amounts of daily radiation but a lack of water

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DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

resources. This region has substantial potential to benefit from the control of solar radiation.

There are two primary aspects of the current research on solar radiation control. One is the effect of shading on crop yield and quality. This research is based on the fact that environmental problems, such as air pollution and an increase in aerosol particles, resulting in a significant reduction in solar radiation reaching the Earth's surface (Li et al., 2010; Mu et al., 2010; Haywood et al., 2011), or crop interplanting, resulting in a reduction in the light intensity received by the crops underneath (Gommers et al., 2013; Xie et al., 2017). Focusing on the Yangtze River Basin and the Huang-Huai-Hai region, many studies have been conducted on the effects of the reduction in radiation on crop yield and quality (Mo et al., 2015; Ili et al., 2017). These studies mostly utilize white or black shading nets with different layers and needles to obtain different shading degrees (SDs). Where the SD gradient is lower, the SD range of the light is relatively centralized, and the SD is generally larger. Thus, the time for shading is shorter and more concentrated on certain growing stages of the crops. Most of the measurements involve the reduction in yield and quality. Recent studies have also shown that when the intensity of the natural light is 88% lower, there is an increase in yield due to the prolonged leaf growth (Mu et al., 2010; Xu et al., 2016). These studies focused on the effects of light intensity on crop growth indicators and yield but did not consider crop water consumption. Besides, changes in the growth of the crop indicate their impact on water consumption.

The effect of shading on economic crops is primarily concentrated in orchards. The results show that the shading nets can reduce solar radiation, prevent fruit burns, and reduce damage from hail (Bogo et al., 2012) and birds (Ashraf and Harris, 2013). A reduction in solar radiation also improves orchard microclimate. At high radiation and temperature, shading can reduce the temperature, increase the relative humidity of the air and reduce the wind speed (Lopez et al., 2018; Mupambi et al., 2018). Thus, this shading reduces water consumption (McCaskill et al., 2016). Shading increased the photosynthesis in fruit trees under a water deficit but also decreased the total absorption of light (Girona et al., 2012). The yield and light absorption increased approximately linearly. In the case of excess radiation, the protection and recovery of photosynthesis will consume photosynthates; thus, increasing the accumulation of dry matter. These studies indicated that the protection of fruit trees by a shading network will reduce water consumption and increase the yield of the fruit. However, for field crops, the use of shading to change their water consumption, as measured by the daily water consumption, water consumption in different growing stages, and the trend of WUE, merits further study.

With the increases in aerosols, air pollutants, and population density, dimming, or shading have become major challenges to crop production in many areas of the world (Mu et al., 2010). Experiments with relative heavy shading treatments were applied, the grain yield decreased. Whether evapotranspiration decrease at the same time needs further research. In this study, the winter wheat (*Triticum aestivum* L.) planting area accounts for nearly 22% of the total sown area of grain crops (NBS, 2014) was selected as the experimental object. As a C3 specie, wheat is more susceptible to photoinhibition under high solar radiation than C4 plants. The photosynthetic rate is relatively low, and the CO_2 concentrating mechanism operates at higher leaf conductance (Sonoike, 2011; Tikkanen et al., 2014; Guidi et al., 2019). Radiation control experiments were conducted to clarify the effects of the long-term control of radiation on wheat

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DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

photosynthetic growth, the trend of wheat evapotranspiration under different shading degrees, the key period of water consumption, and the influence of the law of shading on the wheat yield and water use efficiency.

Materials and methods

Experimental site

Two-season pot experiments were conducted from October 2015 to June 2017 at the experimental site at the Xi'an University of Technology, Xi'an, Shaanxi Province, China (108°93' E, 34°23'N, 416 m H). The soil type was a loam containing 10.0 g kg^{-1} organic matter, 1.30 g kg^{-1} total nitrogen, 22.3 mg kg^{-1} available phosphate, and 110.51 mg kg^{-1} available potassium. The soil bulk density was 1.41 g cm^{-3} with a pH of 8.03. The climate data during the experimental period were shown in *Figure 1*.

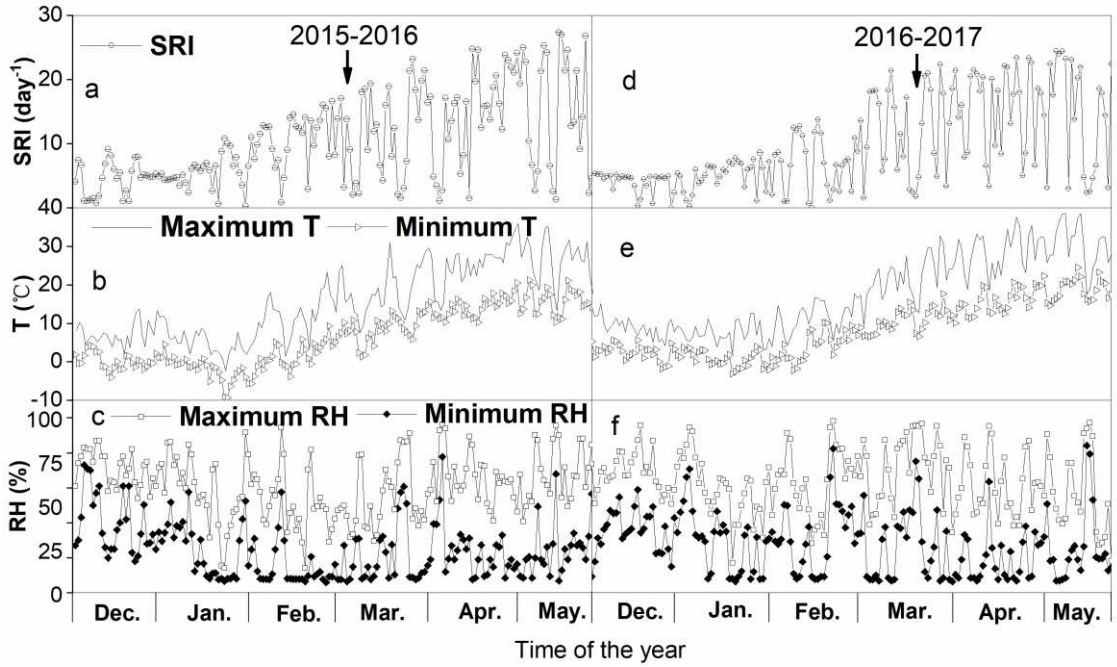

Figure 1. *Solar radiation intensity (SRI, a, d), temperature (T, b, e), relative humidity (RH, c, f) in the atmosphere during the shading period in 2015-2016 and 2016-2017*

Experimental design and field management

One cultivar of winter wheat (Triticum aestivum *L.*) currently used in local production, 'Xinong 979', was chosen for the pot experiments. The top of the wheat canopy was shaded using black polyethylene screens with different needle numbers and layers from the wintering (December 20th 2015, December 18th 2016) to the maturity periods (May 28th 2015, May 24th 2016). The shading net was 20 cm above the wheat canopy. Five treatments were set up: (1) full radiation, CK; (2) 80% of full radiation with 1 layer 2 needles black polyethylene screen, L80; (3) 60% of full radiation with 1 layer 3 needles black polyethylene screen, L60; (4) 40% of full radiation with 1 layer 2 needles and 1 layer 3 needles black polyethylene screen, L40; and (5) 20% of full radiation with 1 layer 6 needles black polyethylene screen, L20. Five treatments were

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DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

established with four replications, 20 treatments in total. The shading experimental culture layout was shown in *Figure 2*.

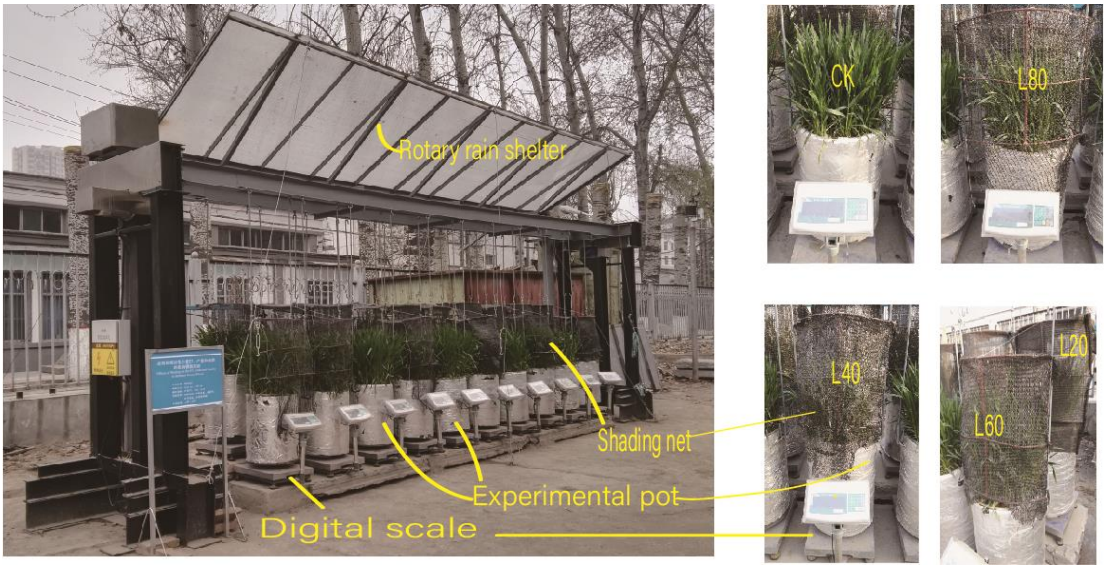

Figure 2. *Image of shading experimental culture. CK refers to the no shading treatment (control), L80, L60, L40, and L20, refer to 80%, 60%, 40%, and 20% of the incident solar radiation, respectively*

The size of each pot was 0.4 m in diameter and 0.6 m in height. The pots were placed under a rotary rain shelter, which could change the rotation angle to keep radiation indirectly, erected in the experimental site. Each pot was uniformly packed with 90 kg soil at a bulk density of 1.41 g cm^{-3}. Fifty wheat seeds were broadcast sown in each pot on October 18, 2015, and October 16, 2016, and four weeks after sowing, thinning was done to obtain the desired density of 40 seedlings per pot. The pot experiments were well irrigated with the soil water content (SWC) ranging from 65% to 95% of field capacity (FC). When the SWC of some treatments was near 65% of FC, all the treatments were irrigated to 95% of FC. The SWC was maintained by weighing the pot and its contents. N, P_2O_5 and K_2O were applied as base fertilizers at 220, 150 and 150 kg ha^{-1}, respectively. A total of 75 kg ha^{-1} N was applied as a top dressing in the wintering period. Fungicides were sprayed to against powdery mildew.

Measurements and methods of the microclimate in the pot experiments

Microclimate in the pot experiments

The temperature and relative humidity (RH) of the canopy under the shading screens were measured using a thermohygrometer (WS-1, Tianjin Fengyang Co. Ltd., China). Photosynthetically active radiation (PAR) was measured using a Li-6400 system (Li-cor, USA). All the data were monitored every 60 min from 8:00 to 20:00 for 30 days after anthesis. The data measured were shown in *Table 1*. The daily meteorological data of the atmosphere, including temperature, RH and total radiation, were recorded every 5 min using a portable automatic weather station (Watchdog 2900ET, Spectrum Technologies Inc., USA).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

Table 1. *Changes in the temperature, relative humidity and photosynthetically active radiation in the canopy under the shading conditions*

Treatment		8:00	9:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00	18:00	19:00	20:00
Temperature (°C)	CK	14.9	16.2	18.4	22.7	22.1	24.6	26.1	25.4	24.8	22.5	21.5	19.5	18.5
	L80	13.9	14.6	15.5	19.8	21.5	22.4	24.0	25.3	25	23.2	20.7	19.4	18.5
	L60	14.2	14.4	15.4	18.7	20.6	21.7	23.4	23.5	24.3	22.5	20.6	19.4	18.3
	L40	14.1	14.4	15.5	18.5	20.7	21.5	23.4	24.1	24.2	22.1	20.4	19.6	18.5
	L20	14.3	14.5	15.3	18.2	20.6	21.6	23.3	24.2	24.1	21.5	20.3	19.4	18.4
Relative humidity (%)	CK	60.8	55.4	53.4	46.5	46.3	42.4	37.8	35.5	33.4	34.7	38.3	40.1	44.9
	L80	62.1	54.7	53.6	49.4	46.3	41.5	39.5	36.4	33.4	36.6	40.5	41	50.8
	L60	61.2	52.6	54.3	51.5	47.7	43.4	39.7	37.8	35.5	36.7	41.8	41.4	50.4
	L40	60.8	55.3	57.6	54.3	48.5	44.5	39.1	37.5	36.8	39.5	40.7	41.8	50.2
	L20	61.4	51.2	53.4	49.7	48.8	45.6	40	38.6	36.7	39.4	41.5	41.9	51
Photosynthetically active radiation (μmol m^2 s^{-1})	CK	425	701	818	1017	1121	1205	1189	1128	903	417	65	7	0
	L20	325	556	649	813	902	961	945	894	700	315	49	5	0
	L40	246	400	478	604	678	718	699	675	530	240	35	4	0
	L60	164	279	321	400	450	474	460	435	350	150	21	2	0
	L80	71	126	152	200	226	246	258	238	145	60	8	2	0

CK refers to the 'no shading' treatment (control), L80, L60, L40, and L20, refer to 80%, 60%, 40%, and 20% of the incident solar radiation, respectively. The data were the average values on March 18th in 2016

Winter wheat growing stages

Winter wheat growing stages were defined base on the Irrigation Experiment Standard (SL13-2015) of China. Winter wheat growth stage was defined as ten percent of pot winter wheat got in one growth stage.

Grain yield components

At the maturity stage of winter wheat, the spike numbers and grain yield (GY) were measured of each pot. Also, 20 representative plants were selected consecutively in each pot, and the kernel numbers per spike and 1000-grain weight were examined in the experiment. All plant parts (leaves, spikes, and stems) were oven dried at 60 ℃for 72 h to calculate dry matter accumulation (DMA). Harvest index (HI) was calculated as the ratio of GY to DMA. Measurement was performed according to the Irrigation Experiment Standard (SL13-2015) of China.

Evapotranspiration and water use efficiency

The experimental pots were weighed every day at 8:00 am using digital scales (TCS-CC, Fuzhou Kedi Electronic Technology Co., Ltd., China) to calculate the water consumption. The water consumption over time was calculated using a water balance equation that incorporates the difference in the weight of the pots with their plants and soil and the mass of water added to them. The ET was calculated as

$$ET = \frac{\Delta W + W_a}{S} \quad \text{(Eq.1)}$$

where ΔW is the pot weight difference, kg; W_a is the mass of added water, kg; and S is the area of the pot, m^2. The daily mean evapotranspiration of each growing stage (ET_{dm})

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

was calculated as divide the evapotranspiration in each growing stage (ET_{gs}) by days of each growing stage. The experimental pots were weighed every 2 h on typical sunny days February 25th, March 22nd, April 7th, April 28th, May 15th 2016 for each growing stage to obtain the typical day ET. The wheat WUE was determined as follows:

$$WUE = \frac{GY}{WET} \quad \text{(Eq.2)}$$

where GY is grain yield of winter wheat, kg ha^{-1}; WET is the whole growth period evapotranspiration of each pot, mm. WET was considered the equivalent of the total amount of irrigation plus the soil water in pots before sowing minus the residual after final harvest.

Statistical analysis

All the data were subjected to a one-way analysis of variance, and Duncan's Range Test was used to determine the significance of the differences between the treatments using SPSS statistical software (SPSS 20.0) (SPSS, Inc., USA).

Results

The microclimate of the pot experiment

Figure 1 shows the changes in solar radiation intensity (*Fig. 1a* and *d*), temperature (*Fig. 1b* and *e*), and relative humidity (*Fig. 1c* and *f*) in the atmosphere during the shading period. The solar radiation intensity (SRI, *Fig. 1a* and *d*) and temperature (*Fig. 1b* and *e*) increased roughly from January to May. The accumulated SRI from January to March in 2016 was higher than that in 2017, but that was lower from April to May. The total SRI with a value of 91.32 MJ m^{-2} in 2016 was higher than that in 2017 during the shading period. SRI under shading nets was shown as PAR, as shown in *Table 1*. In the period of high radiation intensity 10:00-16:00 light control met the design requirements, the rest of the day due to the solar radiation angle was too small, shading rate was higher.

Table 1 displays the change in temperature and RH in the canopy under the shadings condition of March 18, 2016. The canopy temperature decreased as the shading degree increased, compared with the CK. The temperature at 14:00 decreased by 2 °C, 3 °C, 3 °C, and 3 °C under the L80, L60, L40 and L20 treatment, respectively. The RH in the canopy increased with the reduction in solar radiation under the shading treatments. Compared with the CK, the RH increased by 1.0%, 3.0% 4.0% and 5.0% at 14:00 pm for the L80, L60, L40 and L20 treatments, respectively.

Crop growing duration

The days of the whole growth period increased from 215 to 225 with an increase in the intensity of shading (*Table 2*).

Except for the booting stage, shading had a significant effect on crop growing duration, while a significant difference ($p < 0.05$) was observed from sowing to the tillering stage to flowering. However, there was no significant interaction ($p > 0.05$) of shading and year during growth stages. Compared with the CK, the days of the jointing stage were prolonged by 0, 2, 4, and 5 days in 2016 and 1, 2, 3, and 4 days in 2017 for

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the L80, L60, L40, and L20 treatments, respectively. The wintering period in 2016 was longer than that in 2017, while the joining stage was shorter than in 2017.

Table 2. *Days (day) of different wheat-growing stages under different treatments*

Years	Treatment	Sowing to tillering stage	Wintering period	Green stage	Joining stage	Booting stage	Heading to flowering	Filling stage	Mature period	Whole growth period
2015-2016	CK	63 a†	50 b	12 b	28 b	11 a	22 a	16 a	12 b	215b
	L80	63 a	51 ab	13 ab	28 b	12 a	20 b	17 a	13 ab	218 ab
	L60	63 a	52 a	13 a	30 ab	12 a	20 b	17 a	14 ab	221 ab
	L40	63 a	52 a	13 a	32 a	11 a	20 b	17 a	14 a	224 ab
	L20	63 a	52 a	13 a	33 a	11 a	20 b	17 a	14 a	225 a
2016-2017	CK	64 a	41 c	13 a	32 c	14 a	20 a	16 a	14 a	213 c
	L80	64 a	42 b	14 a	33 bc	15 a	19 ab	16 a	14 a	216 b
	L60	64 a	42 b	14 a	34 bc	15 a	19 ab	17 a	14 a	218 ab
	L40	64 a	43 a	14 a	35 ab	15 a	19 ab	17 a	14 a	220 a
	L20	64 a	43 a	14 a	36 a	15 a	18 b	18 a	14 a	221 a
L		0	30.22***	9.688***	19.15***	1.00	11.17***	5.9**	3.56*	11.4***
Y		10.71**	3808.8***	36.13***	67.60***	142.3***	52.0***	0.08	1.13	2.36
L×Y		0	1.83	0.19	0.85	0.21	1.67	0.92	3.31*	0.34

†Data followed by the same letter in a column indicate a nonsignificant difference among the treatments according to the LSD test (P = 0.05)
CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. L, light control; Y, year. *, significance at the .05 level; **, significance at the .01 level; ***, significance at the .001 level

Evapotranspiration and daily mean evapotranspiration of the winter wheat

The evapotranspiration at each growing stage (ET_{gs}) was affected ($p < 0.05$) by the shading from the winter period to the mature period. ET_{gs} were affected ($p < 0.01$) by the year except for the green stage, jointing stage, and filling stage (*Table 3*). And ET_{gs} under different shading treatments were affected ($p < 0.01$) by the growing stages (*Table 4*).

Table 3. *Analysis of variance on evapotranspiration and daily mean evapotranspiration of each growing stage as affected by light control and years*

	Factors	Seeding stage	Tillering stage	Winter period	Green stage	Joining stage	Booting stage	Heading to flowering	Filling stage	Mature period
ET_{gs}	L	2.22	1.41	10.83*	29.75**	79.50***	8.59*	35.48**	28.78**	152.21***
	Y	184.06***	344.11***	81.05***	0.30	0.83	39.82**	471.39***	5.46	233.20***
ET_{dm}	L	0.86	1.51	9.43*	71.32***	807.61***	9.63*	74.56***	35.02**	6.04
	Y	112.97***	344.21***	0.08	15.08*	197.24***	7.95*	1264.57***	5.23	0.55

ET_{gs}, evapotranspiration of each growing stage; ET_{dm}, daily mean evapotranspiration of each growing stage; L, light control; Y, year. *, significance at the .05 level; **, significance at the .01 level; ***, significance at the .001 level

The ET_{gs} from 2015 to 2016 showed that the filling stage > heading to flowering > jointing stage > booting stage > wintering period > mature stage under CK and L80 treatments, filling stage > heading to flowering > booting stage > jointing stage > mature stage > wintering period under L60, L40, and L20 treatments, (*Fig. 3*, *Table 4*). It was similar from 2016 to 2017, except that the ET_{gs} in the booting stage was higher than that in the heading to the flowering stage (*Table 4*). The water consumption of the winter wheat was primarily concentrated in the four growing stages, including the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

jointing, booting, heading to flowering and filling stages, accounting for 78.42% of the whole growth period, and the highest rate of water consumption was in the filling period, accounting for 27.34%.

Table 4. *Evapotranspiration and daily mean evapotranspiration of each growing stage (ET_{gs}, ET_{dm}, mm) of different shading treatments under different growing stages*

Factors		Et_{gs}					ET_{dm}				
Y	**G**	**CK**	**L80**	**L60**	**L40**	**L20**	**CK**	**L80**	**L60**	**L40**	**L20**
2015-2016	Seeding stage	10.71h†	11.01f	12.12 g	12.17 h	12.89 h	0.46h	0.47f	0.52g	0.52g	0.56g
	Tillering stage	16.64g	17.25e	18.28f	17.79g	18.70g	0.47h	0.49f	0.52g	0.50g	0.53g
	Wintering period	33.51e	32.70d	32.04e	30.09f	29.35f	0.67g	0.64f	0.61g	0.57g	0.56g
	Green stage	16.15g	14.18ef	13.12g	11.52h	11.09h	1.34f	1.09e	1.00f	0.88f	0.85f
	Jointing stage	93.24c	79.42c	58.40d	54.13d	57.45d	3.33d	2.63d	1.94e	1.69e	1.74e
	Booting stage	81.18d	75.24c	70.19c	62.96c	59.92c	7.38b	6.27b	5.84b	5.72b	5.44b
	Heading to flowering	150.98b	121.83b	106.42b	106.26b	100.88b	6.86c	6.09b	5.32c	5.31c	5.04c
	Filling stage	180.97a	139.51a	129.95a	119.13a	116.83a	11.3a	8.20a	7.64a	7.00a	6.87a
	Mature period	29.57f	32.15e	33.94e	48.60e	52.78e	2.46e	2.67c	2.42d	4.05d	4.79d
2016-2017	Seeding stage	15.94h	16.21h	16.21f	16.16g	16.56g	0.72f	0.7e	0.7d	0.7f	0.72f
	Tillering stage	23.89g	23.88g	23.88e	23.96f	24.13e	0.68f	0.68e	0.68d	0.68f	0.69f
	Winteringperiod	29.34f	28.26f	25.12e	23.36f	22.12f	0.72f	0.67e	0.6d	0.54f	0.51f
	Green stage	17.04h	14.82h	12.44g	10.69h	9.98d	1.31e	1.06e	0.89cd	0.76f	0.71f
	Jointing stage	95.98c	81.79c	54.9c	51.13d	50.2d	2.8d	2.48d	1.61cd	1.46e	1.39e
	Booting stage	127.01b	109.57b	106.57b	88.63b	83.73b	9.07b	7.3b	7.1a	5.91b	5.58b
	Heading to flowering	83.99d	64.28d	53.53c	49.28e	48.67d	4.2c	3.38c	2.82b	2.59d	2.7d
	Filling stage	209.16a	160.01a	139.74a	120.44a	118.68a	13.07a	9.41a	8.22a	7.08a	6.98a
	Mature period	41.85e	45.56e	42.88d	61.01c	63.97c	2.82d	2.85cd	2.52bc	3.59c	3.55c
G		***	***	***	***	***	***	***	***	***	***
Y		***	***	0.677	***	***	0.001**	0.924	0.496	***	***
G×Y		***	***	***	***	***	***	***	***	***	***

†Data followed by the same letter in a column indicate a nonsignificant difference among the treatments according to the LSD test (P = 0.05)

CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. ET_{gs}, evapotranspiration of each growing stage; ET_{dm}, daily mean evapotranspiration of each growing stage; G, growing stage; Y, year. *, significance at the .05 level; **, significance at the .01 level; ***, significance at the .001 level

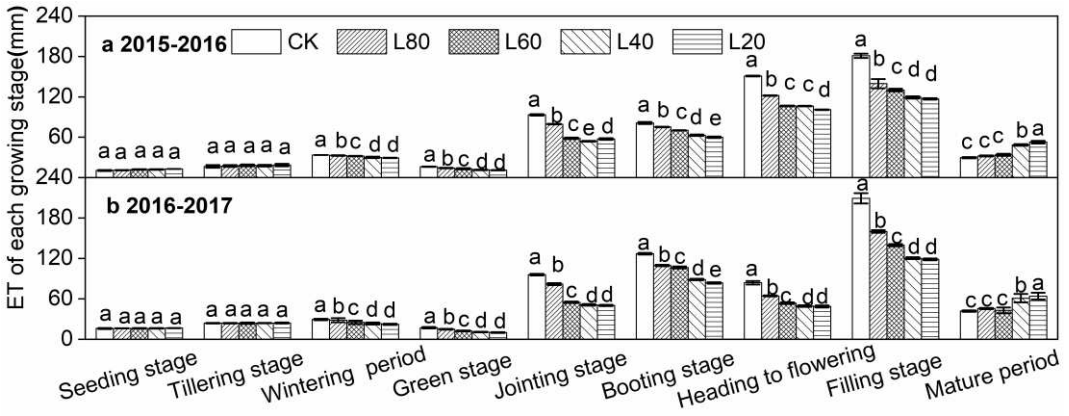

Figure 3. *Evapotranspiration of each growing stage (ET_{gs}) for the shading treatments. CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. Data are means of four replicates. Vertical bars indicate standard error. The same letters within each growing stage in the same year are not significantly different at P < 0.05*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The ET_{gs} decreased gradually as the degree of shading increased in each treatment from the wintering period to the filling stage. There were significant differences in water consumption among the treatments in jointing, booting, heading to the flowering, and filling stages. The ET_{gs} had significant differences among the CK, L80, and L60 treatments, while there were no significant differences among the L60, L40, and L20 treatments. Compared with the CK, the ET_{gs} of the L80, L60, L40, and L20 treatments from the jointing to the filling stages decreased by 16.09%, 27.15%, 32.04%, and 33.30%, respectively. When compared with the CK, the maximum decrease in the ET_{gs} for the L80, L60, L40, and L20 treatments was 22.91%, 28.19%, 34.17%, and 35.44% during the filling period, respectively. However, in the mature stage, the ET of the winter wheat increased gradually with the increase in the intensity of shading.

The daily mean evapotranspiration of each growing stage (ET_{dm}) was significantly affected by shading treatments ($p < 0.05$) from the winter period to the filling stage. ET_{dm} was affected ($p < 0.05$) by the year except for the winter period, filling stage, and mature period (*Table 3*). And ET_{dm} under different shading treatments were affected ($p < 0.01$) by the growing stages (*Table 4*). The ET_{dm} in each growing stage showed that the filling stage > booting stage > heading to flowering > mature stage > jointing stage in two years experiments (*Fig. 4; Table 4*). The ET_{dm} was larger from the booting to the filling stages than that in the other growing stages. The average ET_{dm} of all the treatments from the booting stage to the filling stage was 2.12 times greater than that of the whole growing stages. The maximum of the ET_{dm} in the filling period was 12.51 mm, while the average ET_{dm} of all the treatments was 2.59 times that of the whole growth period.

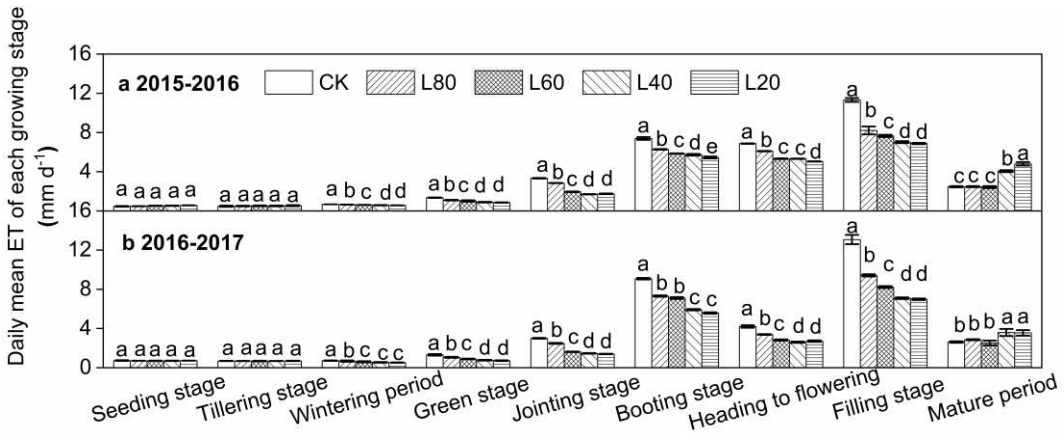

Figure 4. *Daily mean evapotranspiration of each growing stage (ET_{dm}) for the shading treatments. Data are means of four replicates. CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. Vertical bars indicate standard error. The same letters within each growing stage in the same year are not significantly different at P < 0.05*

Except for the mature period, the ET_{dm} decreased with the increase in the shading intensity with significant differences among the different treatments that were the same as the ET_{dm} during the same growing stage. The ET_{dm} for the L80, L60, L40 and L20 treatments decreased by 19.80%, 23.31%, 27.69%, and 30.64%, respectively, from the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

booting to the filling stages and decreased by 27.45%, 32.42%, 38.04%, and 39.24% in the filling stage compared with the CK, respectively.

Typical sunny day evapotranspiration of the winter wheat at different growing stages

The ET of a typical sunny day (ET_{ts}) was divided into the ET of the daytime (from 8:00 to 20:00, ET_d) and nighttime (from 20:00 to 8:00 the next day, ET_n). *Figure 5* shows the natural SRI and 2 h ET (ET_{2h}) of each treatment on a typical sunny day in each growth period from 2015 to 2016. During the daytime, the ET_{2h} changed roughly in a parabolic trend, first increasing and then decreasing, and the change in the trend was the same as that of the solar radiation.

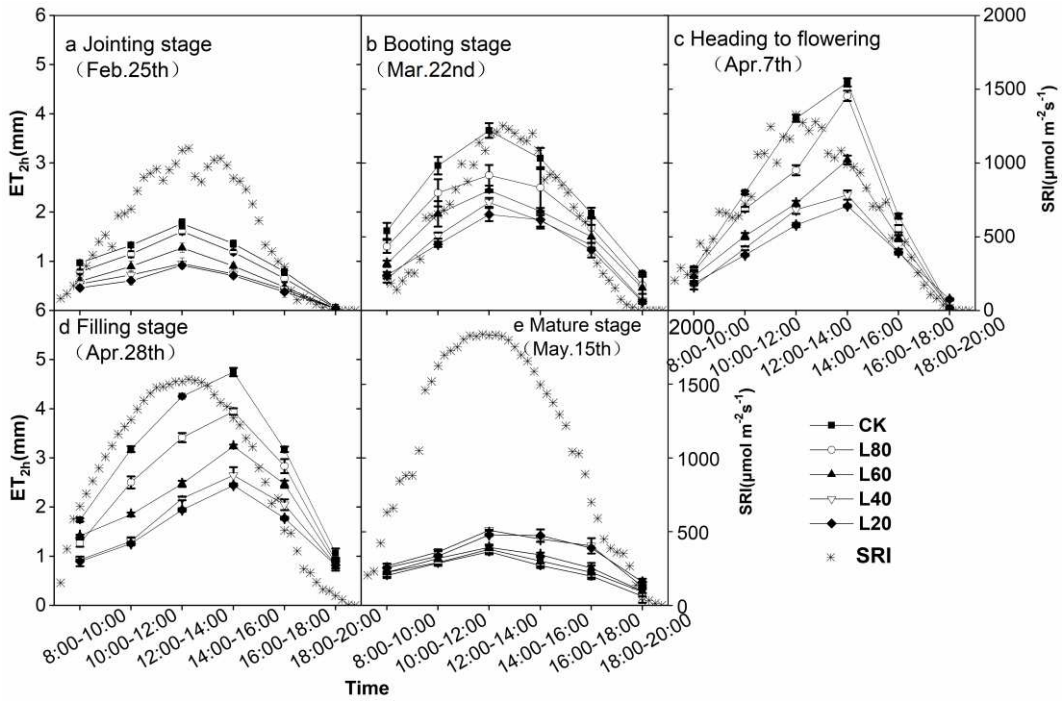

Figure 5. *Evapotranspiration of 2 h (ET_{2h}) of the winter wheat and solar radiation intensity (SRI) from 8:00 to 20:00 on typical sunny days in different growing stages (jointing stage, a, booting stage, b, heading to flowering, c, filling stage, d, mature stage, e). CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. Data are means of four replicates. The data were measured on February 25th, March 22nd, April 7th, April 28th and May 15th, 2016*

Except for the mature stage (*Fig. 5e)*, the ET_{2h} in each growing stage increased gradually in parallel with the increase in the SRI (*Fig. 5)*. The growing stage radiation intensity increased gradually over time, peaking at approximately 13:00. The ET_{2h} peak in the jointing and booting stages was between 12:00 and 14:00, which was the same as the maximum radiation, while in the heading to the flowering and filling growing stages, the peak was between 14:00 and 16:00. The ET_{2h} rapidly increased after 10:00 and rapidly decreased after 16:00.

The ET_d, ET_n, and ET_{ts} of the wheat on typical sunny days in different growing stages are shown in *Figure 6*. The ET_{ts} from the booting to the filling stages (*Fig. 6b, c* and *d*) was larger than that in the other growing stages (*Fig. 6a* and *e*), which was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

consistent with that of the ET_d. The ET_d of the wheat showed that the filling stage > heading to flowering stage > booting stage > jointing stage > mature stage. The proportion of the ET_n to ET_{ts} at the jointing and booting stages (*Fig. 6a* and *b*) was approximately 10%, and it increased from 12% to 19% at the heading to the flowering and filling stages (*Fig. 6c* and *d*), respectively. The proportion of the ET_d to ET_{ts} was more than 80% in all the growing stages, which indicated that the ET_d was the primary part of the ET_{ts}. Except the mature stage, the ET_n from the jointing to the filling stages of the L80, L60, L40, and L20 treatments decreased by 10.37%, 15.33%, 24.06%, and 23.92%, respectively, compared with the CK, which was significantly smaller than that of the daytime.

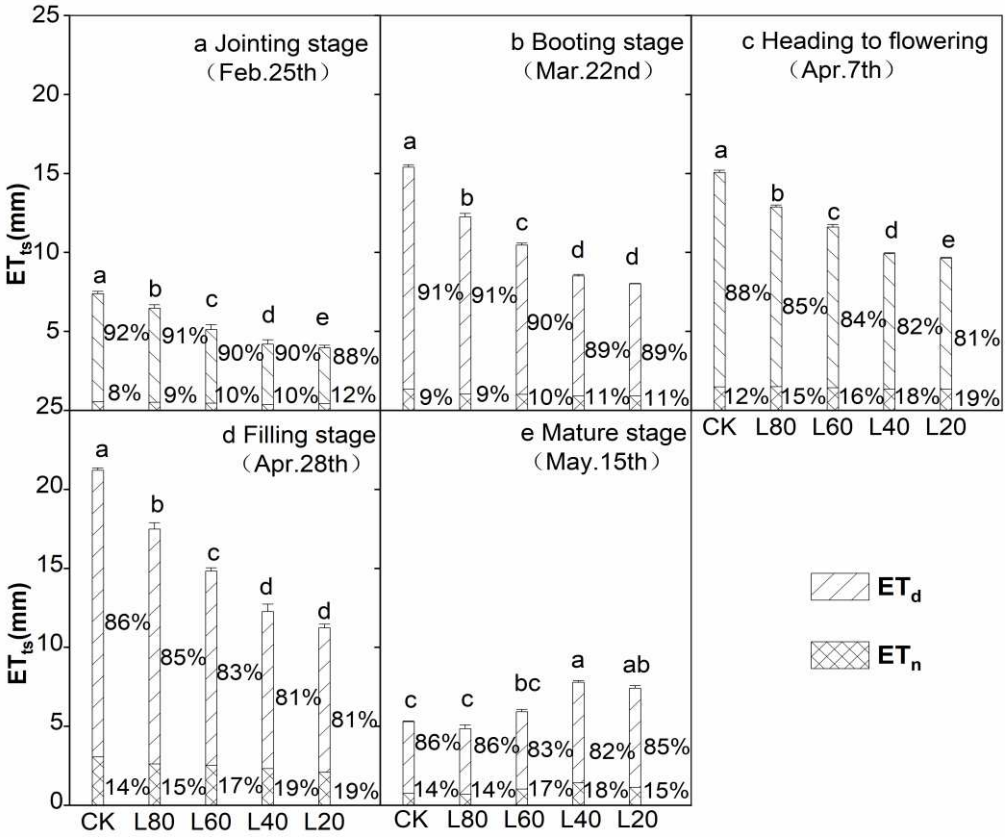

Figure 6. *Typical sunny day evapotranspiration (ET_{ts}), evapotranspiration of day (ET_d) and night (ET_n) of winter wheat on typical sunny days of each growing stage (jointing stage, a, booting stage, b, heading to flowering, c, filling stage, d, mature stage, e). The data were measured on February 25th, March 22nd, April 7th, April 28th and May 15th, 2016. Data are means of four replicates. CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. Vertical bars indicate standard error. The same letters within each growing stage are not significantly different at $P < 0.05$*

From the jointing to the filling stages, the ET_{ts} decreased with the increase in the shading degree. The difference of the ET_{ts} among the CK, L20, and L40 treatments was significant, while that between the L60 and L80 treatments was not significant at the booting and filling stages. At the **mature period** (*Fig. 6e*), the ET_{ts} increased with the increase in the shading degree.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Effects of shading on the grain yield and WUE

The grain yield (GY) was affected ($p < 0.001$) by both shading and year. And, there was significant interaction ($p < 0.001$) of shading and year. Grain yield compomers kernel number per spike (KNPS), spike number, 1000-grain weight were affected ($p < 0.001$) by both shading and year (*Table 5*).

Table 5. *Effect of Shading on the kernel number per stem (KNPS), spike number (spike no.), 1000-grain weight, grain yield (GY), dry matter accumulation (DMA), whole evapotranspiration (WET), water use efficiency (WUE) and harvest index (HI) of the winter wheat in 2015-2016 and 2016-2017*

Years	Treatments	KNPS	Spike no. (10^4 ha^{-1})	1000-grain weight (g)	GY (kg ha^{-1})	DMA (g stem^{-1})	HI (%)	WET (mm)	WUE (kg ha^{-1}mm^{-1})
2015-2016	CK	42.73a†	610.33a	43.93a	11454.3a	3.13a	42.17a	613.01a	18.69a
	L80	40.88b	554.19b	42.300b	9583.85b	2.63b	44.38a	518.26b	18.49a
	L60	26.44c	481.67c	34.40e	4761.52c	1.90c	35.13b	479.60c	9.94b
	L40	25.24d	373.92d	39.26d	3705.54d	1.58cd	35.05b	462.74d	8.01c
	L20	24.87d	292.35e	40.31c	2955.17e	1.46d	33.69b	459.91e	6.43d
2016-2017	CK	49.56a	591.16a	45.78a	13412.2a	3.96a	46.50a	644.20 a	20.83a
	L80	46.22b	536.87b	44.55b	11052.7b	3.67b	48.75a	544.37 b	20.30a
	L60	30.21c	466.68c	38.97d	5495.57c	2.52c	38.75b	475.28c	11.57b
	L40	29.14d	360.69d	39.45c	4146.11d	2.10d	37.75bc	444.67d	9.32c
	L20	27.33d	285.63e	38.65d	3016.16e	1.89e	35.00c	438.02d	6.89d
	L	2538***	3029.0***	611.1***	7367***	348.53***	52.4***	607.9***	2274.45***
	Y	665.9***	51.32***	64.01***	460.1***	306.45***	23.21***	0.92	163.22***
	L×Y	18.6***	0.69	46.44***	63.00***	7.72***	0.69	16.56***	6.16***

†Data followed by the same letter in a column indicate a nonsignificant difference among the treatments according to the LSD test ($P = 0.05$)
CK refers to the 'no shading' treatment (control), L80, L60, L40 and L20, refer to 80%, 60%, 40% and 20% of the incident solar radiation, respectively. Data are means of four replicates. L, light control; Y, year. *, significance at the .05 level; **, significance at the .01 level; ***, significance at the .001 level

There were significant differences in the wheat GY, KNPS, spike number and 1000-grain weight among the treatments, and the yield decreased gradually with the increase in the shading degree. Compared with the CK, the yields of the L80, L60, L40, and L20 treatments decreased by 16.96%, 58.73%, 68.37% and 75.86%, respectively. The yield decreased by more than 50% when the shading degree exceeded 40%. The KNPS and spike number decreased with the increase in the shading degree. The 1000-grain weight decreased first and then increased with the increase in the shading degree, and the minimum value was observed in the L60 treatment.

Dry matter accumulation (DMA) and harvest index (HI) were affected ($p < 0.001$) by both light control and year. The DMA decreased gradually with the increase in the shading degree. There were significant differences for the DMA among the CK, L80 and L60 treatments but no significant difference between the L40 and L20 treatments from 2015 to 2016. With the increase in the shading degree, the HI had increased first and then decreased (*Table 5*). The largest HI was 44.38% in L80 treatment.

The whole growth period ET (WET) and water use efficiency (WUE) were affected ($P < 0.001$) by light control (*Table 5*). The WET and WUE were significant interactions ($p < 0.001$) of light control and year. However, WET was not affected ($P > 0.05$) by year. The WET decreased gradually with the increase in the shading degree. Except for the L40 and L20 treatments in 2017, there were significant differences among the other

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DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

treatments. With the increase in the shading degree, the WET of the L80, L60, L40 and L20 treatments decreased by 15.48%, 24.00%, 27.74% and 28.49%, respectively, relative to the CK, and the reduction in the WET between the treatments was not obvious when the light was shaded to less than 40% of the full radiation.

The WUE decreased gradually with the increase in the shading degree, and the difference of the WUE was not significant between the CK and L20 treatment, but the WUE differed significantly among the other treatments. The WUE with shading over 40% of the full radiation decreased greatly. Compared with the CK treatment, the WUE of the L80, L60, L40 and L20 treatments decreased by 1.80%, 45.64%, 56.20%, and 66.26%, respectively.

Figure 7 shows the fitting curve and equation of the yield, WET and water use efficiency with the natural light transmission degree (NLTD). The GY, WET and WUE all had a close-fitting relationship with the NLTD. The relationship between the yield and NLTD is the "S" curve (*Fig. 7a*). When the NLTD was less than 60%, there was a relatively flat period. The yield increased rapidly when the NLTD was greater than 0.6 and slowed when the transmittance was more than 0.8.

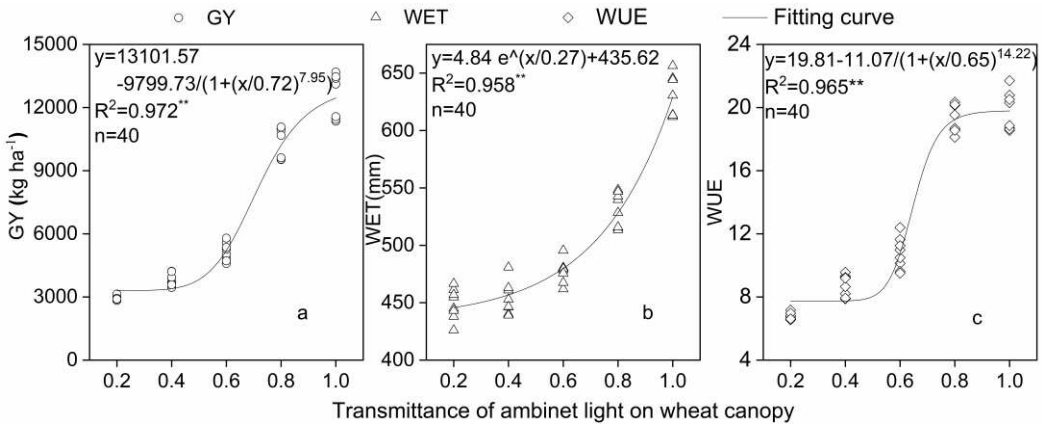

Figure 7. *Regression analyses of the grain yield (GY, a), whole evapotranspiration (WET, b) and water use efficiency (WUE, c) with the transmittance of ambient light on the wheat canopy.*
***Significance at the 1% (P = 0.01) level*

The relationship between the WET and NLTD was fitted using an exponential function (*Fig. 7b*). The growth rate of the WET increased in parallel with the increase in the NLTD. The WET increased slowly with the NLTD of 0.6 and increased approximately linearly with an NLTD of more than 0.8. The relationship between the WUE and NLTD was fitted by the "S" curve (*Fig. 7c*). When the NLTD was less than 0.6, the WUE increased very slowly, and then the WUE increased rapidly in parallel with the NLTD from 0.6 to 0.8. Finally, when the NLTD was more than 0.8, the WUE also increased very slowly.

Discussion

Experiment pots near to each other (25 cm) reduced the shading effect on temperature and RH (*Table 1*). Compared with the CK, the average temperature decreased by 1, 1.6, 1.6, and 1.7 °C under the L80, L60, L40 and L20 treatment,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

respectively. While, the average RH increased by 1.3, 1.9, 2.6 and 2.9% respectively. Therefore, solar radiation became the main factor in determining winter wheat growth and water consumption.

Shading decreased light intensity. Winter wheat photosynthesis time was prolonged to get enough solar radiation to complete winter wheat vegetative and reproductive growth (Yu and Wang, 2010). Phenological stages delayed and growth period prolonged by shading. The difference in phenological stages between treatments gradually increased and got stable after the booting period. Since the radiation amount before the booting period was relatively small, the shading had a greater influence on crop growth. And the later phenological stages radiation amount increased to accumulate enough crop substances (*Fig. 1a* and *d*). With the growth of wheat, the temperature and the radiation increased gradually (*Fig. 1a, b, d* and *e*). The increase in temperature and radiation causes an increase in crop water consumption (Miralles et al., 2011; Sorrentino et al., 1997). Compared with CK treatment, days of mature period under shading treatments condition delayed to get a higher temperature and more energy, it caused ET to increase. As, ET_{gs}, ET_{dm}, ET_{ts} increased with shading degree increased in *Figures 3, 4, 5e,* and *6e*. The extension of growth time was prolonged and the shading treatment was mainly prolonged at the early stages of winter wheat (*Table 2*). To reduce days prolonged by shading, shading should after the booting stage.

With the growth of wheat, solar radiation and temperature was gradually increasing. The ET_{gs}, ET_{dm}, ET_{ts} and WET (except for the mature period) gradually decreased as the shading degree increased (*Figs. 3, 4, 5,* and *6*.), and the difference was significant. The larger ET_{gs}, ET_{dm} and ET_{ts} were concentrated from the booting to the filling stages. The ET_{gs} indicated water consumption intensity. Under supplemental irrigation showed winter wheat water demand law. Booting stage to filling stage is also a critical period of water requirement for wheat (Zhang et al., 2011; Zheng et al., 2014; Zhou et al., 2018). Irrigating during this period can increase the weight of 1000 kernels, crop yield and water use efficiency (Dandan et al., 2013; Zhou et al., 2018). Shading from booting to the filling stages can reduce more ET, while short time shading after booting also has less effect on the growth period.

Winter wheat ET of different growing stages reflects the distribution of water consumption, and ET in typical sunny weather reflects the water consumption potential. Shading degree affects the efficiency of ET in winter wheat, which better reflects the water-saving efficiency of shading (*Table 6*). Water-saving rates higher than shading rates were observed in treatment L80 from the heading stage to the filling stage in the growing period, and from the booting stage to the filling stage under typical sunny weather conditions. The reduction of radiation during rainy days diminished the shade water-saving efficiency. The increase in shading degrees under other treatments also led to a decrease in water-saving efficiency.

Solar radiation has a greater impact on wheat ET. The ET increased rapidly at daybreak and decreased rapidly at sunset (*Fig. 5*). ET_d accounted for more than 80% of the ET_{ts} (*Fig. 6*), while ET_n in each growth period had little change. Because light is a signal for stomatal movement in the absence of water stress, and stomatal regulation is the most important way for plants to adjust their transpiration rate (Yu and Wang, 2010). In day time 6 h of ET accounted for more than 70% of the ET_d. The peak photosynthetic rate of the wheat was from 10:00 am to 12:00 pm (Mu et al., 2009). To improve the water use efficiency, 11:00 to 17:00 can be selected as the daytime light control period by shading.

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DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

Table 6. *Shading effect on winter wheat water reduced rate (%) of growing period and typical sunny day under the different treatments*

	Treatment	Wintering period	Green stage	Jointing stage	Booting stage	Heading to flowering	Filling stage	Mature period
Growing period	L80	3.05	12.63	14.80	10.53	21.39	23.20	-8.79
	L60	9.38	22.90	40.08	14.81	32.89	30.69	-13.62
	L40	15.31	32.98	44.34	26.33	35.47	38.29	-55.06
	l20	18.53	36.40	43.04	30.13	37.62	39.35	-65.66
Typical sunny day	L80	3.95	14.64	16.28	20.41	23.98	22.34	8.41
	L60	10.16	24.09	32.11	32.00	34.14	31.09	-11.92
	L40	17.11	36.30	40.99	44.67	41.36	43.02	-47.03
	l20	15.89	39.92	71.47	47.95	43.18	47.77	-40.00

With an increase in the shading degree, the GY indices gradually decreased, while the 1000-grain weight of the L40 and L20 treatments increased. Compared with the L60 treatment, the decrease in the kernel number per spike was primarily caused by the amount of decrease of the small grains for the L40 and L20 treatments. The yield of the L80 treatment decreased significantly compared with the CK, which is different from the results reported by Mu et al. (2009). This difference may be due to sufficient irrigation in this experiment since there was a larger GY for the CK treatment (Mu et al., 2010). The yield gradually increased with a decrease in the shading degree (*Fig. 7a*), and the yield change rate was different in each treatment.

Shading significantly reduced the GY of L80 treatment compare to CK treatment, but the WUE was not obvious, and there was little difference. The reduction in water consumption weakened the reduction in GY. The increase in HI also promoted the increase in WUE of L80 treatment. The increase in the harvest index of the L80 treatment indicated that the shading could promote the conversion of photosynthates to yield (Zhang et al., 2016). The ET decreased significantly, but the decrease in the WUE was not significant when the shading degree increased. According to Xu et al. (2016), when the shading degree was 12%, the yield increased, indicating that a higher WUE could be achieved under slight shading. In the areas with water shortages and greater light intensities, the crop yield will decrease under inadequate irrigation, and the WUE will be further increased under shading. In terms of the comprehensive yield, ET and WUE, the shading degree should be between 0 and 0.2.

The existing research on the effect of light on crops, crop yield, or the water consumption of fruit trees is based on a certain shading degree of the local natural solar radiation. The target SRI is not quantitative. With quantitative SRI, excessive radiation can be shaded, and rainy-day radiation will be increased to obtain a high yield of grain. However, crop species, growing stages, and geographical locations differ. The saturated light intensity in the different growing stages can be used as a reference for quantitative analysis.

Conclusions

The shading delayed the growth period of the wheat, and 80% of the full radiation increased the transformation of the photosynthates to the yield of grain. With the increase in the shading degree, the ET of the wheat gradually decreased. The ET was concentrated from the jointing to the filling stages. The daily ET was greater from the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

booting to the filling stages with the peak time from 12:00 to 14:00. When the solar radiation was less than 80%, the GY decreased significantly with a similar WUE. To ensure a consistent yield of grain and improve the effect of shading on WUE, shading time and degree should be reduced. Shading time should be from the booting stage to the filling stage, and shading degrees should not more than 20% of natural solar radiation.

Acknowledgments. The project was supported by the National Natural Science Foundation of China (No. 51609197), CAS "Light of West China" Program (No. XAB2016AW06), Programme of Introducing Talents of Discipline to Universities (No. 104-451115012), and Scientific Research Program Funded by Shaanxi Provincial Education Department (No. 16JS084).

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DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77397755

INSECTICIDE RESISTANCE IN *Bemisia tabaci* (GENN.) POPULATIONS COLLECTED FROM THE MEDITERRANEAN AND AEGEAN REGIONS OF TURKEY

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(Received 7th Jul 2020; accepted 17th Sep 2020)

Abstract. In this study, the resistance status to imidacloprid, lambda-cyhalothrin and endosulfan of twenty *Bemisia tabaci* (Genn.) populations collected in Hatay, Adana, Mersin, Antalya, Muğla, Aydın and Denizli provinces in the Mediterranean and Aegean regions of Turkey in 2005-2006 were investigated. The lethal concentration (LC) values for each population were determined by the leaf dipping method. The resistance ratios were calculated by dividing the LC_{50} values of the populations by the LC_{50} values of a susceptible population. The LC_{50} values of the *B. tabaci* populations to imidacloprid, lambda-cyhalothrin and endosulfan were found to be in the ranges of 0.1-8.5, 0.7-232.9 and 0.9-21.4 mg ai (=active ingredients)/l, respectively. The imidacloprid, lambda-cyhalothrin and endosulfan resistance ratios were 1-95, 1-439 and 4-102 times, respectively. Generally, the resistance ratio against the lambda-cyhalothrin were higher than against imidacloprid and endosulfan. In addition, in the Kumluca and Demre *B. tabaci* populations from greenhouses, resistance to the 3 insecticides was found to be between medium and high levels. Based on the results, we determined that a significant proportion of the *B. tabaci* populations in the Mediterranean and Aegean regions are resistant to the active substances in neonicotinoids, pyrethroids and cyclodiene organochlorines.
Keywords: *cotton whitefly, resistance management, imidacloprid, lambda-cyhalothrin, endosulfan*

Introduction

The development of insecticide resistance in pest populations has become one of the main problems in agricultural production in recent years. Resistance has been recorded in 597 arthropod species for 336 active substances and in 14,644 cases from 1914 to 2015 (IRAC, 2016). However, in agriculture both worldwide and in Turkey, the usage of insecticides in significant quantities is apparent in the statistical data. The annual pesticide usage amount in the world was 2,285,881 tonnes in 1990, and by 2017, it had increased to 4,113,591 tonnes (FAO, 2020). The pesticide usage amount in Turkey in 1990 was 29,918 tonnes, and it had increased to 54,098 tonnes by 2017 (FAO, 2020). When only considering the amount of insecticides used, the USA is in the first place in the world, with 86,726 tons, and Turkey is ranked seventh, with 12,435 tonnes. In Turkey, the five provinces that use the most pesticides and their usage rates are as follows: Antalya (10.1%), Manisa (9%), Adana (9%), Mersin (5.7%) and Aydın (5.7%) (Anonymous, 2020). To sum it up, the Mediterranean and Aegean regions are the regions with the most intensive agricultural insecticide use in Turkey. Therefore, greenhouse and field

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

populations in these regions are constantly exposed to high selection pressure due to frequent insecticide use.

The cotton whitefly *Bemisia tabaci* is among the main species for which chemical control is insufficient due to insecticide resistance. *B. tabaci* is one of the most significant agricultural pests and is common worldwide, including Turkey (EPPO, 2020). The adult and larval stages of the pest cause serious damage by sucking plant sap. It decreases the quality and market value of the product by causing sooty mould. It is also a vector of certain important viruses that cause serious economic losses in agricultural production, such as tomato yellow leaf curl virus (TYLCV). It is a polyphagous pest, and its number of hosts is very high. It causes serious economic losses by feeding on many industrial plants, ornamental plants, vegetables, especially tomato, eggplant, pepper, cucumber, etc., and cotton (Göçmen and Özgür, 1990; Hoddle, 1999; Oliveira et al., 2001). In Turkey and other countries whitefly management is still based largely on insecticides. However, insecticide resistance is a major problem in *B. tabaci* populations worldwide (Wool and Greenberg, 1990; Dittrich et al., 1990; Cahill et al., 1996; Elebert and Nauen, 2000; Palumbo et al., 2001; Kranthi et al., 2002; Ahmad et al., 2002; Nauen et al., 2002; El Kady and Devine, 2003; Javed et al., 2003; Horowitz et al., 2004, 2005; Roditakis et al., 2005; Nauen and Denholm, 2005; Erdoğan et al., 2008; Basij et al., 2010; Gorman et al., 2010; Ma et al., 2010; Schuster et al., 2010; Wang et al., 2010; Ünal Bahşi et al.,2012; Şahin and İkten, 2017; Ahmad and Akhtar, 2018; Satar et al., 2018; Bielza et al., 2019). Resistance to 56 different active substances has been reported in *B. tabaci* populations (APRD, 2020). Among these active ingredients, the best known are imidacloprid, acetamiprid, thiamethoxam (neonicotinoids); cypermethrin, deltamethrin, (pyrethroids); chlorpyrifos, malathion (organophosphates); methomyl, carbosulfan (carbamates); pyriproxyfen (juvenile hormone mimics); spiromesifen, spirotetramat (tetronic and tetramic acid derivatives); and endosulfan (cyclodiene organochlorines) (APRD, 2020).

It has been reported that insecticides should be used within the scope of "resistance management programmes", and the frequency of insecticide application should be reduced to combat the insecticide resistance problem (Soderlund and Bloomquist, 1990). Resistance screening studies reveal populations' resistance to insecticides, and routine monitoring is essential for insecticide resistance management (Croft, 1990).

In this study, twenty *B. tabaci* populations were collected in Hatay, Adana, Mersin, Antalya, Muğla, Aydın and Denizli provinces in the Mediterranean and Aegean regions of Turkey. The resistance status of these populations to imidacloprid (neonicotinoids), lambda-cyhalothrin (pyrethroids) and endosulfan (cyclodiene organochlorines) was investigated. The findings obtained in this study on *B. tabaci* provide important information for comparison to past records or for recording the initial phase of insecticide resistance in populations from Turkey. The data presented here can be a valuable resource for researchers on this subject.

Materials and Methods

Populations

Twenty population of *Bemisia tabaci* were collected from south-west region of Turkey. These sampling locations were indicated on the map in *Figure 1*.

Furthermore, the location, host and collection dates of *B. tabaci* populations tested within the scope of the study are given in *Table 1*.

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DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

Figure 1. *Sampling locations of the Bemisia tabaci populations were indicated on the map*

Table 1. *Sampling locations, host plants and biotypes of the Bemisia tabaci (Dağlı et al., 2020)*

Location	Host	Sampling Date	Biotype
HATAY			
Kırıkhan-(Hassa)	Cucumber	03.09.2005	Qe
Samandağ	Eggplant	03.09.2005	B
ADANA			
Mihmandar	Cotton and soybean	02.09.2005	Qe
Mustafabeyli	Cucumber	02.09.2005	Qe
MERSİN (İÇEL)			
Mahmutağa (Tarsus)	Eggplant	04.09.2005	B
Gümüşkum	Eggplant	04.09.2005	B
Ovacık (Silifke)	Eggplant	04.09.2005	B
Melleç (Anamur)	Nightshade	01.09.2005	B
ANTALYA			
Yurtpınar (Aksu)	Eggplant	30.09.2005	B
Kampus (Uncalı)	Cotton	28.11.2005	B
Konaklı (Alanya)	Eggplant	30.09.2005	B
Çobanlı (Gazipaşa)	Cucumber	30.09.2005	B
Demirtaş (Alanya)	Eggplant	30.09.2005	B
Kumluca	Pepper	05.07.2006	Qe
Demre	Pepper	05.07.2006	Qe
MUĞLA			
Kınık	Cotton	17.08.2006	B
Fethiye	Eggplant	18.08.2006	B
Ortaca	Cucumber	18.08.2006	B
AYDIN			
Koçarlı	Cotton	18.08.2006	Qw
DENİZLİ			
Beylerbeyi	Cotton	19.08.2006	Qw

Insecticides

Detailed information about the active substances used in the study is given in *Table 2*. The neonicotinoid imidacloprid and the pyrethroid lambda-cyhalothrin are still registered in Turkey. However, the use of the cyclodiene organochlorine endosulfan was banned in Turkey.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

Table 2. *Active ingredients and commercial names for insecticides and their mode of action*

Active substance	**Commercial name and formulations**	**Chemical classification and mode of action (IRAC, 2020)**	**Recommended label dose** (mg ai)/l
imidacloprid	Confidor SC 350, Bayer	Neonicotinoids, 4A, Nicotinic acetylcholine receptor (nAChR) competitive modulators	350 mg ai/l
lambda-cyhalothrin	KarateZeon CS, Syngenta	Pyrethroids, 3A, Sodium channel modulators	25 mg ai /l
endosulfan	Hektionex 36 EC, Hektaş	Cyclodien organochlorine, 2A, GABA-gated chloride channel blockers	540 mg ai /l

Insect Rearing

Bemisia tabaci adults were collected with a mouth aspirator from the locations specified in *Table 1*. The adults were released on cotton and eggplant seedlings in 20 litre plastic containers covered with tulle to lay. Populations were kept in a climate-controlled room with a temperature of 26 ±2 °C and 16:8 (light:dark) hours per day.

Insecticide Bioassay

The leaf dipping method was used to determine the LC_{50} and LC_{90} values of the populations (Roditakis et al., 2005). Briefly, the method is as follows: To obtain a distribution of death between 0% and 100% in *B. tabaci* populations, 4-6 different doses of these insecticides were prepared in distilled water containing 0.2 g/l Triton X-100. Discs with a diameter of 48 mm were taken from insecticide-free cotton leaves. These leaf discs were immersed in distilled water containing insecticide concentrations or the control (only 0.2 g/l Triton X-100) for 5 seconds and allowed to dry for approximately 2 hours. These leaf discs were placed in Petri dishes containing 1.5% agar poured onto the base of the dish. Adult individuals were shaken directly into these Petri dishes, and each Petri dish was covered with a cotton cloth. Each dose was conducted with 3 replicates. Bioassays were performed on 3 days for imidacloprid and on 2 days for lambda-cyhalothrin and endosulfan.

Data Analysis

In the study, a *Bemisia tabaci* population was collected from a cotton field in Koçarlı where no pesticides were used for a long time to be used as a susceptible population. The probit analysis program POLO-PC was used to calculate the lethal dose values (LC_{50}, LC_{90}) and the related parameters of the populations, and the LC_{50} values of the populations were divided by LC_{50} values of a susceptible (Koçarlı) population to calculate the resistance ratio. Any two LC50 values were considered significantly different if their respective 95% confidence limits (CL) did not overlap.

Results

The lethal dose values (LC), slopes, resistance ratios and related parameters obtained from *B. tabaci* populations in the screening for imidacloprid, lambda-cyhalothrin and endosulfan resistance are given in *Tables 3*, *4* and *5*, respectively. The results showed that there were significant differences between the LC values of the populations. Considering the LC_{50} and LC_{90} values of all populations, Koçarlı (Aydın) was determined to be the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

most susceptible population. Insecticides stopped being used many years ago in cotton fields in which the Koçarlı population was collected. In this population, the LC_{50} values (0.2 and 0.09 mg ai/l) obtained for endosulfan and imidacloprid were lower than the LC_{50} values of the reference susceptible population (SUD-S) (1.65 and 0.36 mg ai/l) determined by Roditakis et al. (2005) for the same insecticides with the same method. Based on these data, Koçarlı was considered a susceptible population, and the LC_{50} values of the other field and greenhouse populations were divided by the LC_{50} values of the Koçarlı population to obtain their resistance ratios.

Table 3. *The resistance ratios to imidacloprid in Bemisia tabaci populations collected from Mediterranean and Aegean regions of Turkey*

Population	**n**	**slope±se**	**LC_{50} (mg ai/l)** 95% confidence limits	**LC_{90} (mg ai/l)** 95% confidence limits	**RR**
Koçarlı (Susceptible)	301	1.47±0.22	**0.09** 0.02-0.18	**0.65** 0.34-2.15	-
Mustafabeyli	188	0.92±0.14	**0.08** 0.01-0.30	**2.09** 0.55-34.02	0.9
Mahmutağa	332	1.00±0.14	**0.39** 0.07-0.99	**7.28** 2.80-50.56	4.3
Gümüşkum	186	1.76±0.39	**1.39** 0.58-2.55	**7.46** 3.93-24.09	15.4
Ovacık	351	0.71±0.09	**0.06** 0.00-0.21	**4.12** 1.15-58.43	0.7
Melleç	182	0.87±0.16	**0.10** 0.00-0.41	**3.07** 0.72-918.12	1.1
Yurtpınar	265	1.17±0.14	**0.24** 0.08-0.59	**2.99** 1.10-20.97	2.7
Kampus	275	1.04±0.11	**0.57** 0.37-0.88	**9.92** 5.38-23.30	6.3
Konaklı	227	0.94±0.11	**1.11** 0.32-3.52	**25.74** 6.81-675.00	12.3
Çobanlı	154	1.45±0.32	**0.69** 0.23-1.28	**5.33** 2.95-14.53	7.7
Demirtaş	157	3.22±0.81	**8.51** 0.83-17.64	**21.29** 8.42-67.13	94.6
Kumluca	223	1.41±0.19	**2.22** 1.26-3.71	**17.95** 9.93-42.05	24.7
Demre	209	1.19±0.18	**1.61** 0.79-2.96	**19.18** 9.51-55.23	17.9
Kınık	326	1.46±0.18	**0.87** 0.49-1.45	**6.59** 3.56-18.23	9.7
Fethiye	430	1.41±0.17	**0.88** 0.38-1.91	**7.18** 3.08-33.46	9.8
Ortaca	410	1.23±0.13	**0.72** 0.14-2.30	**7.98** 2.46-129.53	8.0
Beylerbeyi	178	3.04±0.72	**0.10** 0.07-0.15	**0.26** 0.17-0.72	1.1

n: Total number of insects used in the experiment
Resistance Ratio (RR): LC_{50} values of the populations / the LC_{50} values of a susceptible population

Resistance to Imidacloprid

Imidacloprid, which has been registered in Turkey since 1994, is a systemic insecticide and is widely used. Within the scope of this research, 17 populations were assayed to imidacloprid (*Table 3*). The LC_{50} value range to imidacloprid in populations was found

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DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

to be 0.1-8.5 mg ai/l. The resistance ratios were between 1-95 times the resistance of the susceptible population. In the Demirtaş, Kumluca, Demre, Gümüşkum, Konaklı, Kınık and Fethiye populations, resistance between moderate and high levels was detected. In other populations, resistance was at a low level, below 10 times the resistance of the susceptible population.

Resistance to Lambda-cyhalothrin

Lambda-cyhalothrin, which is in the pyrethroid class, has been registered since 1988 in Turkey. It can be used alone as a single active ingredient or in mixture with buprofezin.

Fifteen populations were tested to lambda-cyhalothrin (*Table 4*). The LC_{50} values of the populations for lambda-cyhalothrin were found to be between 0.7-232.9 mg a.i./litre. The resistance ratios were between 1-439 times. In most populations taken from greenhouses, resistance to this insecticide was between medium and high levels. On the other hand, resistance was under 10 times the resistance of the susceptible population in the Kırıkhan, Samandağı, Mihmandar, Mahmutağa and Beylerbeyi populations (*Table 4*).

Table 4. *The resistance ratios to lamda-cyhalothrin in Bemisia tabaci populations collected from Mediterranean and Aegean regions of Turkey*

Population	**n**	**slope±se**	**LC_{50} (mg ai/l)** 95% confidence limits	**LC_{90} (mg ai/l)** 95% confidence limits	**RR**
Koçarlı (Susceptible)	171	1.56±0.42	**0.53** 0.19-1.04	**3.51** 1.65-21.82	**-**
Kırıkhan	174	1.72±0.39	**3.48** 0.63-7.33	**19.19** 9.02-134.84	**6.6**
Samandağ	78	1.19±0.35	**1.04** 0.16-2.78	**12.39** 4.47-145.95	**2.0**
Mihmandar	302	0.90±0.15	**1.05** 0.14-2.92	**27.72** 10.54-147.32	**2.0**
Mahmutağa	308	0.59±0.07	**1.19** 0.07-8.62	**195.88** 24.30-11606.28	**2.2**
Melleç	423	1.31±0.22	**12.39** 5.20-21.38	**118.44** 70.81-254.34	**23.4**
Yurtpınar	284	0.60±0.06	**40.91** 5.23-400.36	**5585.62** 526.10-2019588.89	**77.2**
Kampus	351	0.75±0.09	**33.19** 5.13-110.57	**1730.56** 497.56-14037.95	**62.6**
Çobanlı	370	2.02±0.59	**232.86** 21.97-447.62	**1005.07** 548.23-3301.06	**439.4**
Kumluca	231	0.95±0.13	**42.61** 11.26-128.30	**955.18** 279.24-11230.23	**80.4**
Demre	289	0.58±0.08	**140.77** 22.55-1200.94	**23715.74** 2196.34-41168269.34	**256.6**
Kınık	493	1.28±0.19	**43.92** 11.94-91.39	**442.41** 221.55-1307.91	**82.9**
Fethiye	315	1.11±0.15	**5.22** 2.52-11.27	**75.28** 30.69-284.94	**9.8**
Ortaca	595	1.36±0.13	**14.74** 8.00-28.86	**128.42** 57.35-538.80	**27.8**
Beylerbeyi	234	0.77±0.13	**0.73** 0.02-4.21	**32.98** 5.47-9980.88	**1.4**

n: Total number of insects used in the experiment
Resistance Ratio (RR): LC_{50} values of the populations / the LC_{50} values of a susceptible population

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

Resistance to Endosulfan

Endosulfan, which is in the cyclodiene class, has been used as a registered insecticide since 1954. It is one of the oldest insecticides used in Turkey and worldwide. Its use was banned in Turkey in 2010. In this study, 19 populations were assayed to endosulfan (*Table 5*). The LC_{50} value range of the populations for endosulfan was found to be 0.9-21.4 mg ai/l. The resistance ratios were between 4-102 times. Resistance was high in the Kumluca, Demre, Ortaca, Kınık and Fethiye populations. Resistance was at moderate levels in the Mahmutağa, Gümüşkum, Yurtpınar, Kampüs and Konaklı populations.

Resistance was low in the Beylerbeyi, Demirtaş, Kırıkhan-Hassa, Melleç, Mihmandar, Ovacık and Samandağ populations (*Table 5*).

Table 5. *The resistance ratios to endosulfan in Bemisia tabaci populations collected from Mediterranean and Aegean regions of Turkey*

Population	**n**	**slope±se**	**LC_{50} (mg ai/l)** 95% confidence limits	**LC_{90} (mg ai/l)** 95% confidence limits	**RR**
Koçarlı (Susceptible)	342	1.02±0.13	**0.21** 0.07-0.45	**3.89** 1.86-11.16	**-**
Kırıkhan	309	4.33±0.51	**1.83** 1.53-2.19	**3.62** 2.95-4.79	**8.7**
Samandağ	269	2.21±0.41	**1.37** 0.49-2.11	**5.19** 3.66-9.21	**6.5**
Mihmandar	313	1.85±0.22	**1.98** 1.26-2.95	**9.72** 5.87-23.73	**9.4**
Mustafabeyli	178	4.88±0.96	**2.25** 1.54-3.08	**4.13** 3.02-6.51	**10.7**
Mahmutağa	284	2.07±0.30	**3.80** 2.35-7.95	**15.80** 7.67-260.26	**18.1**
Gümüşkum	306	4.95±1.41	**3.51** 2.27-4.43	**6.38** 4.96-13.73	**16.7**
Ovacık	102	2.95±0.78	**1.50** 0.34-2.44	**4.08** 2.51-13.52	**7.1**
Melleç	240	2.72±0.38	**0.85** 0.57-1.21	**2.51** 1.66-5.63	**4.0**
Yurtpınar	254	1.49±0.22	**3.27** 1.00-7.54	**23.65** 9.65-322.10	**15.6**
Kampus	286	2.28±0.26	**3.66** 2.40-5.83	**13.36** 7.87-35.88	**17.4**
Konaklı	680	8.96±1.19	**2.68** 2.28-3.10	**3.73** 3.20-6.13	**12.8**
Demirtaş	590	1.77±0.20	**1.51** 0.80-2.31	**7.99** 4.67-27.20	**7.2**
Kumluca	123	2.67±0.47	**21.42** 14.18-34.27	**64.80** 39.43-153.35	**102.0**
Demre	345	1.05±0.12	**13.70** 3.77-34.69	**229.53** 78.74-2565.15	**65.2**
Kınık	284	1.05±0.12	**7.23** 4.41-11.10	**23.08** 14.32-62.74	**34.4**
Fethiye	526	2.75±0.41	**7.85** 5.78-10.91	**22.94** 15.33-49.88	**37.4**
Ortaca	586	2.94±0.36	**16.77** 10.41-23.72	**45.74** 32.46-71.89	**79.9**
Beylerbeyi	230	1.30±0.2	**1.08** 0.44-2.39	**10.43** 4.28-56.20	**5.1**

n: Total number of insects used in the experiment
Resistance Ratio (RR): LC_{50} values of the populations / the LC_{50} values of a susceptible population

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DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

Discussion

In general, resistance against lambda-cyhalothrin in the pyrethroid class in populations was higher than resistance to imidacloprid and endosulfan. In *B. tabaci* populations taken from greenhouses, such as the Kumluca and Demre populations, resistance to all 3 insecticides was higher than that in other field populations (*Tables 3-5*). The main reason for this situation is that the more frequent insecticide applications in greenhouses result in higher selection pressure on populations. Most greenhouses in Turkey are located in Antalya province. In terms of the rates of insecticide use, Antalya takes first place in Turkey, with a rate of 10.1%. These data show that greenhouse populations are exposed to more insecticides than other field populations in other regions. Whitefly populations continue to exist in greenhouses almost throughout the year. In addition, due to the virus vectors, insecticide application is carried out at very short intervals. As a result, the more frequent insecticide applications in greenhouses cause an increase in selection pressure, which provides an important advantage to resistance development. In addition, the fact that greenhouses are relatively closed areas can prevent susceptible individuals from entering from the outside, preventing a decrease in the resistance of the current population from occurring. On the other hand, low levels of resistance were generally found in the Eastern Mediterranean (Kırıkhan, Samandağ, Mihmandar, Mustafabeyli, Ovacık) and Western Aegean (Koçarlı, Beylerbeyi) populations. These populations are field populations, and they are exposed to lower insecticide applications than the greenhouse populations. Another reason why these populations are less resistant is that they are almost always in interaction with susceptible individuals in the natural vegetation around them, so some level of resistance dilution occurs.

In this study, it was determined that a significant number of the tested field and greenhouse populations were highly resistant compared to the susceptible population. The resistance levels in populations increased to 95, 439 and 102 times for imidacloprid, lambda-cyhalothrin and endosulfan, respectively. However, the extent to which the resistance ratios determined how impact insecticide practices should also be evaluated. To analyse to what extent resistance will negatively affect the success of insecticide applications in practice, the label-recommended doses of insecticides were compared with the LC_{90} dose values (cause 90% mortality in populations). The LC_{90} dose values of imidacloprid and endosulfan for all populations (for imidacloprid: 0.3-25.7; for endosulfan: 3.6-229.5 mg ai/l) were below the label doses of these insecticides (imidacloprid: 350 mg ai/l; endosulfan: 540 mg ai/l) (*Tables 3 and 5*). Accordingly, it can be expected that these two insecticides can still be successful in controlling *B. tabaci*. However, the detected high resistance indicates that there are significantly resistant individuals among the populations. The frequent use of these insecticides can lead to a rapid increase in the frequency of resistant individuals due to selection, and the LC_{90} values for the populations can easily exceed the label doses of these insecticides. In this case, the application of these insecticides will lead to control failure. For this reason, the frequency of imidacloprid and endosulfan applications in greenhouse locations with highly resistant populations must be limited. On the other hand, except for that in 2 populations, the LC_{90} dose value (12.4-23715.7 mg ai/l) obtained for lambda-cyhalothrin was well above the label dose of this insecticide (25 mg ai/l) (*Table 4*). Accordingly, when lambda-cyhalothrin is used alone, it will be ineffective against *B. tabaci* in almost all sampling locations. This active ingredient is also used in combination with the IGR class buprofezin. Using lambda-cyhalothrin this mixture can increase the chances of its success in practice. Endosulfan, one of the world's oldest insecticides, was banned years

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ago. Even if endosulfan was banned years ago, endosulfan-resistant populations may not disappear in a short time. These resistant populations can maintain their populations longer in places such as greenhouses where susceptible insect migration is limited. Therefore, these resistant populations should be considered in the implementation of any resistance management program.

Some research has been done on the insecticide resistance of *B. tabaci* populations in Turkey. Four *B. tabaci* populations were collected from Adana, Antalya, İzmir and Tarsus in 2000-2001, and the resistance levels of these populations to bifenthrin, fenpropathrin (pyrethroids), formothion, triazophos (organophosphates) and buprofezin (insect growth regulator) active substances were determined (Erdoğan et al., 2008). All of the populations showed significant resistance to active substances with pyrethroids (57-360 times) and organophosphates (20-310 times). However, only the İzmir population was found to be resistant to buprofezin (Erdoğan et al., 2008). The resistance status of *B. tabaci* populations collected from Antalya and its districts between 2007 and 2009 was investigated in terms of resistance to these three insecticides in the populations (Ünal Bahşi et al.,2012) and were found to be 6-299, 2-16 and 1-22 times, respectively. In addition, the recommended label doses of these 3 insecticides remained below the the population LC_{90} values. In short, these active substances will be ineffective for *B. tabaci* population control in the sampling locations (Ünal Bahşi et al.,2012). In another study, *B. tabaci* populations from five different locations in Antalya were collected in 2011, and the resistance status of these populations to acetamiprid and thiamethoxam was determined (Şahin and İkten, 2017). The resistance ranges to these insecticides in populations were found to be 4-30 times and 9-32 times the resistance of a susceptible population, respectively. In this study, as in the previous study, it was reported that the label doses of these 2 active substances were below the population LC_{90} values. Briefly, these active substances will also fail in *B. tabaci* control at the locations that were sampled (Şahin and İkten, 2017). In another study conducted in Turkey, in Mersin, Adana, Antalya and Hatay in the Mediterranean region, the resistance levels of the provincial *B. tabaci* populations to the neonicotinoid class active substances acetamiprid, imidacloprid, thiacloprid and thiamethoxam were investigated (Satar et al., 2018). It has been reported that most of the tested populations are highly resistant to these active substances. The highest resistance was 2060 times for imidacloprid in the Antalya (Kumluca) population, and the lowest resistance was 5.36 times for thiamethoxam in the Hatay (Samandağ) population (Satar et al., 2018).

Insecticide resistance in *B. tabaci* populations is a serious problem in Turkey and around the world. The United States, Israel, Greece, Spain, Iran, Pakistan, India, Morocco, Egypt and some other countries have reported resistance in *B. tabaci* to active substances from many classes (Wool and Greenberg, 1990; Dittrich et al., 1990; Cahill et al., 1996; Elebert and Nauen, 2000; Palumbo et al., 2001; Kranthi et al., 2002; Ahmad et al., 2002; Nauen et al., 2002; El Kady and Devine, 2003; Javed et al., 2003; Horowitz et al., 2004, 2005; Roditakis et al., 2005; Nauen and Denholm, 2005; Basij et al., 2010; Gorman et al., 2010; Ma et al., 2010; Schuster et al., 2010; Wang et al., 2010; Ahmad and Akhtar, 2018; Bielza et al., 2019). In the APRD (Arthropod Pesticide Resistance Database), it has been recorded that *B. tabaci* resists 56 different active substances. The best known of these active ingredients are imidacloprid, acetamiprid, thiamethoxam (neonicotinoids); cypermethrin, deltamethrin, (pyrethroids); chlorpyrifos, malathion (organophosphates); methomyl, carbosulfan (carbamates); pyriproxyfen (juvenile hormone mimics); spiromesifen, spirotetramat (tetronic and tetramic acid derivatives) and

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DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

endosulfan (cyclodiene organochlorine) (APRD, 2020). Greece is one of the geographically closest countries to Turkey and has also carried out a similar survey in the same time period (Roditakis et al., 2005). In this study, high levels of resistance were found in *B. tabaci* greenhouse populations from Greece to neonicotinoid, cyclodiene, pyrethroid and organophosphate insecticides. Resistance levels for bifenthrin, α-cypermethrin, pirimiphos-methyl, endosulfan and imidacloprid in greenhouse populations have been reported to reach 23, 80, 18, 58 and 730 times, respectively. However, a *B. tabaci* population taken from open melon fields was found to be more sensitive than the referenced susceptible population (SUD-S) (Roditakis et al., 2005). The results of this research are largely in line with our findings. It was also determined in our study that susceptible populations may be present in areas where insecticides are used little or not at all. On the other hand, insecticide resistance in greenhouse populations where insecticides were used frequently was generally detected at high levels.

Conclusion

According to the results of this study, most of the *B. tabaci* populations from the Mediterranean and Aegean regions of Turkey had significant levels of resistance to imidacloprid, lambda-cyhalothrin and endosulfan. In other studies, *B. tabaci* populations from Turkey were noted to be resistant to other active substances. Worldwide resistance screening of *B. tabaci* shows that resistance is a common problem at the global level and is not limited to Turkish populations. All of these results indicate that chemical management may be inadequate against *B. tabaci* populations due to their insecticide resistance. Unfortunately, this situation is not limited to only *B. tabaci*. The same problem has been observed in other important pests, such as *Frankliniella occidentalis* and *Tetranychus urticae*. In summary, it should be taken into consideration that chemical management alone against pests is currently insufficient. More attention should be paid to other options, such as biological control and cultural measures. To reduce the selection pressure on pests, reducing the pesticide application frequency or rotating insecticides with different action mechanisms is necessary. The resistance levels of populations should be routinely monitored, and chemical management should be performed within the scope of resistance management programmes that include preventive tactics.

Acknowledgement. We thank the TUBITAK for their contribution to the TOVAG-104O312 project.

Conflict of interests. The authors declare that they have no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77577768

DROUGHT RESPONSE OF YOUNG PEAR TREES (*PYRUS COMMINUS)*

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(Received 9th Jul 2020; accepted 17th Sep 2020)

Abstract. This experiment was conducted at Fruit Research Institute, MAREM, Eğirdir-Isparta, Turkey to detect the effects of drought stress on young pear trees in 2017. Deveci, Ankara and Margarita pear varieties grafted onto OHxF 333 rootstocks were used. The young pear trees were one year old and they were planted into 18-litre pots with 1:1:0.5 ratio of soil:peat:manure. During the experiment, three different water stress level were applied to all trees D_0: the soil was fully irrigated to reach field capacity in each irrigation, full irrigation, no stress; D_1: 50% of D_0, moderate stress; D_2: 25% of D_0, severe stress. Drought stress treatments were applied from July 3 to September 11 2017. Photosynthesis (P_n) and stomatal conductance (g_{sw}) measurements were performed three times after starting drought stress applications. Trunk diameter and leaf area were measured as vegetative development. SPAD measurements were also measured three times during experiment. All pear varieties were affected by drought stress but Deveci variety was determined to be a variety more resistant against to drought stress according to Ankara and Margarita varieties. Ankara and Margarita varieties had similar response to drought stress.
Keywords: *water stress, gas exchange, stomatal conductance, vegetative parameters*

Introduction

Available water resources not only for agricultural production but also the other sectors have been decreasing day by day. Therefore, we should be more careful when we use water. Because the most using rate of water is used in agricultural production (FAO, 2011), the studies including water stress is getting be more important. Scarcity of water is a severe environmental constraint to plant productivity (Farooq et al., 2009).

Fruits are important for agricultural production and also for human health. Pear is a fruit commonly cultivated after apple. According to the production yield data of 2018, total pear production is 23.852.421 tons and Turkey is at fifth rank with annual pear production of 519.451 tons (FAO, 2020). In recently years, different rootstocks and new varieties have been starting to use in fruit growing. Pear growing has also different rootstocks having different growth vigor. Pear trees need water to grow and development. Therefore, they need to be studied water stress relationships between rootstocks and varieties. OHxF 333 is one of the rootstock used commonly in pear growing (Hepaksoy, 2019). So, we used this rootstock with different varieties in this study.

The one of the most important factors in ensuring the commercial sustainability in pear cultivation is water. In recent days, the countries having threat of drought such as Turkey, studies related with water stress have begun to gain momentum. Not only rootstocks used in fruit growing can affect response of fruit trees to drought stress, but also varieties can affect their responses. Therefore, in addition to rootstocks, researchers should study also relationships between varieties and drought stress.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77697781

The drought resistance of a plant is not only related with the area of root system (rootstock), but also the growth and development rate, the state of the stem and leaf structure, the stomatal conductivity, and the plant's activity status. However, plants cannot overcome drought only by the root development and suction power alterations resulting from the soil moisture conditions. The organs over scion area of plants (trunk, shoot etc.) also play an important role in providing drought resistance (Eriş, 2007).

It is aimed to determine the responses of Deveci, Ankara and Margarita pear varieties grafted onto OHxF 333 rootstock against to different levels of drought stress, in this study.

Materials and methods

Experimental area and plant material

The study was carried out in semi-open (non-heated) greenhouse on the experiment field at Fruit Research Institute (Eğirdir, Isparta-Turkey) in 2017. The young pear trees used in the study were one-year-old and Deveci (*Pyrus Comminus L.* "Deveci"), Ankara (*Pyrus Comminus L.* "Ankara") and Margarita (*Pyrus Comminus L.* "Margarita") varieties grafted onto OHxF 333 rootstock were used. The reasons for that they were selected for this study, Ankara and Deveci pear varieties are cultivated as high rate in Turkey (Özaydın and Özçelik, 2014; Sakaldaş and Gündoğdu, 2016). Margarita is a new variety in comparison with Ankara and Deveci. Trees were planted in pots in early April. We selected the pear trees having similar growth vigour before this experiment started. Except from the experiment subjects, five unplanted pots with mixed soil were put into use in order to determine the field capacity. They are placed into a greenhouse with clear plastic cover on the top, sides open in order to prevent the pots to be effected by the rainfall. The temperature and humidity values are recorded with Hobo data logger. Values of relative humidity and temperature of the green house during the study are given in *Figure 1*. Relative humidity values ranged between 35% and 78% but average relative humidity values were about 50%.

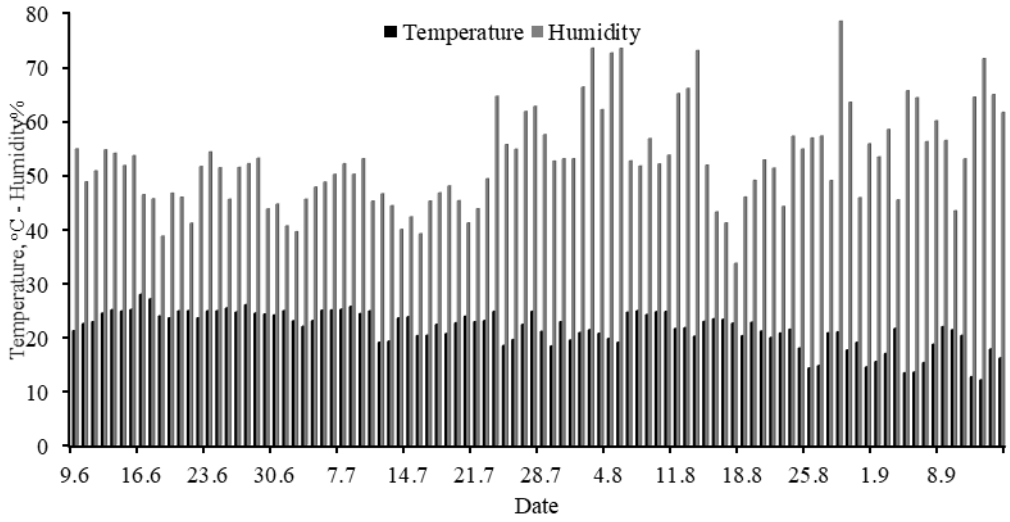

Figure 1. *Average daily temperature (black columns) and relative humidity (grey columns) inside of the greenhouse*

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The mixed soil in the pots and irrigation water

The mixed soil (18 liters) of soil:peat:manure (1:1:0.5 ratios) ~~of~~ was placed into 18 liters pots. Irrigation water (EC=0.3 dS m^{-1} and SAR=1.04) used for the trees was supplied from a well at Fruit Research Institute. Classification was realized according to the US Salinity Laboratory Graphical System. According to this system, the salinity values of the irrigation water, which are in 250-750 $ECx10^6$ range, are included in category C_2, and in category S_1 in terms of SAR value (USSL, 1954). Irrigation water was C_2S_1 class, which is suitable for irrigation.

Irrigation treatments

Before the study started, the mixed soil in the five pots without plant were saturated with water. Then the pots were covered with aluminum foil to prevent evaporation. After no leaking was observed from the pots, the pot weight was considered as field capacity. Irrigation water was applied to the potted trees every four days as long as the soil water reached to field capacity until July 3rd. The water stress treatments started on July 3rd when the temperatures were higher and finished on September 11. Water stress treatments lasted 70 days. There were three different irrigation treatments in the experiment. Treatments were; D_0 treatment: available soil water was reached to field capacity for each irrigation, 100% (control), D_1 treatment; irrigated 50% of water used in D_1 treatment (50% water deficit, moderate stress), D_2 treatment; irrigated 25% of water used in D_1 treatment (75% water deficit, severe stress). The field capacity value of the mixed soil in the pots is determined in order to figure out the irrigation water amount used in every irrigation treatment.

Before each treatment, the pots in the D_1 treatment were weighed, and the missing water was given to the pots by using a tape (with a 2-litre volume and 50 ml accuracy) to assure that the pots reach the field capacity. To calculate the amount of water used in the other subjects, the average water amount used in the first treatment was taken into consideration. The irrigation water that leaked into the base plate was added back into the pots.

Plant water consumption

Until programmed irrigations started (July 3rd), because irrigation water was applied to reach the available water up to field capacity in each irrigation, irrigation water amounts were taken into consideration as plant water consumption. After programmed irrigations started the plant water consumption was calculated for 10-day periods and *Equation (1)* was used.

$$ET_{10\ days} = T_1 + I - T_2 \qquad \text{(Eq.1)}$$

In Equality;

ET_{10days} = 10-day period plant water consumption (g),

T_1 = Previous weight value of pot (g),

I = The water amounts applied between two weight measurements (g),

T_2 = The weight value of pot at the last weighing (g).

The plant water consumption values calculated in weight is converted to volume and expressed as l / plant.

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Photosynthetic rate (Pn) and stomatal conductance (gsw)

Plant photosynthetic rate (Pn) and stomatal conductance (g_s) were measured by portable Li-Cor 6800 Photosynthesis System (LI-6800XT Portable Photosynthesis System, LI-COR, USA) in three times after starting water stress treatments (July 12nd, August 10th, September 3rd). One plant was selected from each replication and totally three different young pear trees were used for each treatment. The leaves were used from the sun-exposed mature leaves of one year old shoots from different sides of the selected trees in each treatment. At least 3 leaves per plant were sampled between 11:00-14:00 on the day before irrigations. The measurement conditions were leaf chamber PAR (photosynthetically active radiation), 1100 μmol m^{-2} s^{-1}; leaf to air vapor deficit pressure, 1.6–2.7 kPa and chamber CO_2 concentration 400 μmol mol^{-1}.

SPAD measurement

The leaf greenness of the young pear trees was determined by a portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan) in three times after initiation water stress treatments. The results were given as leaf chlorophyll values. SPAD measurements were made on the leaves having similar characteristics (Khan et al., 2003).

Trunk diameter and leaf area

Trunk diameter were measured in three times after initiation of water stress treatments by using digital caliper. Trunk diameter was measured on east-west and north-south at 15 cm upper level from grafted point and average of their values was calculated and considered as trunk diameter.

For leaf area measurements, three leaves were picked up from each tree with thirty leaves totally at the end of the water stress treatments (September 3) and the samples were taken from one year old shoot and fully developed leaves. Leaf areas were measured by digital planimeter (Koizumi KP-90 N) as cm^2.

Experimental design and statistical analyses

This study was designed according to Factorial Experimental Design at Randomized Plots with three replications. Each treatment had three replications and there were three plants in each replication. The analysis of variance (ANOVA) test for the data was conducted with JUMP software program and the differences among treatments were compared by means using LSD test.

Results and discussion

Plant water consumption (ET, evapotranspiration)

Table 1 shows ET (plant water consumption) values of the teratments. The highest ET was obtained from Deveci variety (46.9 liters) and the others had similar values (42.4 and 42.0 liters). Tree structers and growth vigour may cause these differences (Küçükyumuk et al., 2015a). According to decreasing rates, Ankara and Margarita varieties had higher ET decreasing rates (35.0% and 34.8%) than Deveci variety. *Figure 2* presents the 10-day ET graphs. Just after starting drought stress applications (July 3rd) ET values decreased for all varieties.

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Table 1. *ET Decreasing rates of treatments*

Variety/rootstock combination	D_0		D_1		D_2	
	ET (l/plant)	***Decreasing rates (%)***	**ET (l/plant)**	***Decrease rates (%)***	**ET (l/plant)**	***Decrease rates (%)***
Deveci/OHxF 333	46.9	0	30.5	13.2	23.2	30.9
Ankara/OHxF 333	42.4	0	27.3	15.4	20.3	35.0
Margarita/OHxF 333	42.0	0	26.9	17.7	21.0	34.8

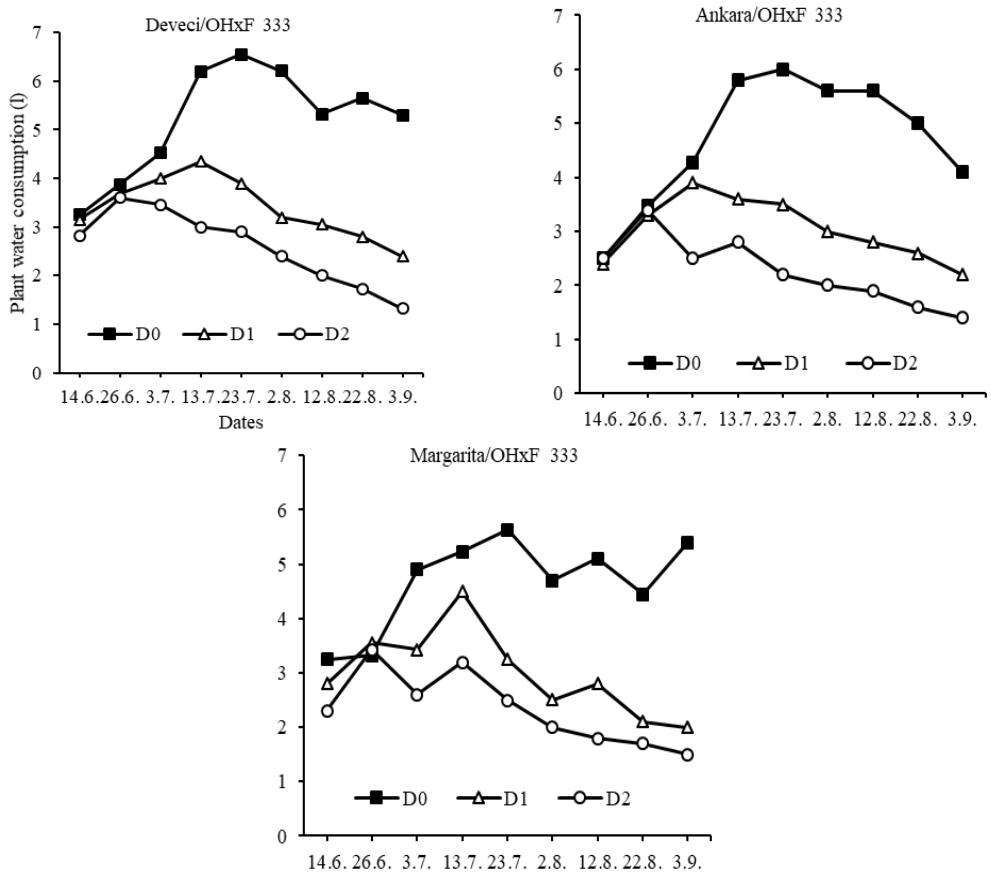

Figure 2. *Plant water consumptions of pear trees during growing period*

Photosynthetic rate

The last Pn measurements (September 3rd ~~8th~~) were made statistical analysis. There were no differences among varieties for trees in no-stress level (vertical column) (*Table 2*). It means that Pn values of different varieties were similar in no-water stress conditions but their responses to drought responses were different. While drought stress (water deficit) was increasing, each variety showed different response. Each drought level had different statistical class. Deveci variety had the highest value in all drought treatments. Margarita variety had the lowest values in drought treatments (4.50 and 1.40 μmol m^{-2} s^{-1} in D_1 and D_2, respectively. Ankara and Margarita were in similar group in D_2 treatment.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77697781

Table 2. *Photosynthetic rate (Pn) values of drought treatments (µmol m⁻² s⁻¹)*

Variety/rootstock combination	Drought treatments		
	D_0	**D_1**	**D_2**
Deveci/OHF-333	13.86 A**a^{ns} *(1.02)^{SD}*	7.05 Ba* *(0.31)*	3.00 Ca* *(0.17)*
Ankara/OHF-333	12.34 A**a *(0.89)*	5.26 Bab *(0.45)*	1.90 Cb *(0.49)*
Margarita/OHF-333	13.45 A**a *(0.21)*	4.50 Bb *(0.91)*	1.40 Cb *(0.18)*

Capital letters indicate drought stress treatments differences (horizontal); Small letters indicate variety, Differences (vertical). Means followed by the same letter are not significantly different, **p<0.01, *p<0.05, ns: no significance, SD: Standard deviation (in brackets)

Figure 3 shows fluctuations of Pn values after beginning drought stress applications. The Pn values were measured for three times in July 12nd, August 10th ~~16th~~ and September 3rd ~~8th~~. Pn values of no-stress treatments showed fluctuating during growing period but Pn values decreased towards the end of the season in drought stress treatments.

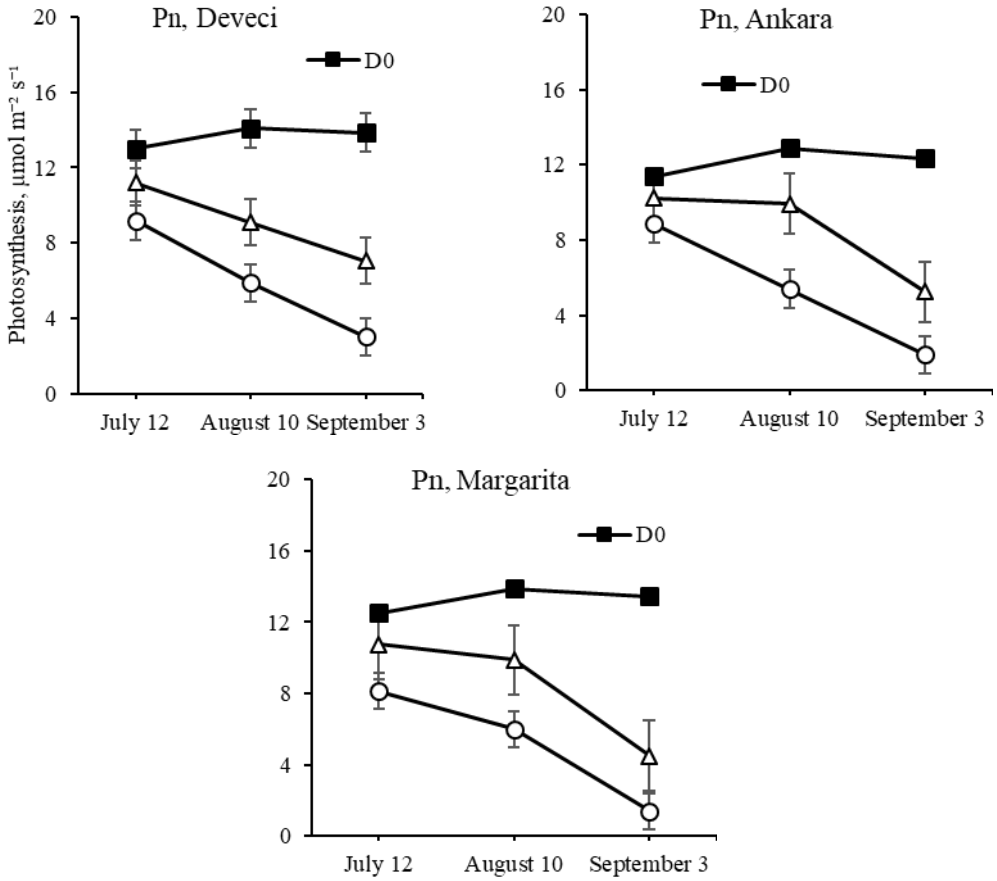

Figure 3. *Variation of photosynthetic rate measurements after beginning drought stress applications*

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Photosynthetic productivity is an important physiological parameter and it has been widely used to evaluate the plant growth vigor (Kalaji and Pietkiewicz, 2004; Kalaji et al., 2011). The photosynthetic rate (Pn) is one of these parameters (Pérez et al., 1997; Liu et al., 2012). The varieties which belongs to same species can shows different response to drought stress. Zarafshar et al. (2014) stated that drought period decreased Pn values. Tozzi et al. (2018) studied two different pear genotype (PremP009 and Hosui). They found that Pn values were different considered different varieties and PremP009 were higher than Housi.

Stomatal conductance

The last gsw measurements (September 3rd ~~8th~~) were made statistical analysis. When drought treatments were considered for each different variety (*Table 3*, horizontal line), it can be seen each treatment has different statistical group ($p<0.01$). It was obtained that the pear varieties used in this study had different responses against different drought levels. It can be said that each different drougth level had different effect on pear trees.

Table 3. *Stomatal conductance (gsw) values of drought treatments (mol m^{-2} s^{-1})*

Variety/rootstock combination	Drought treatments		
	D_0	**D_1**	**D_2**
Deveci/OHF-333	0.194 A**a^{ns} *(0.012)^{SD}*	0.106 Ba* *(0.006)*	0.069 Ca* *(0.004)*
Ankara/OHF-333	0.186 A**a *(0.009)*	0.076 Bb *(0.007)*	0.052 Cb *(0.002)*
Margarita/OHF-333	0.180 A**a *(0.008)*	0.075 Bb *(0.006)*	0.048 Cb *(0.004)*

Capital letters indicate drought stress treatments differences; Small letters indicate variety differences, Means followed by the same letter are not significantly different, **p<0.01, *p<0.05, ns: no significance, SD: Standard deviation (in brackets)

Deveci variety had the highest gsw for all treatments. While Deveci variety is at the first statistical group (0.106 and 0.069 for D_1 and D_2, respectively) another two varieties were in the similar group. It means that Ankara and Margarita varieties had similar responses according to gsw.

The gsw were measured three times during growing period (*Fig. 4*). While D_0 treatments showed fluctuations, D_1 and D_2 treatments decreased towards to end of the growing period.

The first adaptation mechanism in plants which are exposed to drought stress is the narrowing or closing of stomata in order to prevent water loss (Osakabe et al., 2014). When the soil moisture decreased in root zone, stomatal conductance decreased because stomas closed and there were limited gas exchange (Kalefetoğlu and Ekmekçi, 2005; Kocaçalışkan, 2005). Some researchers informed increasing stomatal conductance with increasing amount of irrigation water (Pouyafard, 2013; Lepaja et al., 2019). Zarafshar et al. (2014) reported that drought period decreased stomatal conductance.

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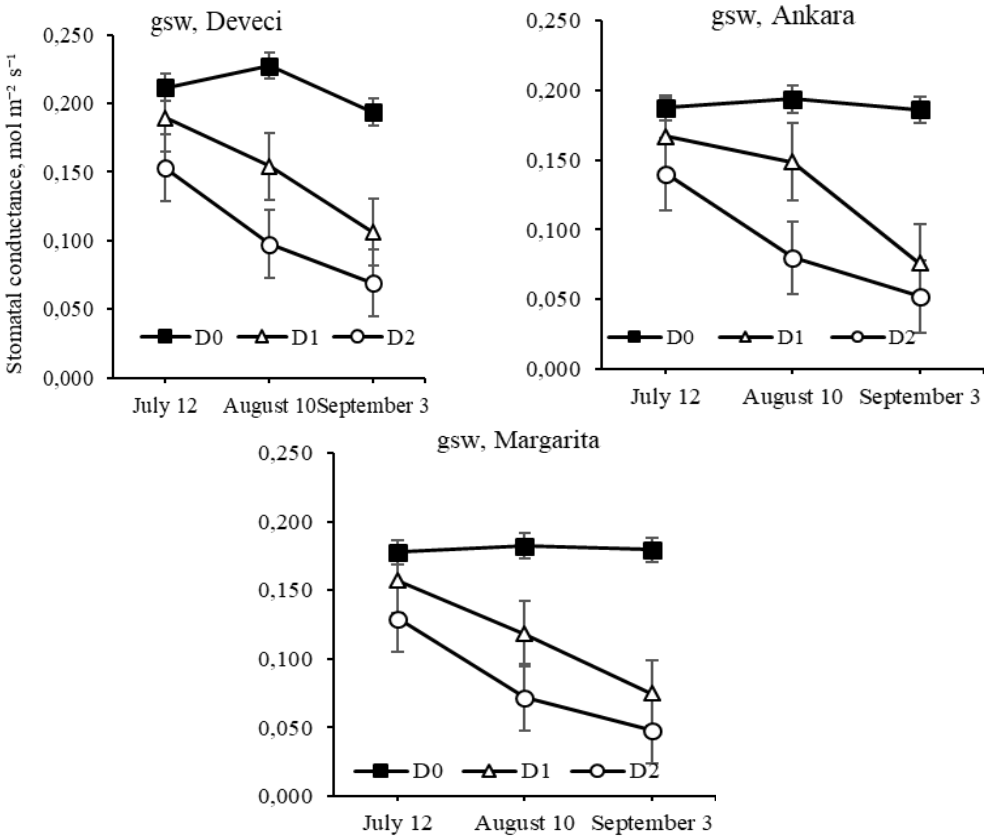

Figure 4. *Stomatal conductance (gsw) measurements after beginning drought stress applications*

SPAD

SPAD measurements were significant both for varieties and treatments ($p<0.01$) (*Table 4*). The last SPAD measurements (September 3rd ~~8th~~) were made statistical analysis. Water deficit was effective for all varieties except D_1 treatment (50% water deficit) of Deveci variety. The highest value in drought treatments were obtained in D_1 treatment of Deveci variety, Margarita had the lowest value (41.2). *Figure 5* shows SPAD variation after beginning drought stress applications. SPAD values in all D_0 treatments increased during growing period. Only D_1 treatment of Deveci variety increased like D_0 treatments.

Table 4. *SPAD values of drought treatments*

Variety/rootstock combination	**Drought treatments**		
	D_0	**D_1**	**D_2**
Deveci/OHF-333	65.4 A**a** *(2.6)^{SD}*	61.6 Aba** *(2.0)*	54.0 Ba** *(9.8)*
Ankara/OHF-333	58.0 A** b *(3.7)*	53.3 ABb *(4.1)*	48.6 Bab *(2.6)*
Margarita/OHF-333	52.6 A**c *(4.1)*	45.6 Bc *(2.3)*	41.2 Cb *(1.7)*

Capital letters indicate drought stress treatments differences; Small letters indicate variety differences, Means followed by the same letter are not significantly different,
**p<0.01, SD: Standard deviation (in brackets)

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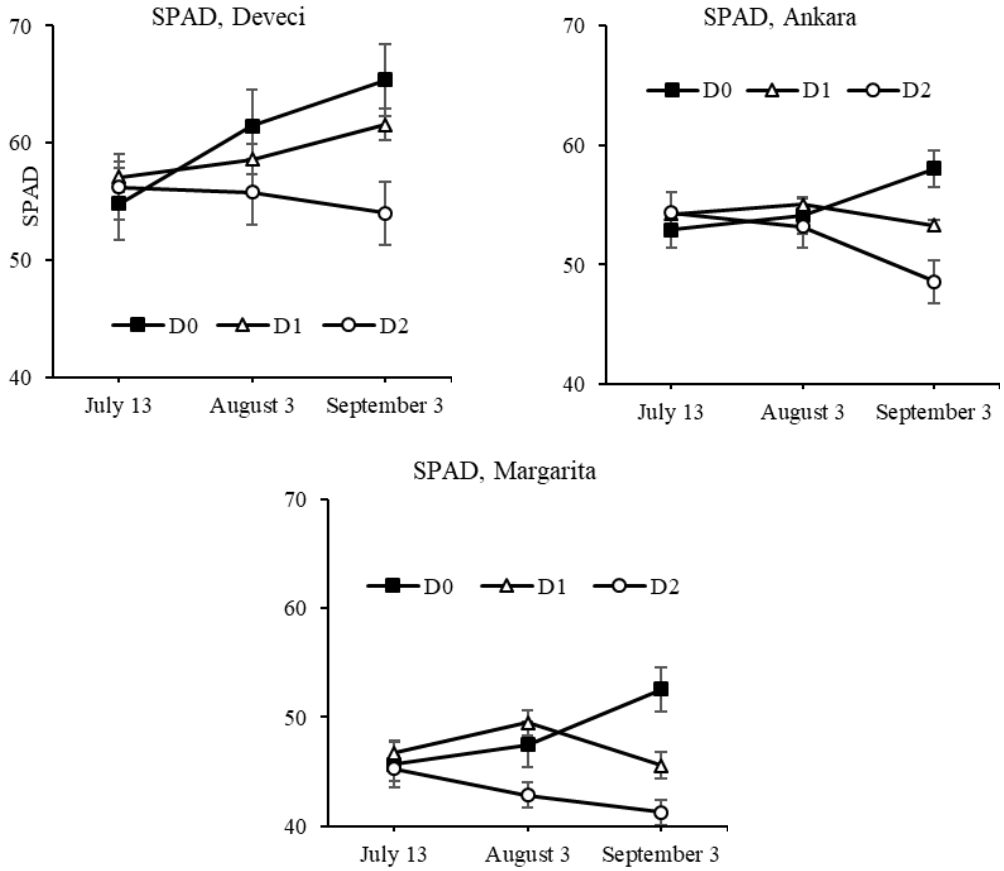

Figure 5. *Variation of SPAD values after beginning drought stress applications*

Effect of water stress decreases amounts of chlorophyll in plant and leaves and it also prevents chlorophyll occurring (Özer et al., 1997; Kırnak and Demirtaş, 2002; Tatari et al., 2019).

Leaf area

The leaf areas of all drought treatments were ~~e~~affected negatively (*Table 5*). D_1 and D_2 drought treatments had similar effects on Ankara and Margarita varieties. Leaf areas decreased between 16.3%-25.5 (*Table 6*). The highest decreasing rate was determined in ~~D~~D_2 treatment~~s~~ of Margarita variety with 25.5%. Leaf area development is one of the important indicator of water stress in plants (Küçükyumuk et al., 2015b). Fernandez et al. (1996) stated that water stress causes decreasing leaf area up to 50% in plants. In order to prevent water loosing from leaves, plants reduce their leaf areas (Kocaçalışkan, 2005). The aim of this is to adapt to drought conditions. Alizadeh et al. (2011) and Kamiloğlu et al. (2014) reported that drought stress had negative effects on leaf areas of fruit trees.

Trunk diameter

All the results were found to be significant according to statistical analysis ($p<0.05$ and $p<0.01$) in *Table 7*. Different water deficit rates affected trunk diameter and drought treatments had different effects on trunk diameter values. While D_1 treatment in Ankara

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variety didn't effect on trunk diameter, D_1 and D_2 treatments had similar effects on pear trees in other varieties. As seen in *Figure 6*, all the trunk diameter values increased except D_2 treatment of Ankara variety. Although water deficit affected pear trees, trunk diameter increased except Ankara variety. Increasing rates ranged from -2.6% (D_2-Ankara) to 21.5% (D_0-Deveci) in *Table 8*.

Table 5. *Leaf area values of the treatments (cm^2)*

Variety/rootstock combination	Drought treatments		
	D_0	**D_1**	**D_2**
Deveci/OHF-333	28.4 A*b *(3.0)^{SD}*	23.8 ABb *(5.1)*	21.4 Bb *(3.4)*
Ankara/OHF-333	27.1 A**b *(2.7)*	22.0 Bb *(1.9)*	20.7 Bb *(1.5)*
Margarita/OHF-333	36.2 A**a** *(4.9)*	30.3 Ba** *(2.4)*	27.0 Ba** *(1.4)*

Capital letters indicate drought stress treatments differences; Small letters indicate variety differences, Means followed by the same letter are not significantly different,
**p<0.01, *p<0.05, SD: Standard deviation (in brackets)

Table 6. *Decreasing rates of leaf areas*

Varieties	Treatments	September 3	Decreasing rates*
Deveci	D_0	28.38	0.0
	D_1	23.76	16.3
	D_2	21.40	24.6
Ankara	D_0	27.10	0.0
	D_1	22.02	18.7
	D_2	20.66	23.8
Margarita	D_0	36.22	0.0
	D_1	30.26	16.5
	D_2	27.00	25.5

*Each treatment was compared with D_0 treatment for same variety

Table 7. *Trunk diameter (cm) of the treatments (at the end of the study, September 11th)*

Variety/rootstock combination	Drought treatments		
	D_0	**D_1**	**D_2**
Deveci/OHF-333	13.7 A*b *(0.5)^{SD}*	11.7 Bb *(2.9)*	11.1 Bb *(1.0)*
Ankara/OHF-333	16.8 A*a *(1.4)*	16.0 Aba** *(1.1)*	15.0 Ba *(0.9*
Margarita/OHF-333	16.8 A*a** *(1.3)*	15.7 Ba *(0.8)*	15.5 Ba** *(1.1)*

Capital letters indicate drought stress treatments differences; Small letters indicate variety differences, Means followed by the same letter are not significantly different,
**p<0.01, *p<0.05, SD: Standard deviation (in brackets)

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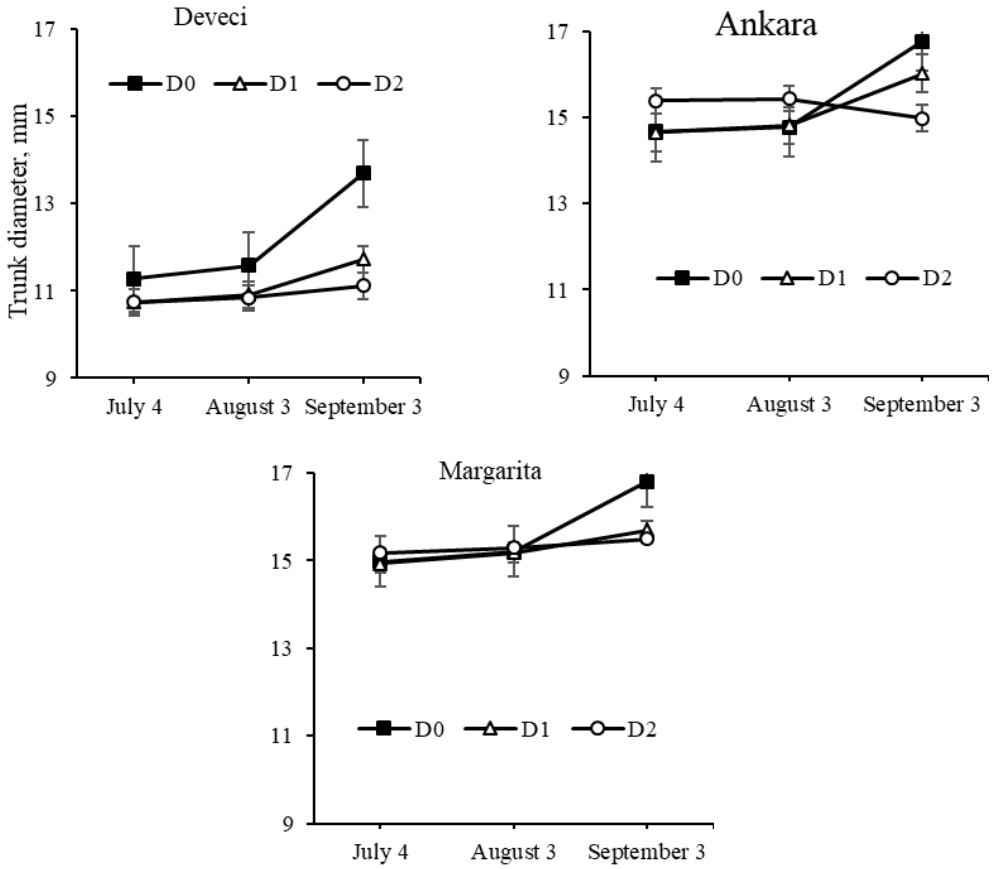

Figure 6. *Variation of trunk diameter values during growing period*

Table 8. *Increasing rates of trunk diameter values*

Varieties	Treatments	July 4	September 11	Increasing rates
Deveci	D0	11.28	13.69	21.5
	D1	10.75	11.73	9.1
	D2	10.75	11.12	3.4
Ankara	D0	14.67	16.77	14.3
	D1	14.66	16.03	9.4
	D2	15.39	14.99	-2.6
Margarita	D0	14.98	16.81	12.2
	D1	14.94	15.69	5.1
	D2	15.18	15.50	2.2

Kaya (2012) reported that there was a linear relationship between amounts of irrigation water and trunk diameter in young olive trees. Water deficit applications decrease trunk diameter development (Alizadeh et al., 2011; Parlak, 2014).

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Conclusions

Different pear varieties grafted onto same rootstock (OHxF 333) had different responses against water deficit. According to plant water content indicators such as Pn and stomatal conductance values, it was determined that Deveci/OHxF 333 pear trees were less affected by water stress. When the other parameters were also considered like SPAD and trunk diameter measurements, Deveci variety was less affected.

When all results were evaluated together, the most resistance one was determined as Deveci/OHxF 333 and it was followed by Ankara/OHxF 333 and Margarita/OHxF 333.

This study was conducted young pear trees. I highly recommend that studies having similar drought levels should be conducted on pear trees in yield age.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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EFFECT OF INTERCROPPING ON SOIL PHYSICAL AND CHEMICAL PROPERTIES IN AN OLIVE ORCHARD

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(Received 27th Jul 2020; accepted 6th Oct 2020)

Abstract. Conservational agricultural practices need to be implemented for improving agricultural productivity and protecting soils in a changing climate. Intercropping systems indicating multiple cropping within the same space may be used as an alternative production system which involves various plant species. This system reduces mineral N-fertilizer use and also improves soil physical and chemical properties. Thus, this study was carried out to investigate the effects of four different intercrops (i- barley+pea, ii- barley+vetch, iii- triticale+pea, and iv- triticale+vetch) in an olive orchard on biomass yield, and some physical and chemical soil properties in a two-year field experiment in South-East Turkey. The biomass yield, soil porosity, bulk density, penetration resistance, aggregate stability, and soil organic matter content have been determined at the end of each harvest. The highest biomass yield was recorded in triticale+pea, while the lowest yield was obtained under barley+vetch treatment. A significant positive relationship was determined between aggregate stability and biomass yield, in contrast significant negative relationship was obtained between penetration resistance and biomass yield. The results of the study clearly showed that intercrops (specifically triticale+pea) can be used to produce additional biomass for animal husbandry and improve soil quality in olive tree-plantations.
Keywords: *cover crop, forage crops, soil tillage, orchard, porosity, mixed cropping*

Introduction

Land use change and inappropriate agricultural practices caused severe soil erosion and land degradation in semi-arid regions of Turkey (Budak et al., 2018). Approximately 85% of lands in the country are under the threat of erosion. Topographic structure and climatic conditions are natural erosion causing factors, while the lack of vegetation is a major anthropogenic factor of erosion. Therefore, establishment of vegetation on soil surface is extremely important to reduce wind and water erosion. Intercropping in orchards outside of the fruit season may contribute to alleviate the erosion, improve soil quality and meet the forage demand of livestock husbandry.

In intermediate farming with cover crops, mixing the remaining part after harvest as a green manure significantly increases soil organic matter content (Montagnini and Nair, 2004). Whyte et al. (1955) stated that intercropping of leguminous forage crops and the cereals enrich the soil organic matter. Tarman (1972) stated that the nine-year alfalfa cultivation contributed an average of 37 ton ha^{-1} of organic matter to soil. Soil organic matter improves soil fertility by improving the physical and chemical properties of soils. The effect of the intercropping on soil organic matter accumulation varies depending on plant type, soil tillage and some other factors. Cultivation of forage crops with abundant above- and underground components and reduced tillage significantly increase organic matter content of soils. Cover crops in semiarid environments improve soil quality compared to frequently tilled management, by increasing organic matter content, improving the chemical and physical fertility of soils, and enhancing soil

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biological activity. Higher water extraction of cover crop plants could affect negatively the orchard growth and/or productivity; however, early cover crop removal would minimize possible yield losses. In contrast, cover crop treatments significantly increased the apricot yield compared to the control (Ramos et al., 2010).

Forage crops in intercropping have an important potential to meet roughage demand of livestock husbandry. Legumes and cereals are a source of protein and carbohydrates in animal nutrition (Ağtürk, 2010). The water infiltration of surface layer in no-tillage system is better than the that of compacted layer in the tilled lands. The plough pan constrains infiltration of water into the soil, thus water run off on the surface causes erosion. Therefore, compacted plough layer is recommended to break up every 3 to 4 years (Öztürkmen and İnce, 1998).

The porous structure of soils is lost with the tight arrangement of coarse particles due to the pressure exerted by tire or agricultural equipment and a compacted layer is formed on soil surface. Penetration resistance is closely related to pore size distribution of soils and a compacted layer contain few large pores, less total pore volume and, consequently, a greater density (Korucu et al., 2009). In general, the penetration resistance in 0-30 cm soil layer is lower than the subsurface layers (30-60 cm depth). The subsurface layers contain fewer macro porous due to the pressure of upper layer soil. Penetration resistance is closely related to air and water movement in soils (Şeker, 1997). Wagner et al. (2000) indicated a positive relationship between aggregate stability, organic matter and clay content of soils. Here, researchers stated that the aggregation process is accelerated with the addition of organic matter. Soil aggregate formation is closely related to the properties of surface soils. The speed of the aggregation process in surface layer has a positive effect in minimizing the erosion threat.

Soil microorganisms decompose organic matter and turn the nutrients into plant available forms. In legume planted soils, soil mineral N content may increase due to the leakage form the nodules where microorganisms living symbiotically within nodules in the root systems of leguminous forage crops. Therefore, soil fertility in intercropping systems may be greater than that in monoculture cultivation (Anıl et al., 1998).

Hay yield in Turkey varies between 3 and 10 tons per hectare depending on ecological conditions. Vetch is an excellent legume forage plant that can be used as cover crop, green manure, meadow, silage or hay. Vetch has a high dry matter content and moderately resistant to cold, thus can also be sown in the winter. Polat et al. (1999) carried out a research in Ceylanpınar district of Sanlıurfa province to investigate the effect of barley (*Hordeum vulgare* L.) + vetch (*Vicia sativa* L.) mixtures, grown as intercropping under rainfed conditions in Pistachio (*Pistacia vera* L.) orchards, on yield components. Five different forage crops sowing ratios (pure barley, 75% barley + 25% vetch, 50% barley + 50% vetch, 25% barley + 75% vetch, pure vetch) were tested in the study and the highest hay yield (1020 kg ha^{-1}) was obtained from 25% barley + 75% vetch mixture.

The aim of this study was to investigate the effects of different cover crop treatments tested as intercropping in an olive orchard on some soil quality parameters and biomass yield. Soil organic matter, total nitrogen, electrical conductivity, basal soil respiration, structural stability index, aggregate stability, saturated hydraulic conductivity, bulk density, permanent wilting point, field capacity and available water content of soil samples were determined as soil quality indicators.

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Materials and Methods

The study site is located in the Harran Plain, Şanlıurfa, Turkey (37° 9.7′ N Lat, 38° 58.7 E Long). The field site has a semi-arid climate with a mean annual temperature, precipitation, and evaporation of 17.2 °C, 365.2 mm, and 1848 mm, respectively (from 2000 to 2015). Elevation of the study area ranges from 358 to 530 m. This two-year study was carried out in an olive orchard consisted of 12 plots, where 15-16 year old olive trees planted at 6 x 6 m distances in the Kısas village, representing the Harran Plain of Şanlıurfa, Turkey (*Fig. 1*). The field was tilled with a small garden plow and leveled with a cultivator. A hand-held scythe was used for moving the weeds.

Figure 1. *Experimental site and soil sampling*

Disturbed and undisturbed soil samples were collected from 0-20 cm depth prior to soil tillage, and soil properties of the olive orchard were given in *Table 1*.

Table 1. *Soil properties of experimental field prior to tillage*

Soil Properties	Depth (0 – 20 cm)
Moisture content (%)	13.21
Bulk density (g cm^{-3})	1.31
Porosity (%)	50.45
Penetration resistance (MPa)	0.90
pH	7.98
Lime (%)	21.25
Electrical conductivity (dS m^{-1})	0.275
Organic matter (%)	1.14

Four different mixtures, which were widely used in the region were tested as forage crop mixtures.

1. Mixture: Common vetch + barley (10 kg common vetch +5 kg barley)
2. Mixture: Common vetch + Triticale (10 kg common vetch +5 kg Triticale)
3. Mixture: Forage pea + Barley (10 kg Forage pea + 5 kg Barley)
4. Mixture: Forage pea +Triticale (10 kg Forage pea + 5 kg Triticale)

Forage crops were planted in early November 2017 and 2018 and harvested in the first week of May. Soil samples were collected following the harvest of each year at the same time and biomass yield was also calculated for each year. The common vetch,

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barley, triticale and forage pea mixtures, which are widely preferred in the region, were used in experiment. The experiment was laid out in randomized blocks with 3 blocks and 4x6 m plots between the olive trees. Fertilizers usually is not applied for winter forage crops (Carr et al., 2004), therefore we did not use any nitrogen and phosphorus fertilizers in this study. Seeds were sown manually following two tillage operation with a cultivator in November. The seeds were not planted at a distance of 1 m to the olive trees. The plants were harvested manually at the beginning of May, when 1/3 of the plants were flowering, dried in the field and removed.

Soil Sampling and Preparation to Laboratory Analysis

Three soil samples representing the experimental field were taken prior to the planting. After the harvest in each year, soil samples were collected from 0-30 cm depth of each plot (12 plots). Soil samples were air dried under room temperature, rocks and roots were removed, grinded by a wood roller and sieved through 2 mm sieve for laboratory analysis.

Soil Analysis

Soil reaction (pH) (Richards, 1954), calcium carbonate content (%) (Allison and Moodie, 1965), electrical conductivity (EC, dS m^{-1}) (Jackson, 1962), organic matter content (%) (Kacar, 2009), bulk density (Blake and Hartge, 1986), water content at field capacity, permanent wilting point and available water content (Klute, 1986), aggregate stability (Kemper and Rosenau, 1986) of soil samples were determined. Penetration resistance was measured using a penetrometer (Say and Işık, 1996). Plant available phosphorus content was determined in spectrophotometer (Olsen et al., 1954). Extractable magnesium, potassium and calcium contents (Warncke and Brown, 2011), and DTPA extractable zinc, iron, manganese and cupper contents (Lindsay and Norvell, 1978) were determined in an atomic absorption spectrophotometer (Olsen et al., 1954).

Statistical Analysis

The effects of cover crop treatments on biomass yield and some of physical and chemical properties of soils were evaluated using a one-way analysis of variance (ANOVA). Post-Hoc LSD test was carried out where ANOVA indicated a statistically significant differences. All statistical analyses were carried out using SPSS 21 (SPSS Inc., Chicago, IL, USA) statistical software.

Result

Biomass Yield

Mean biomass yield in intercropping treatments during 2018 and 2019 seasons were presented in *Fig. 2*. The biomass yield of respective treatments varied between 1940 to 2610 kg fresh dry matter ha^{-1}. The highest biomass yield in both years was obtained in triticale + pea treatment. The forage pea mixtures in the intercropping caused a higher yield compared to the common vetch mixtures. The lowest yield in both years was recorded in barley + vetch treatments. Biomass yield in 2019 was slightly higher than that in 2018, however, relative differences among treatments were similar.

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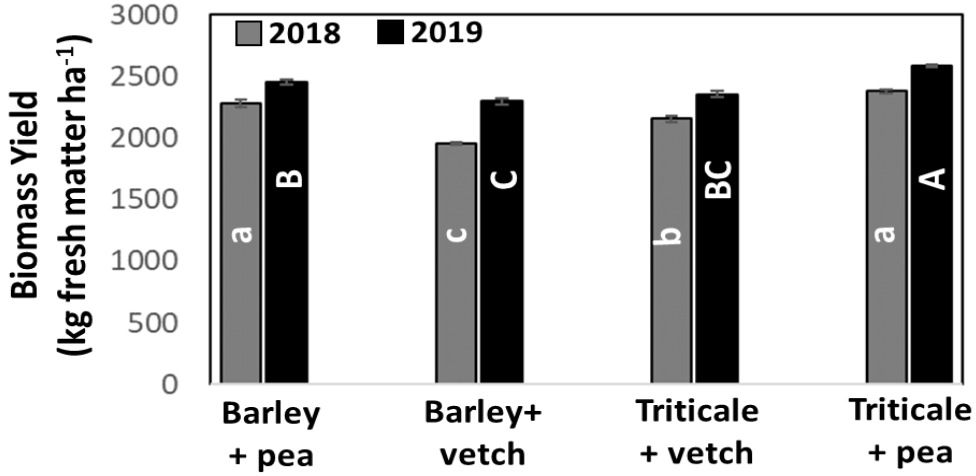

Figure 2. *Biomass yield of various intercrop treatments in 2018 and 2019. Data presented as the mean value of 4 replicates (n=3). Error bars represent the standard error of 3 replicates. Small or capital letters represent the statistical differences among treatments within each year*

Aggregate Stability

Aggregate stability measured at the end of each season was presented in *Table 2*. There was a clear differences among treatments in both years with similar trends. The aggregate stability was higher in pea mixed intercropping as compared to the vetch mixture. The highest aggregate stability was measured barley+pea treatment, while the lowest was measured in triticale+vetch treatment. The statistical analysis showed no differences in aggregate stability between the years. The aggregate stability of the respective treatments followed the trend, barley+pea > triticale+pea > barley+vetch > triticale+vetch.

Table 2. *Effect of cover crops on some soil physical and chemical properties determined in 2018 and 2019 growing seasons*

Year	Crop type	Aggregate stability	Porosity	Bulk Density	Field Capacity	Wilting Point	Available Water	Penetration Resistance	Organic Matter
		%	(cm^3 cm^{-3})	(g cm^{-3})	(%)	(%)	(%)	Mpa	(%)
2018	**Barley +pea**	24.7±0.01a	0.62±0.01b	1.14±0.02*	37.09±0.88*	27.16±0.35*	9.93±0.56b	0.82±0.02*	1.25±0.01*
2018	**Barley +vetch**	19.9±0.38c	0.59±0.02ab	1.18±.0.01	37.69±0.41	24.46±0.36	13.23±0.18a	0.87±0.02	1.21±0.01
2018	**Triticale +vetch**	13.85±0.15d	0.58±0.01ab	1.19±0.01	37.22±0.18	28.18±0.22	9.04±0.28b	0.85±0.03	1.29±0.01
2018	**Triticale +pea**	23.23±0.36b	057±0.02a	1.20±0.04	36.52±1.02	27.21±.0.63	9.32±0.71b	0.85±0.03	1.21±0.01
2019	**Barley +pea**	26.66±0.37a	0.64±0.01*	1.18±0.01*	37.30±0.23*	27.33±0.51*	9.98±0.41b	0.72±0.02*	1.55±0.05*
2019	**Barley +vetch**	21.24±0.50c	0.61±0.02	1.21±0.01	37.70±0.14	25.88±0.28	11.82±0.42a	0.77±0.02	1.54±0.01
2019	**Triticale +vetch**	15.82±0.23d	0.72±0.11	1.21±0.01	37.19±0.29	27.68±0.45	9.50±0.42b	0.75±0.03	1.66±.001
2019	**Triticale +pea**	24.35±0.40b	0.65±0.09	1.21±0.01	37.42±0.44	27.73±.0.42	9.70±0.18b	0.77±0.02	1.54±0.01

Data presented as the mean value of 4 replicates (n=3). * indicates no significant differences

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DOI: http://dx.doi.org/10.15666/aeer/1806_77837793

Porosity

The effect of various intercropping treatments on porosity measured at the end of each growing season was presented in *Table 2*. Soil porosity varied significantly among the treatments in both years with similar trends. Similar to the aggregate stability, porosity in 2018 was higher in pea mixed barley treatment as compared to the others. The porosity of barley included treatments was generally higher in 2018, however no differences was found in 2019. The porosity in 2019 was slightly higher in all treatments as compared to that in 2018.

Available Water

Plant available water content measured in various cover crop treatments was given in *Table 2*. Plant available water content measured in barley+pea mixture was significantly higher as compared to the other treatments in both years. The available water content in all other treatments remained similar without any significant differences. The highest mean available water content (13.2±0.18%) in 2018 was recorded in barley+pea treatment, which was ranged from 9.0 to 9.9% in other treatments. The mean available water content in 2019 was obtained in barley+pea treatment (11.8±0.18%) and varied between 9.5 to 10.0 in other treatments.

Other Soil Parameters

Bulk density, field capacity and wilting point water contents, penetration resistance and organic matter content of soil samples were presented in *Table 2*. The values of these parameters were slightly different among the treatments, however the differences were not statistically significant among the treatments. Soil organic matter content in 2019 was slightly higher as compared to that in 2018. The difference in soil organic matter content between the years can be attributed the biomass incorporation of cover crop treatments. The above ground biomass were not removed from the field, in contrast incorporated to the soil, which likely caused such temporary increase in organic matter content.

Discussion

Most cropping systems in the world are dominated by a low number of crop species and genotypes. For example, 60% of arable land is devoted to a set of three cash crops (maize, winter wheat and oilseed rape). In addition, the grasslands are under high pressure of livestock grazing and the number of relevant species are decreasing by time. Many forests and other woody stands such as short rotation forests are still managed as monocultures, although during the last three decades many mature pure stands have been converted to mixed stands. In our approach we suggest, based on proven expertise, that mixed or multi species stands could be considered as an element to facilitate both agrobiodiversity and productivity. Diverse crop stands is expected to improve resilience and increase overall yield compared to the corresponding monoculture. Unfortunately, general valid explanation and verification across land-use systems have not been provided yet; thus the biodiversity and productivity relationship has not been explained clearly. Moreover, in those cases where mixed cropping systems were established, breeding and especially official testing have been intended to improve the performances in pure stand (single crop stands, mono culture).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77837793

Intercropping is a rather prominent farming system in conservational agriculture, due to the significant advantages in yield and its beneficiary effects on soil properties. The main advantages of the intercropping system are the increase in yield potential without additional input (such as mineral fertilizer) and the increase in buffering against stress and other negative circumstances. Intercropping receives growing interest in temperate climate zones (Lauk and Lauk, 2008). The review on intercropping revealed that the mixtures had a favorable mixing effect in 60% of 344 cases (Li et al., 2006). Intercrop components do not compete with the identical ecological niches. Two important mechanisms were reported between legume and non-legume intercrops. The first mechanism is the rhizo-deposition of nitrogen in legumes and subsequent nitrogen transfer can promote yield of the non-legume crops. Similar transfer mechanism have been reported for phosphorous (Li et al., 2006). Hauggaard-Nielsen et al. (2001) observed faster and deeper growing barley roots in intercrop stands with pea compared to single barley cultivation. Thus, a greater soil volume was available for the mixed intercrop compared to the single crops. Similarly, soil porosity and other soil physical parameters were positively affected by the mixed crop stands. The barley + cereal mixture caused a significant increase in porosity and aggregate stability which likely increased the available soil water content. In addition, positive relationship was obtained between biomass yield of mixed stands and aggregate stability (*Fig. 3A*). Penetration resistance is an important indicator of root growth and storage of soil water. Significant correlation was recorded between yield and penetration resistance of soil.

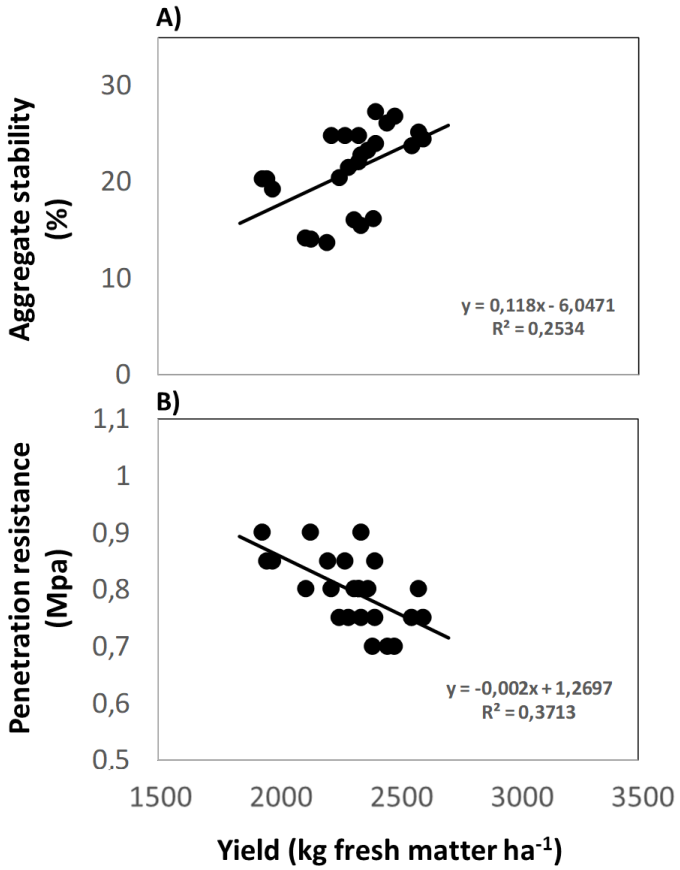

Figure 3. *Relationship between aggregate stability (A), penetration resistance (B) and biomass yield of various intercrops (data pooled for 2018 and 2019)*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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Microorganisms in soils use organic matter as food and excess nutrients are released while decomposition of organic matter by microorganisms. Fixation of atmospheric nitrogen by microorganisms, especially through symbiosis with legume forage crops, enhances the nitrogen content of soils. Healthy growth of forage crops increases phosphorus and potassium uptake of roots and improves the availability of nutrients in soils (Barber, 1989). Therefore, intercropping is more productive than monoculture (Anıl et al., 1998). Forage crops produced in intercropping have an important potential in meeting the forage demand of livestock husbandry. Legume and cereals are important sources of protein and carbohydrates in animal nutrition (Ağtürk, 2010). In this study, approximately 2-3 tons per hectare forage obtained from legume + cereal mixture grown between olive trees shows that intercropping may be a good opportunity to develop animal husbandry in the region. In addition, high nitrogen content (due to the legume mixture) of forage increases the feed quality in animal nutrition. Hay yield in Turkey varies between 3 to 10 tons per hectare depending on the ecological characteristics of the region (Hatipoglu et al., 2009). Vetch is an excellent legume forage plant that can be used as cover crop, green manure, meadow, silage and hay. The vetch, which has a high dry matter content, fixes nitrogen similar to the other legumes, is grown in the winter, and is moderately resistant to cold. The vetch can be grown by mixing with other forage crops or can be grown alone in the field, or the vetch can be cultivated by mixing or alone as intercropping in vineyards and orchards. Polat et al. (1999) carried out a research in Ceylanpınar district of Sanlıurfa province to investigate the effect of barley (*Hordeum vulgare* L.) + vetch (*Vicia sativa* L.) mixtures, grown as intercropping under rainfed conditions in Pistachio (*Pistacia vera* L.) orchards, on yield components. Five different forage crops sowing ratios (pure barley, 75% barley + 25% vetch, 50% barley + 50% vetch, 25% barley + 75% vetch, pure vetch) were tested in the study and the highest hay yield (1020 kg ha^{-1}) was obtained from 25% barley + 75% vetch mixture. The highest yield in this study was obtained in triticale + pea mixture.

Southeastern Anatolia Region of Turkey has 6 million ha land planted by pistachio, olive, peach, apple and apricot. The results of this study indicated that the orchard fields can be used to grow forage crops as intercropping. The lack of vegetative cover on soil surface causes severe soil erosion and land degradation; and prevents the production of forage crops, which is one of the most important inputs of livestock husbandry. Soil water content ranged from 7.1 to 34.5% during the intercropping growth season. The porosity of soils was between 48 and 57% which indicates that the water saturation of soils is at most between 61 and 72%. The results reveals that especially the sub-surface layers are mostly not saturated. Soil water content of surface soils in barley treatments varied between 11.3 and 19.4%, and the lowest soil water content was recorded in single barley treatment, followed by vetch and mixture of vetch + barley. The results of the first year revealed no serious competition for water use between the forage crops and olive trees until the end of the growth season. However, reliable evaluation for perennial plants can be made only by taking the results of long-term studies into account. The results of current study indicate that cultivation of barley + pea or triticale + vetch mixture as intercropping can contribute to agricultural production, soil quality and sustainable agriculture in the semi-arid regions.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77837793

Conclusion

Overall, the present study clearly demonstrates that the cultivation of barley + pea or triticale + vetch mixture as intercropping may significantly improve soil quality in the semi-arid regions. Cereal-legume mixed-intercropping caused a significant change in porosity and aggregate stability which likely affect the available soil water content. A significant positive relationship was determined between aggregate stability and biomass yield, in contrast significant negative relationship was obtained between penetration resistance and biomass yield. The results of the study clearly showed that intercrops (specifically triticale+pea) can be used to produce additional biomass for animal husbandry and improve soil quality in olive tree planted lands plantations. Further research is needed to understand the mechanisms on how soil rhizosphere processes controls soil chemical and physical parameters in mixed cropping systems.

Acknowledgement. Authors would like to thank Assoc. Prof. Dr. Mehmet ŞENBAYRAM in Harran University for his help during the organization of the field studies and editing the manuscript. This study was funded by Harran University Scientific Research Coordination Unit (HÜBAK) (Project No: 17091).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77837793

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77837793

COST AND BENEFIT ANALYSIS OF ORGANIC MULCHING AND INTERCROPPING IN MAIZE CULTIVATION

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(Received 23rd May 2019; accepted 31st Oct 2019)

Abstract. The aim of this article was to investigate the effect of different mulching and intercropping techniques in maize cultivation with particular focus on weed control, using a randomized complete block design with four replicates to determine the financially optimum method. Field experiment was performed at two different geographical locations which were Nkakom, Nwabiagya District in the Ashanti region of Ghana and Changchun, Jilin-China during 2017 and 2018 cropping seasons, respectively. The experiment consisted of 4 treatments which were control (no mulch), maize (*Zea mays* L.) straw-maize, green gram (*Vigna radiate*)-maize intercrop and groundnut (*Arachis hypogaea*)-maize intercrops. Measured agronomic parameters were weed biomass, maize yield and legume yields. Green gram-maize intercrop recorded the lowest weed biomass and the control (no mulch) recorded the highest weed biomass. There were no significant differences between maize yields measured. Economic analysis of data was carried out with partial farm budgeting. The highest financial net return was obtained in the green gram-maize intercrop whiles the lowest was recorded in control (no mulch) treatment at the two different geographical locations.
Keywords: *legumes, organic mulch, partial budgeting, weed*

Abbreviations: WAP: weeks after planting; GG: green gram; GN: groundnut; LSD: least significant difference; NS: no significant; R_{MC}: revenue from maize earned in the control plot; R_{MGG}: revenue of maize earned in the green gram-maize intercrop plot; R_{MGN}: revenue of maize earned in the groundnut-maize intercrop plot; R_{MMS}: revenue of maize earned in the maize straw plot; R_{GG}: revenue earned from green gram; R_{GN}: revenue earned from groundnut; Y_{MC}: grain yield of maize earned in the control plot; Y_{MGG}: grain yield of maize earned in the green gram-maize intercrop plot; Y_{MGN}: grain yield of maize earned in the groundnut-maize intercrop plot; Y_{MMS}: grain yield of maize earned in the maize straw-maize plot; Y_{GG}: grain yield earned from green gram; Y_{GN}: grain yield earned from groundnut; MP_M: market price of maize; TC_C: total cost on the control plot; $TC_{GG\text{-}M}$: total cost on green gram-maize intercrop plot; $TC_{GN\text{-}M}$: total cost on the groundnut-maize intercrop plot; $TC_{MS\text{-}M}$: total cost on the maize straw-maize plot; C_{MS}: cost of maize seeds planted; C_{MP}: cost of planting maize seeds; C_{FA}: cost of fertilizer and its application; C_{MH}: cost of harvesting maize; C_{WC}: cost of weeding the control plot; C_{GGS}: cost of green gram seeds planted; C_{GGP}: cost of planting green gram seeds; C_{WGG}: cost of weeding green gram-maize intercrop plot; C_{GGH}: cost of harvesting green gram; C_{GNS}: cost of groundnut seeds planted; C_{GNP}: cost of planting groundnut seeds; C_{WGN}: cost of weeding groundnut-maize intercrop plot; G_{GNH}: cost of harvesting groundnut; C_{MA}: cost of maize straw application/mulching; C_{WMS}: cost of weeding maize straw-maize plot; TR_C : total revenue earned in the control plot; $TR_{GG\text{-}M}$: total revenue earned in the green gram-maize intercrop plot; $TR_{GN\text{-}M}$: total revenue earned in the green gram-maize intercrop plot; $TR_{MS\text{-}M}$: total revenue earned in the maize straw maize plot; P_C: profit earned in the control plot; $P_{GG\text{-}M}$: profit earned in the green gram-maize intercrop plot; $P_{GN\text{-}M}$: profit earned in the groundnut-maize intercrop plot; $P_{MS\text{-}M}$: profit earned in the maize-straw maize plot; Eq: equation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

Introduction

Maize is one of the most important crops worldwide. It is grown throughout the world, with the United States, China, and Brazil being the top three maize-producing countries in the world producing approximately 563 of the 717 million metric tons/year (Ranum et al., 2014). Maize accounts for over 50% of the total cereal production in Ghana and annual yield have been reported to be growing around 1.1% (IFPR, 2014). Maize contains about 72% starch, 10% protein, and 4% fat, supplying an energy density of 365 Kcal/100 gas (Nuss and Tanumihardjo, 2010) compared to rice and wheat, but has lower protein content. In Ghana maize account for 62% of total grain output. It is the largest staple crop in Ghana and the mainstay of the diet of the majority Ghanaians. Maize is also an increasingly important component of poultry feed, and to a lesser extent, the livestock feed and brewing industry. The agro-ecological zones for maize in Ghana is grouped into four, namely the Coastal savannah zone, Forest zone, Transitional zone, and Guinea savannah zone. Maize production happens in almost every part of Ghana however output differs among these agro-ecological zones (Morris et al., 1999). The Ashanti region is among the five major principal areas in maize cultivated.

China's average per capita meat consumption has quadrupled Nuss and Tanumihardjo (2010) and Schneider and Sharma (2014). In terms of the numbers of animals, China has seen a five-fold increase in pig stocks and an almost 9-fold increase in chicken since 1961 (FAO, 2013). As maize is the country's primary feed crop (Shihuang and Kaijian, 2014) the rapid expansion of maize and meat production and consumption are intrinsically linked. The principal maize production areas in China are situated in a belt of very diverse environments traversing China from northeast to southwest. Production environments can be classified into six agro-ecological regions: Northeast China, North China, Yellow-Huai River Valley, Northwest China, Southwest China, and South China. The provinces and prefectures included in each agro-ecological region are; Heilongjiang, Jilin, Liaoning, Inner Mongolia, Hebei, Shanxi, Shandong, Henan, Shanxi, Shaanxi, Sichuan, Guizhou, Yunnan, and Guangxi (Meng, 2008).

Because of this, serious attention should be on the sustainable production of maize and supply to curb higher prices, malnutrition, poverty, and hunger for its direct and indirect consumers. One major factor contributing to the decline in maize production arises from weed competition and its associated management constraints. Of all crop pest, weed is the commonest blooming each year on almost every farm in most areas of the world competing with crops for growth requirements (Obuo et al., 1997). Major problems caused by weeds include interfering and competing with cultivated and desirable plants for space, light, water and soil nutrients, interference with cultural operations and harvest, serving as alternate hosts for other crops pests and diseases and reducing crop output. Therefore, to attain optimal yield, weeds need to be controlled before planting a crop and be monitored during the growing season until harvesting. Inefficient weed management methods are a crucial factor in the overall decline in the yield of maize (Gianessi, 2013).

To obtain optimum growth and yield of maize, weed management becomes a pivotal factor to consider. Numerous research has identified essential techniques that could suppress weed competition but there is little attention to the economic, biological, environmental and health effects of these techniques (Omovbude and Udensi, 2012). Attention is mostly shifted towards cultural (hand-or-hoe -weeding), mechanical (slashing), chemical (pre-plant, pre or post-emergence herbicide (Omovbude and Udensi, 2012). Although all the above practices could yield positive weed management results,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

yet, associated with significant environmental, biological, health and economic setbacks when applied intensively.

The approach used in this research such as the selection of treatments and the mathematical expressions executed in calculating the total cost, total revenue and profit/net revenue has not been used elsewhere therefore making this research peculiar.

This research was based on three objectives: (1) effects of organic mulch on weed growth, (2) whether organic mulch and intercropping could reduce the cost of weed management and boost net revenue, and (3) whether the legumes used for the intercrop used could affect maize yield.

Literature Review

Intercropping is defined as the farming practice of growing two or more crops in the same space at the same time. Intercropping method ensure productivity per unit area of land. Intercropping system ensures maximum utilization of soil and environmental resources. Intercropping system is associated with socio-economic, biological and ecological returns. Besides maintaining the soil health (Prasad et al., 2008) further reported an efficient utilization of growth resources with intercropping when with maize-black gram intercrop.

This practice is an attractive strategy to smallholder farmers for increasing productivity and labor utilization per unit of area of available land through intensification of land use (Seran and Brintha, 2010). Intercropping cereals with legumes have a huge capacity to replenish soil mineral nitrogen through its ability to biologically fix atmospheric nitrogen (Giller, 2001). Intercropping works better by ensuring maximum yield when crops have different growth requirements such as moisture, light, nutrients and space for growth and yield. The features of an intercropping system differ with soil, climatic conditions, economic situations and preferences of the local community (Steiner, 1982).

Maize-legume intercropping system reduces the risk of total crop failure; thus when one crop fails due to pest and disease attack, there is still hope of yield gain. Maize-legume intercrop system also proves to have financial advantage over maize mono-cropping. Commonly, maize is intercropped with some legume crops such as cowpea, soybean, pigeon pea, groundnut and green gram.

Organic mulching, either life or dead mulch, besides its weed control ability, could make available several benefits including; increasing microbial activity in the soil, limiting soil erosion, permitting symbiotic nitrogen fixation, nutrient conservation and enhancement of biodiversity (Hartwig, 2002; Gerhards, 2018). Live mulch through intercropping compete with weeds and very effectively prevent or suppress them from growing. Dependence on the organic mulch as a weed control agent plays a significant role in integrated weed management techniques because living mulches could deal with weed control over an extended period, from the early growth stages until harvesting. Mulch and legume crops provide food and habitat for beneficial insects. They could also retain moisture, regulate soil temperature, ensure weed suppression, improve soil structure and add soil nutrients such as nitrogen (Kahangi et al., 2014). The most important attributes required for species used as living mulches are quick emergence and soil covering, short height, low water and nutrients demands (Kolota and Adamezewska, 2013).

Deepening farmer's knowledge on mulching and intercropping as weed control agent could reduce the cost of production through reducing weed competition. It could also deal

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DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

with matters of malnutrition and prevent some conventional practices such as burning of residues after harvesting which leads to land degradation and pollution.

Materials and Methods

Experimental Sites

Field experiment was performed in two different geographical regions. The experiment was carried out in the year 2017 and 2018 at Young Adults Training Center, Nkwakom, Nwabiagya District in the Ashanti region of Ghana and Jilin Agricultural University, Changchun, Jilin-China respectively. *Figures 1 and 2* represents the maps of the study areas.

Sites Description of Atwima Nwabiagya-Kumasi, Ghana

Location

In 2017, the experiment was performed at the Young Adults Training Center, Nkakom Nwabiagya District in the Ashanti region of Ghana. The area lies approximately between latitude 6˚ 32'N and 6˚ 75'N, and between longitude 1o 36˚ and 2˚ 00' West. It is situated in the Western part of the Ashanti Region and shares boundaries with Nkawie (to the West), Afari (to the East), Foase (to the North) and Nkoran (to the south).

Topography and Drainage

The area has an undulating topography. The lands have average heights of about 77 m above sea level. The high land has gentle to steep slopes. There are several wider valleys with no evidence of stream-flow. These valleys provide opportunities for rice, sugarcane and vegetable cultivation.

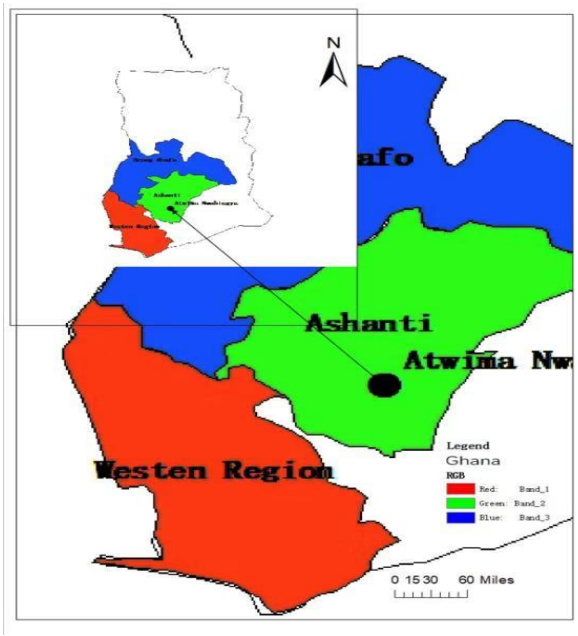

Figure 1. *Map of the study area at 2017. The Atwima Nwabiagya District is in the Ashanti Region of Ghana*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
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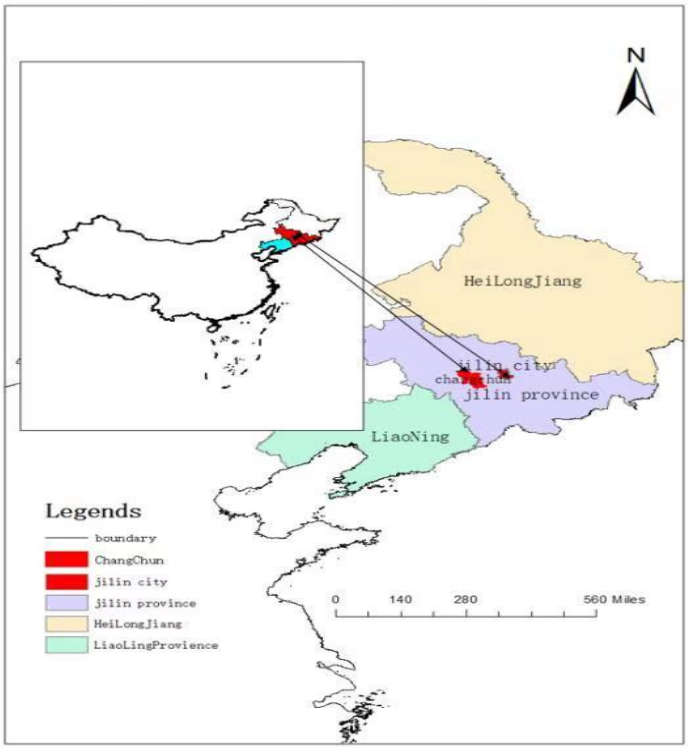

Figure 2. *Map of the study area at 2018. Changchun is in Jilin Province of China*

Climate

The area lies within the wet semi-equatorial zone, which is marked by double maximum with annual rainfall ranging between 170 cm and 185 cm. Major rainfall season is from Mid-March to July, and the minor season is between September and mid-November. Temperature is reasonably uniform, ranging between 27ºC (August) and 31ºC (March). Mean relative humidity of about 87 to 91 per cent is characteristic of the area. The lowest relative humidity usually occurs in February/April when they are between 83-87 in the morning and 48-67 in the afternoon.

Vegetation

The vegetation found in the area is predominantly the semi-deciduous type. The vegetation type has primarily been disturbed by human activities (logging, farming, bush fires, etc.), thus, depriving it of its original valuable tree species, soil fauna and other forest products.

Soil and Characteristics

The soil has a high water holding capacity. The soil is marginal for mechanical cultivation. Hand cultivation is recommended. The soil is good for agriculture. They are suitable for tree and arable crops such as cocoa, citrus, oil palm, mangoes, guava, avocado, maize, cassava, yams. Their moisture-holding capacity is reasonably high, although surface layers are susceptible to dry season drought. The soils are moderately suitable for agriculture.

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Pest and Diseases

Pest and disease condition in the district is not all that serious, apart from the usual caterpillars, grasshoppers, aphids and mites, which affect some crops. Armyworm attack on maize occurs seldom in the area with the highest incidence recorded in 2017. Common diseases are fruit drop in citrus and black pods on cocoa. The common pests in animals include ticks, worms and lies.

Site Description of Changchun, Jilin-China

Location

The test site is located in the long-term positioning test field of Jilin Agricultural University, Changchun City Jilin Province northeastern China (43°47′N, 123°20′E).

Topography and Drainage

The terrain is flat and open, which is a transitional zone between the eastern mountain area and the western Songliao Plain. The river runoff in Changchun City is mainly caused by atmospheric rainfall with annual total of about 11.4 billion cubic meters, surface water resources of about 1.287 billion cubic meter and groundwater resources of about 1.238 billion cubic meter.

Climate

Changchun is located in the center of the Song Liao Plain and has a semi-wet monsoon type climate. The temperature varies throughout the year. The season is defined with spring being dry and windy; summer being short and cool; autumn being sunny and warm during the day but cold at night; winter is cold, with a permanent covering of snow. The average annual temperature of Changchun is about 4.8˚C. January is the coldest month, with an average temperature of -17.2˚C. The lowest temperature is -39.8˚C. July is the hottest with an average temperature of about 23˚C. With the highest recorded temperature being 39.5˚C.

Vegetation

The natural vegetation is prairie grass in the western plains and mixed conifer and broad-leaved deciduous forest in the eastern mountainous area. The vegetation in the east of mountains includes tree species such as Japanese red pine, Manchurian ash, fish-scale pine, larch, birch, oak, willow, elm and the Manchurian walnut.

Soil and Characteristics

There are two main types of soils in the province: podosols in the eastern mountainous region and black earth in the western plains. The podosols occur in several forms and are of both high and low fertility. Central and west Jilin are the areas of the black earths of the northeastern plains. It is of high productivity and contains a high percentage of organic matter, and they form good arable land.

Pest and Diseases

Diseases are mainly silk smut and pest are mostly aphids, army worm and white star.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
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Soil Parameters Tested

Land on which the experiment was performed in Ghana was initially tilled conventionally through residue burning and plowing with the use of a hoe. From early 2016, the area was gradually transitioning from conventional tillage to conservation tillage through soil cover, minimum disturbance and crop rotation. In China the land was under continuous tillage with the use of bigger machinery for fertilizer broadcasting.

The parameters of soil we tested were pH, temperature, moisture, soil organic matter, total nitrogen, alkali hydrolyzed nitrogen, available phosphorus, total phosphorus, available potassium and total phosphorus. We measured soil pH on 1:10 (w/v) ratio in distilled water using pH meter, we tested soil moisture and temperature with a hand-held moisture meter and thermography, respectively. We determined soil organic matter by dichromate oxidation with external heat and titration with ferrous ammonium sulphate. We measured soil alkali nitrogen using the Illinois soil nitrogen test diffusion. We determined soil total potassium calorimetrically using the molybdate method. We determined soil available phosphorus (AP) calorimetrically based on the Olsen method. We extracted soil total potassium by incubation with sodium hydroxide, and we extracted soil available potassium by incubating with 1.0 mol L^{-1} ammonium acetate for 0.5 h, followed by filtration. *Tables 1 and 2* below summarizes the soil parameters tested at each experimental site before the commencement of the study. Soil parameters tested in 2018 (China) were more as compared to that in 2017 (Ghana) due to the availability of laboratory equipment that was in China.

Treatments and Experimental Design

Treatment involved in the research consisted of organic mulching and different intercropping methods. The treatments were green gram-maize intercrop, groundnut-maize intercrop, maize straw-maize and control (no mulch). We arranged the treatments in a randomized complete block design (RCBD) with four replications. Each one plot occupied a total land area of 3.9 m x 8 m (31.2 m^2) with an alleyway of 1 m way among plots and 1 m among replicates given a total land area of 19.6 m x 36 m (705 m^2) approximately (0.07 ha). All plots were kept clean of weeds before we planted. We applied all mulching procedures the same day as maize seeds were planted. We sowed maize seeds with between and within row spacing of 65 cm and 25 cm, respectively. Maize varieties we used for the experiment in Ghana and in China were obatampa and Ji nong yu 885, respectively. With green gram-maize and groundnut-maize intercrops, we established two rows of the legumes spaced 21 cm between rows and 25 cm within rows between two rows of maize (2:2). We kept the maize straw obtained between and within the maize rows which completely covered the entire maize straw-maize plot. In 2017, the maize straw we used for the experiment in Ghana was obtained from 2016 minor season maize residues which we gathered to mulch the plot. Also in 2018, maize straw we used for the experiment in China was obtained from residues left on the field after 2017 harvest. We kept the control plot bare before planting maize.

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Table 1. *Chemical properties of the experimental site before the commencement of the study 2017 season at the Young Adults Training Center Nkaakom-Ashanti, Ghana*

Soil Properties	Values
Ph (1:10 w/v)	5.54
Moisture (%)	18.5
Temperature (˚C)	22.3

Table 2. *Chemical properties of the experimental site before the commencement of the study 2018 season at Jilin Agricultural University, Changchun City Jilin Province China*

Soil Properties	Values
Ph (1:10 w/v)	6.1
Moisture (%)	20.6
Temperature (˚C)	21.1
Organic matter (g/kg)	22.8
Total Nitrogen (g/kg)	1.399
Total Phosphorus (g/mg)	0.53
Total Potassium (g/kg)	23.19
Alkali Hydrolyzed Nitrogen (mg/kg)	125
Available Phosphorus (mg/kg)	35.1
Available Potassium (mg/kg)	156

We performed the experiments at both locations under rain-fed condition but due to shortage of rainfall during the first and second week in 2018 (China), we watered the experimental plots through sprinkler irrigation. We performed thinning 20 days after planting to obtain one plant per stand for maize and two plants per stand for both legumes. Total plant populations were 62, 500 plants/h, 400,000 plants/h and 400,000 plant/h for maize green gram and groundnut respectively. *Table 3* further explains the sowing rate of seeds in each treatment. Sowing rates were similar in both geographical regions. We applied NPK fertilizer in a ratio of 27:12:11 to the maize in a localized manner after thinning. *Figures 3, 4, 5, 6* represent the various experimental treatments. Images were taken at 12 weeks after planting during 2018 season at Jilin Agricultural University, China. We could not repeat the experiment at both locations due to high incident of armyworm attack on maize in the minor season in Ghana and limited time for the experiment in China.

Table 3. *Sowing rate of maize, green gram, and groundnut 2017 (Ghana) and 2018 (China) seasons*

TREATMENT	MAIZE	GREEN GRAM	GROUNDNUT
CONTROL	62, 500 (52 Kg/ha)	-	-
GG-MAIZE INTERCROP	62, 500 (52 Kg/ha)	400, 000 (56 Kg/ha)	-
GN-MAIZE INTERCROP	62, 500 (52 Kg/ha)	-	400, 000 (225 Kg/ha)
MS-MAIZE	62, 500 (52 Kg/ha)	-	-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
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Figure 3. *Green gram-maize intercrop*

Figure 4. *Maizestraw-maize*

Figure 5. *Control plot (no mulch)*

Figure 6. *Groundnut-maize intercrop*

Figures of the various treatments are presented above (images were taken at 12 weeks after planting during 2018 season at Jilin Agricultural University, China)

Data Collection and Analysis

We recorded data on weed biomass and maize yield. We also recorded grain yield of green gram and groundnut. We recorded weed biomass at 6 weeks and 12 weeks after planting from randomly selected three central rows from each experimental unit and we averaged to get weed kg/m^2. In determining maize kernel weight, were we harvested two rows in the middle of each plot to achieve results. We detached maize ears, threshed the seeds and weighed. We converted values to kg/ha. Other maize yield parameter we analyzed were ear length, cob diameter, number of rows per cob, number of kernels per cob and maize grain. We selected 10 ears randomly from each plots making a total of 40 ears per treatment for the analysis. We harvested two central rows of both groundnut and green gram for the analysis of kg/ha of grain. Results of maize, green gram and groundnut grain yields at the two different geographical locations are presented in *Tables 4 and 5* below.

Table 4. *Maize, green gram and groundnut grain yields during 2017 season (Ghana)*

TREATMENT	MAIZE (Kg/ha)	GREEN GRAM (Kg/ha)	GROUNDNUT (Kg/ha)
CONTROL	10, 759.4	-	-
GG-MAIZE INTERCROP	10,724.5	1, 848.3	-
GN-MAIZE INTERCROP	10, 749.5		2, 444.5
MS-MAIZE	10, 741.5	-	-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

Table 5. *Maize, green gram and groundnut grain yield during 2018 season (China)*

TREATMENT	MAIZE (Kg/ha)	GREEN GRAM (Kg/ha)	GROUNDNUT (Kg/ha)
CONTROL	11, 219.2		-
GG-MAIZE INTERCROP	11, 199	1, 949.58	-
GN-MAIZE INTERCROP	11, 201.6		2, 305.2
MS-MAIZE	11, 207.5	-	-

Data Analysis

We subjected the data collected to Fisher's analysis of variance technique and LSD test at 0.05 P was used to compare the differences among treatment means.

Economic Assessment

An economic evaluation of the different weed control methods was carried out using partial farm budget. The formula we used for calculating the various total cost, the various total revenue and the profit of the treatments are presented in the mathematical expression below. We considered these variables for the calculations:

Total cost in the control treatment involved (cost of maize seeds planted, cost of planting, cost of fertilizer application, cost of weeding the control plot and cost of harvesting maize all added together), total cost involved in maize straw-maize treatment involved (total cost of maize cultivation, cost of mulch application and cost of weeding all added together) and total cost involved in legume-maize intercrops involved (total cost of maize cultivation, cost of legume seeds planted, cost of planting legumes seeds, cost of weeding, and cost of harvesting legumes all added together). Total income earned from the control treatment involved only the revenue earned from maize, total revenue earned from maize straw-maize treatment also involved only the revenue earned from maize, total revenue earned from maize-legume intercrops involved (revenue earned from maize and the revenue earned from legume added together).

The profits earned from each treatment were calculated by subtracting the costs involved in each treatment from the revenues obtained from each treatment.

Results

Results obtained at the two different geographical regions at the different growing seasons are presented below. Results of weed biomass at the different locations are presented in *Table 6*. The results of maize yield parameters are presented in *Tables 7, 8, 9, 10, 11, and 12*. Results of cost and benefit analysis obtained from the treatments are presented in *Tables 13 and 14*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

Table 6. *Effects of different organic mulching procedures on the weed biomass (kg/m2) during 2017 and 2018 seasons*

	2017		2018	
Treatments	**6 WAP**	**12 WAP**	**6 WAP**	**12 WAP**
Control (no mulch)	12.83a	11.56a	10.26a	8.67a
GG-maize intercrop	4.76c	3.48d	4.53c	2.61d
GN-maize intercrop	8.73b	6.16c	7.24b	4.62c
Maize straw-maize	2.57d	8.16b	2.08d	6.45b
LSD (5%)	1.99	1.64	1.59	1.23

Table 7. *Effects of different mulching methods on maize cob diameter (cm), ear length (cm) and ear weight during 2017 season*

Treatments	**cob diameter (cm)**	**ear length (cm)**	**ear weight (kg/m2)**
Control (no mulch)	3.01a	21.19b	3.51c
GG-maize intercrop	3.05a	21.31b	3.44c
GN-maize intercrop	2.98a	21.36b	3.43c
Maize straw-maize	3.06a	21.54b	3.61c
LSD (5%)	NS	NS	NS

Table 8. *Effects of different organic mulching methods on the number of kernel rows/cob, number of kernels/row and number of kernel /cob of maize during the 2017 season*

Treatments	**rows/cob**	**kernels/row**	**kernels/cob**
Control (no mulch)	18a	38b	618c
GG-maize intercrop	17a	38b	616c
GN-maize intercrop	17a	38b	661c
Maize straw-maize	18a	39b	677c
LSD (5%)	NS	NS	NS

Table 9. *Effects of different organic mulching methods on 100 seed weight and maize grain during 2017 season*

Treatments	**100 seed weight (wet)(g)**	**100 seed weight (dry)(g)**	**maize grain kg/m^2**
Control (no mulch)	44.38a	32.77b	10.28c
GG-maize intercrop	40.34a	32.09b	10.24c
GN-maize intercrop	40.79a	31.41b	10.22c
Maize straw-maize	40.98a	31.64b	10.25c
LSD (5%)	NS	NS	NS

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

Table 10. *Effects of different mulching methods on maize cob diameter (cm), ear length (cm) and ear weight during 2018 season*

Treatments	cob diameter (cm)	ear length (cm)	ear weight (kg/m2)
Control (no mulch)	3.07a	20.98b	3.40c
GG-maize intercrop	3.11a	21.09b	3.34c
GN-maize intercrop	3.04a	21.44b	3.32c
Maize straw-maize	3.67a	21.32b	3.51c
LSD (5%)	NS	NS	NS

Table 11. *Effects of different organic mulching methods on rows/cob, kernels/row and kernel/cob of maize 2018 season*

Treatments	rows/cob	kernels/row	kernels/cob
Control (no mulch)	16a	38b	592c
GG-maize intercrop	16a	36b	576c
GN-maize intercrop	16a	36b	576c
Maize straw-maize	16a	37b	592c
LSD (5%)	NS	NS	NS

Table 12. *Effects of different mulching methods on 100 seed weight and maize grain yield during 2018 season*

Treatments	100 seed weight (wet)	100 seed weight (dry)	maize grain kg/m^2
Control (no mulch)	43.04a	31.66b	11.18c
GG-maize intercrop	39.14a	29.16b	11.15c
GN-maize intercrop	39.75a	29.72b	11.17c
Maize straw-maize	39.74a	31.16b	11.17c
LSD (5%)	NS	NS	NS

Weed Biomass

Results obtained showed similar results at the two different geographical locations. Weed biomass obtained showed significant differences at each sampling times at the two geographical locations where the experiment was performed. In both sampling times, the control treatment which had no mulch recorded the highest weed biomass at both locations. At 6 WAP, maize straw-maize treatment recorded the least weed biomass compared to both legume-maize intercrops at the two locations. At 12 WAP maize-green gram treatment recorded the least weed biomass followed by maize-groundnut treatment.

Maize Yield Parameters

Maize yield parameters measured showed no significant differences between treatments at the two geographical locations.

Economic Assessment (Cost and Benefit Analysis)

The results obtained in 2017 presented the highest net revenue under green gram-maize inter-crop followed by groundnut-maize intercrop whiles the control (no mulch) treatments recording the least net revenue. Similarly, in 2018 green gram-maize recorded the highest net income followed by groundnut-maize whiles the control (no mulch) recorded the least net income.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

Table 13. *Cost and benefit analysis during the 2017 cropping season (Nwabiagya-Ashanti-Ghana)*

Treatments	Cost of legume seeds planted GHC (USD)/ ha	Time of Planting legumes / mulching (man-hr / ha)	Cost of Planting legumes/ mulching GHC (USD) /ha	Time of Weed Control (man-hr / ha)		Cost of Weed Control GHC (USD)/ ha		Time of Harvesting legumes man-hrs/ha	Cost of harvesting legumes GHC (USD)/ha	Total Cost of weed control GHC (USD) /ha	Total cost of maize cultivation GHC (USD) /ha	Legume yields obtained Kg/ha	Revenue from legumes GHC (USD)/ ha	Maize yield obtained Kg/ha	Revenue from maize GHC (USD)/ ha	Total Revenue GHC (USD)/ ha	Profit (Net Revenue) GHC (USD)/ha
				6 WAP	12 WAP	6 WAP	12 WAP										
Control (zero mulch)	-	-	-	186	116.00	1, 953 (399 USD)	1, 218 (249 USD)	-	-	3, 171 (647 USD)	3, 136 (640 USD)	-	-	10, 759. 4	21, 518.8 (4, 391 USD)	21, 519 (4, 392 USD)	15, 212 (3, 104 USD)
Green gram-maize intercrop	847 (171 USD)	79	829.5 (169 USD)	85	37	893 (182 USD)	389 (79 USD)	89	935 (191 USD)	3, 893 (795 USD)	3, 136 (640 USD)	1, 848.3	18, 483 (3, 772 USD)	10, 724.5	21, 449 (4, 377 USD)	39, 932 (8, 149 USD)	32, 903 (6, 715 USD)
Groundnut-maize intercrop	900 (181 USD)	79	829.5 (169 USD)	93	51	977 (199 USD)	536 (109 USD)	89	935 (191 USD)	4, 177 (853 USD)	3, 136 (640 USD)	2, 444.5	7333.5 (1, 496 USD)	10, 749.5	21, 499 (4, 388 USD)	28, 833 (5, 884 USD)	21, 520 (4, 392 USD)
Maize straw-maize	-	56	588 (120 USD)	61	98	641 (131 USD)	1, 029 (210 USD)	-	-	2, 258 (461 USD)	3, 136 (640 USD)	-	-	10, 741.5	21, 483 (4, 384 USD)	21, 483 (4, 384 USD)	16, 089 (3283 USD)

**Kg of Groundnut planted per ha = 225 Kg
**Cost of 1 Kg of Groundnut seeds planted = 4 GHC (0.8 USD)
**Price of 1Kg of Groundnut sold = 3 GHC (0.6 USD)
**Kg of Green gram planted per ha= 56.5Kg
**Cost of 1Kg of Green gram seeds planted = 15 GHC (3.1 USD)
**Price of 1Kg of Green gram seeds sold= 10 GHC (2 USD)
**Cost of Labor = 10.5 GHC/hr (2 USD)
**Kg of maize planted per ha = 52 Kg
**Cost of 1Kg of Maize seeds planted = 5 GHC (1 USD)
**Price of 1 Kg of Maize seeds sold = 2 GHC (0.4 USD)
**Kg of fertilizer applied per ha = 419.87 Kg
**Cost of 1Kg of fertilizer=4 GHC (0.8 USD)
**Time of planting maize (man-hr/ha)= 39 hrs
**Time of fertilizer application= 30 hrs
**Time of harvesting = 45 hrs

Table14. *Cost and benefit analysis during the 2018 cropping season (Changchun, Jilin-China)*

Treatments	Cost of legume seeds planted RMB (USD)/ha	Time of Planting legumes / mulching (man-hr / ha)	Cost of Planting legumes / mulching RMB (USD) / ha	Time of Weed Control (man-hr / ha)		Cost of Weed Control RMB (USD)/ha		Time of Harvesting legumes man-hrs/ha	Cost of harvesting legumes RMB (USD)/ha	Total Cost of weed control RMB(USD)/ha	Total cost of maize cultivation RMB (USD)/ha	Legume yields obtained Kg/ha	Revenue from legumes RMB (USD)/ha	Maize yield Kg/ha	Revenue from maize RMB (USD)/ha	Total Revenue RMB (USD)/ha	Profit (Net Revenue) RMB (USD)/ha
				6 WAP	12 WAP	6 WAP	12 WAP										
Control (zero mulch)	-	-	-	179	128.00	2, 685 (389 USD)	1, 920 (298 USD)	-	-	4, 605 (667 USD)	3, 649 (529 USD)	-	-	11, 219.2	22, 438 (3,251 USD)	22, 438 (3, 251 USD)	14, 184 (2, 055 USD
Green gram-maize intercrop	989 (143 USD)	79	1, 185 (172) USD)	54	30	810 (117 USD)	450 (65 USD)	89	1, 335 (193 USD)	4, 769 (691 USD)	3, 649 (529 USD)	1, 949.58	13,647 (1,978 USD)	11, 199	22, 398 (3, 246 USD)	36, 045 (5, 223 USD)	27, 627(4, 004 USD)
Groundnut-maize intercrop	3, 038 (440 USD)	79	1, 185 (172 USD)	71	52	1, 065 (154 USD)	780 (113 USD)	89	1, 335 (193 USD)	7, 403 (1, 073 USD)	3, 649 (529 USD)	2, 305.21	9220.8 (1,336 USD)	11. 201.6	22, 403 (3, 247 USD)	31, 624 (4, 583 USD)	20, 572 (2, 981 USD)
Maize straw-maize	-	56	840 (122 USD)	33	102	495 (72 USD)	1, 530 (222 USD)	-	-	2, 865 (415 USD)	3, 649 (529 USD)	-	-	11, 207.5	22, 415 ((3, 249 USD)	22, 415 (3, 249 USD)	15, 901(2, 304 USD)

**Kg of Groundnut planted per ha = 225 Kg
**Cost of 1 Kg of Groundnut seeds planted = 13.5 RMB (2 USD)
**Price of 1 Kg of Groundnut seeds sold = 4 RMB (0.6 USD)
**Kg of Green gram planted per ha= 56.5 Kg
**Cost of 1Kg of Green gram seeds planted = 17.5RMB (2.5 USD)
**Price of 1 Kg of Green gram seeds sold = 7 RMB (1 USD)
**Cost of Labor = 15 RMB/hr (2.2 USD/hr)
**Kg of maize planted per ha = 52 Kg
**Cost of 1 Kg of Maize seeds planted = 25 RMB (3.6 USD)
**Price of I kg of Maize seeds sold = 2 RMB (0.3 USD)
**Kg of fertilizer applied per ha = 419.87 Kg
**Cost of 1Kg of fertilizer=4 RMB (0.6 USD)
**Time of planting maize (man-hr/ha)= 39 hrs
**Time of fertilizer application= 30 hrs
**Time of harvesting = 45 hrs

Discussion

Effects of Mulching and Intercropping on Weed Biomass

Intercropping and mulching produced the least weed weight as compared to the control plot where no mulch was applied. The weed dry matter in intercrop was statistically significantly lower than sole maize. The results obtained at 6 WAP was due to the initial uniform cover created by the maize straw which hindered weed growth requirement for emergence and establishment and also slow initial growth of legumes. At 12 weeks after planting, the results obtained was due to better legume establishment and decomposition of maize straw. The reason for this was that living cover crop competed with emerging

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

and growing weed for the essential resource and prevented weed emergence and establishment more than dead mulch. The control plot (no mulch) recorded the highest weed biomass at each sampling times. The results obtained are in line with the findings of (Bilalis et al., 2010) who both obtained highest weed dry matter in maize mono cropping as compared to intercrop systems thus considering maize-bean and maize-cowpea and other maize-legume intercrops even though different from the treatments observed in this experiment. Bilalis et al. (2010) observed that intercropping maize and legumes reduce weed density when compared with maize mono-cropping as available light needed for weed to emerge decreases with intercropping. Mehmood et al. (2018) found lower weed biomass when rice straw was used as mulch in maize cultivation under rain-fed conditions. Considerable results were observed with other mulch materials in dealing with weed biomass.

Also in their findings weed biomass increased on the plot where live mulch (soya bean-maize intercrop) was used as weed control agent during the early stages of growth as compared with the weed biomass of rice straw mulch which is in direct connection with our findings. Wayayok et al. (2014) also found weed biomass density with rice straw as compared to no mulch application, which concurs with our findings even though the crop sown was rice. The suppression of weed at the early stages of growth observed in the maize straw-maize plots and later stages observed in the legume-maize plots was due to the decrease in available light for weed to emerge. Moreover, leguminous crops have a rapid canopy development which aids them competes with weeds for growth requirements such as light, water, air and nutrients hence leading to stunted and slow weed growth.

Maize Yield Parameters

Effects of Intercropping on Maize Grain Yield

An intercrop is mostly grown for the purpose of making use of interspace which is not fully utilized by main crop in early growth period. Practicing intercrop system may reduce yield of main crop, based on the species, spatial arrangement of component crops, and environmental conditions.

Maize yield parameters measure showed no significant differences among treatments at the two locations where the experiments were performed even though maize grain yield on the control plot (no mulch) was higher as compared to the other treatments. The results are in agreement with the finding of Legwaile et al. (2012) who reported no significant differences in the number of maize cobs and 100 seed weight when sole maize and maize-cowpea were compared.

The insignificant difference obtained would suggest that maize that will be planted in the subsequent season in the same field will benefit from the residual nutrients set free by the leguminous crops (Nyasasi and Kisetu, 2014). The results obtained by Patel et al. (2018) disagree with the results of this research. In their findings, maize grain yield obtained from sole maize treatment was significantly higher when compared to the maize-green gram and maize-cowpea intercropping systems. Also, the results of the research performed by Nyasasi and Kisetu (2014) also dispute our findings. In their study, there was a significant difference in the yield of sole maize as compared to that of maize-cowpea intercrop. A reduction in maize yield, when inter-cropped with legumes, could be attributed to the nature of leguminous plant considered for the intercropping. Moreover, environmental factors could also lead to yield reduction as legume crops could

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

compete with maize for growth and yield conditions when such conditions are not sufficiently available.

Economic Assessment

The results obtained at both locations show that a maize farmer in Ghana and China respectively could earn an additional income of 17, 691 GHC (3, 610 USD) and 13, 443 RMB (1, 923 USD) more when he/she invests in green gram-maize instead of cultivating maize alone without mulching (control treatment). A maize farmer in Ghana and China could also earn an additional income of 16, 814 GHC (3, 431 USD) and 11, 726 RMB (1, 699 USD) when he/she invest in green gram-maize instead of cultivating maize under maize straw mulch (maize straw-maize treatment). Again a maize farmer could earn an additional income of 6, 308 GHC (1, 287 USD) and 6, 388 RMB (926 USD) in Ghana and in China respectively when he/she invest in groundnut-maize instead of cultivating maize alone without mulching (control treatment). The farmer could more also earn an additional income of 5, 431 GHC (1, 108 USD) and 4, 671 RMB (677 USD) in Ghana and China higher than cultivating maize under maize straw mulch (maize straw-maize treatment).

The results clearly show that, growing maize and intercropping with legumes such as green gram and groundnut could result in higher net returns. The higher net returns earned from legumes was due to lower cost incurred for the control of weeds and the income gain from selling the harvested seeds. Even though at both locations, green gram-maize recorded the highest net returns. These results in line with the findings of Kheroar and Patra (2013) who recorded a higher monetary advantage of legume-maize intercrop over maize mono-cropping. However, in their findings-groundnut intercrop recorded the highest net returns followed by maize-green gram intercrop with sole maize, giving the lowest profit. Patel et al. (2018) found a higher maize equivalent yield and monetary advantage with maize-green gram intercrop over sole maize and maize-cowpea intercrop due to higher price of green gram. The results also agree with the findings of Kermah et al. (2017) who also found a higher monetary advantage of intercropping over sole maize cultivation; however, their focus was on cowpea-maize intercrop. Seran and Brintha (2010) also found higher cash returns with intercropping over mono- cropping. The results also concur with the findings of Yusuf et al. (2014) who found a higher net benefit with maize-soybean intercrop than sole maize and sole soya bean.

Conclusions

We concluded from the experimental findings that, the two intercropping treatments namely, green gram-maize and groundnut-maize, proved to be profitable as compared to the other treatments at both locations. This result is due to their suitability for weed control and the extra income gained from the sales of the grains harvested. Therefore, legume-maize intercrop mulching should be part of weed management techniques for smallholder maize farmers in most parts of the world as it reduces labor input, ensures land-use efficiency and provides extra income. In addition to that, organic residues added to the soil could improve the physical, biological and chemical condition of the soil by reducing soil erosion, improving the soil structure, aggregate stability, water holding capacity, porosity of the soil, enhances microbial organism multiplication and boost soil nutrients through the decomposition of organic residues. Anyway, the adoption of a specific mulching method may depend on some factors such as socioeconomic,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

environmental and mental needs and knowledge about the practices and individual perception about the methods used to achieve those needs. Culture also could influence attitude and behavior intention towards innovation which has been shown to affect the decision to adopt.

Recommendations

Further research should be done on different leguminous crops and different organic mulch material at different regions of the world to test the efficiency of leguminous crop and mulch as a general weed control agent in maize cultivation. More also, further research should consider herbicide application as one of the treatments. Finally, further research should test the residual effects of the leguminous crops and maize straw on the soil physicochemical properties.

Acknowledgments. This work was supported by funding from the National key R&D project sub-project (2017YFD0300405--4), Natural Science Foundation of Jilin Province, China (20170101077JC).

Conflict of interests. The authors declare that they have no conflict of interests.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors. The article is an original paper, is not under consideration by another journal, and has not been published previously. All authors read and approved the final manuscript.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

APPENDIX

Mathematical Expressions:

$$R_{MC} = Y_{MC} \times MP_M \quad \text{(Eq.1)}$$

$$R_{MGG} = Y_{MGG} \times MP_M \quad \text{(Eq.2)}$$

$$R_{MGN} = Y_{MGN} \times MP_M \quad \text{(Eq.3)}$$

$$R_{MMS} = Y_{MMS} \times MP_M \quad \text{(Eq.4)}$$

$$R_{GG} = Y_{GG} \times MP_{GG} \quad \text{(Eq.5)}$$

$$R_{GN} = Y_{GN} \times MP_{GN} \quad \text{(Eq.6)}$$

$$TC_C = C_{MS} + C_{MP} + C_{FA} + C_{MH} + C_{WC} \quad \text{(Eq.7)}$$

$$TC_{GG-M} = C_{GGS} + C_{GGP} + C_{WGG} + C_{GGH} + C_{MS} + C_{MP} + C_{FA} + C_{MH} \quad \text{(Eq.8)}$$

$$TC_{GN-M} = C_{GNS} + C_{GNP} + C_{WGN} + C_{GNH} + C_{MS} + C_{MP} + C_{FA} + C_{MH} \quad \text{(Eq.9)}$$

$$TC_{MS} = C_{MA} + C_{WMS} + C_{MS} + C_{MP} + C_{FA} + C_{MH} \quad \text{(Eq.10)}$$

$$TR_C = (Eq.1)$$

$$TR_{GG-M} = (Eq.2) + (Eq.5)$$

$$TR_{GN-M} = (Eq.3) + (Eq.6)$$

$$TR_{MS-M} = (Eq.3)$$

$$P_C = (Eq.1) - (Eq.7)$$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_77957812

PHYTOSOCIOLOGICAL ASSESSMENT OF BROWSING VEGETATION IN ASSOCIATION WITH EDAPHIC FACTORS IN THE CHOLISTAN RANGELANDS OF PAKISTAN

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(Received 9th Jan 2020; accepted 17th Sep 2020)

Abstract. Rangelands are temporally and spatially unique socioecological systems supporting natural vegetation and livestock production. The browsing vegetation of Cholistan rangelands in Pakistan is degrading due to multiples stresses therefore a phytosociological survey was carried to determine the browse community structure and their association with edaphic features. In this study a total of 25 browse species belonging to 12 families and 17 genera were identified among which Chenopodiaceae, Mimosaceae, and Rhamnaceae were found to be the dominant families. According to vegetation analysis, twenty browse communities were documented based on the Importance Value Index (IVI) of each species. Multivariate analysis has delineated three vegetation associations inhabiting the sand-dune, inter-dune sandy and clayey saline habitats. Detrended Correspondence Analysis (DCA) of sites has maintained the coherency with vegetation groups identified by cluster analysis (CA). In Canonical Correspondence Analysis (CCA) soil electrical conductivity, sodium, organic matter, phosphorus, potassium were most important variables influencing range site distribution. Soil physicochemical analysis revealed that texture of sand-dune habitat was sandy; inter-dune was sandy loam while clayey saline was clayey. This study has revealed that browsing vegetation pattern was predominantly associated to soil attributes. Results showed that organic matter, and soil nutrients were better at inter-dune sandy habitat whereas pH, electrical conductivity, sodium, and soil moisture were high at clayey saline habitat. The outcome of this research suggested that browse cover of Cholistan rangelands was under stress and varied according to seasons. Consequently, plant species with low IVIs need priority measures for conservation and those with high IVIs need monitoring ultimately to make these rangeland resources sustainable.

Keywords: range vegetation, degradation, physicochemical, multivariate, conservation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78137827

Introduction

Understanding the associations among environmental variables in each ecosystem has strong applications in conservation and management (Zhou et al., 2016). In Range ecosystems, describing environmental variables which affect ecological process has been identified as main issue in plant ecology (Kong et al., 2015). Environmental factors perform vital role in determining spatial variation of biodiversity on broad geographical extent. Arid and semi-arid areas are mainly described by their sparse vegetation which makes them vulnerable to external or internal factors (Gharechelou et al., 2016). To examine vegetation structure and relationships between communities and their environment, quantitative classification and ordination have been broadly used (Li et al., 2018).

Assessing and monitoring are the first steps in understanding the ecological position of rangeland vegetation. Investigating the relationship between range plants and environmental variables has been the aim of many ecological studies. Ecologists have documented that environmental variables may control rangelands species composition and distribution (Briske et al., 2015). Rangelands cover about 40% of global land surface and are essentially the larger tracts of natural vegetation, used to support livestock (Holechek et al., 2011). Range livestock production at present supports the livelihood of an estimated 01 billion people and estimated 02 billion people depend on products from rangelands (Sayre, 2017). Majority of these rangelands are in vegetation biomes such as grasslands, shrublands, savannas, and deserts which are often characterized by arid climate (Friedel et al., 2000).

Pakistan is a sub-tropical country, and out of its total area (80 million ha) 49 million ha has been classified as rangelands which are almost consisting of arid to semiarid condition. The rangelands of Pakistan show a great diversity of species composition, structure, and productivity to support livestock (Majeed et al., 2002). Rangelands are very important because they provide vegetation cover, protection for soil, which also ensures sustainable production of animal feed. Especially browse plants (shrubs and tree foliage) beside grasses compose one of the cheapest sources of feed for animals in many parts of the World. Mostly browses have advantage of maintaining their nutrition and greenness during the dry season when grasses decline in both quantity and quality (Abdullah et al., 2013a).

In Pakistan, previous policies have always supported crops production over livestock, leading in the misuse of rangelands. Rangelands of Pakistan are degrading due to short growth period, over grazing, droughts, and marginal availability of productive species. The vegetation of these rangelands only flourishes in monsoon season, these problems are common everywhere in world where arid or semiarid rangelands exist. Therefore, developing countries like Pakistan face same situation in their rangeland's health (Harris, 2010). The rangelands of Pakistan are not managed by scientific approaches and presently, only 10-15% of their actual potential is being documented (Ali et al., 2001).

The desertification of rangelands does not refer to the spreading out of existing deserts, but to the process of land degradation in these natural ecosystems (Kong et al., 2015). Deserts are unique habitats, categorized by punishing temperature, extreme wind velocity, lack of moisture, high solar radiations and coarse topography where very limited species of animals and plants are adapted to stay alive (Van-Auken, 2000; McIntosh et al., 2019). A good percentage of human population in the subcontinent depend upon desert resources and increasing human population and communication links has extended the human misuse of these biotic resources (Middleton and Sternberg, 2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78137827

The Cholistan desert was formerly a prosperous area but now largely converting into an abandoned patch. The productivity of its rangelands is degrading because the livestock number is increasing; ultimately, carrying capacity is decreasing. Sustainability of life in this hot desert rotates around the rainfall. During summer season, weather is tremendously severe and harsh; certain xeric plant species survive but suffer high grazing pressure. Resultantly, the palatable species are diminishing and unpalatable species with less nutritious properties are becoming abundant. Continuous increase in human population for livelihood and multiplying number of livestock is adding towards desertification (Arshad et al., 2001; Farazmand et al., 2019).

In Cholistan rangelands during summer season, nomadic pastoralists migrate with their cattle, sheep, and goats towards nearby canal-irrigated areas. However, a few male members of some clans remain in desert for their camel herds. These camels are seen everywhere in desert, browsing on different shrubs and tree species (Abdullah et al., 2017a). Due to year-round stress, the browses of Cholistan rangelands are under severe threat and need detail assessment. Therefore, it was urgently required to collect the base line data about browsing vegetation and based on this information to chalk out their conservation strategy.

To better manage the rangelands, it is very important to study the relation between vegetation and environmental factors (Brinkmann et al., 2009). Despite some previous work, mostly data are missing about the ecological aspects of Cholistan rangelands. Equally lacking are quantitative data about distribution of plant species, and their response to environment. The aim of this study was to analyses the browsing species composition and structure in Cholistan rangelands along environmental gradients. We hypothesize that environmental factors discriminate best between the vegetation types identified by a multivariate analysis and explain a high proportion in variability of plant species composition and structure.

Material and methods

Research area

This study was conducted in Cholistan desert of Pakistan which is a part of Great Indian desert (longitudes 69° 52′ and 75° 24′ E and latitudes 27° 42′ and 29° 45′ N) covering an area of about 2.6 million ha (*Fig. 1*). It is an arid sandy desert where mean annual rainfall varies from less than 100 mm in west to 200 mm in east, mostly received in monsoon season (July to September). The mean summer temperature (May-July) is 34-38 °C with the highest reaching over 51.6 °C. The soil of Cholistan desert is mostly alkaline, saline, and gypsiferous composed of schists, gneiss, granites, and slates. Sand dunes are common in Cholistan and reach an average height of about 30 m to 100 m. Cholistan contains large alluvial saline flats (locally called dahars) alternating with low sandy ridges. These soils are classified as either saline or saline sodic, with pH ranging from 8.2 to 8.4 and from 8.8 to 9.6, respectively. The vegetation of this desert consists of xerophytes, adjusted to low moisture, extremely hot temperature, and more salinity with wide variation of edaphic factors (Abdullah et al., 2017b).

Phytosociological survey and analysis

A reconnaissance survey was conducted in January 2014, to have an impression of site conditions, accessibility, overview of plant assemblages and to determine the sampling

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78137827

and data collection methods. According to schedule, whole research project was carried for two consecutive years i.e. 2014 and 2015. After going through the topographic map of area followed by frequent visits during initial stages of study, research area was divided into 20 sites to cover the variations of physiognomy and physiography (*Fig. 1*). Name of each sampling site, topography, and coordinates are given in *Table 1*.

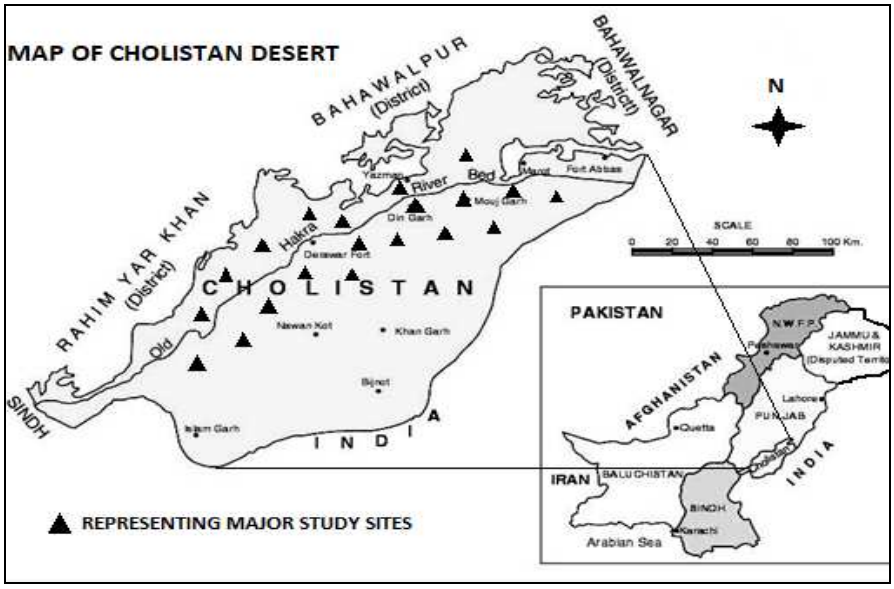

Figure 1. *Map of study area; the Cholistan desert of Pakistan*

Table 1. *Name, location, and topography of each survey site in study area*

Sr. No.	Site name	Topography	Elevation	Latitude	Longitude
1	Mansora	Sand-dune	121 m	N: 29°12.161′	E: 072°15.427′
2	Kalapahar	Clayey saline	117 m	N: 29°10.430′	E: 072°05.569′
3	Chaklihar	Inter-dune sandy	119 m	N: 29°11.315′	E: 071°57.648′
4	Januwali	Inter-dune sandy	124 m	N: 29°05.056′	E: 072°09.933′
5	Khirsir	Sand-dune	119 m	N: 29°10.339′	E: 072°08.749′
6	Haider wali	Clayey saline	116 m	N: 29°02.672′	E: 072°10.200′
7	Mojgarh Fort	Sand-dune	119 m	N: 29°01.059′	E: 072°08.106′
8	Chelanwala Toba	Inter-dune sandy	112 m	N: 28°57.261′	E: 072°03.089′
9	Khanser	Sand-dune	107 m	N: 28°59.227′	E: 071°55.299′
10	Aldin Mor	Inter-dune sandy	104 m	N: 28°47.988′	E: 071°45.770′
11	Dingarh Fort	Clayey saline	111 m	N: 28°57.454′	E: 071°51.910′
12	Dingarh Fort	Sand-dune	113 m	N: 28°57.182′	E: 071°49.362′
13	Nidamwala Toba	Clayey saline	108 m	N: 28°52.963′	E: 071°44.270′
14	Mehmodwala Toba	Inter-dune sandy	102 m	N: 28°47.939′	E: 071°45.770′
15	Lakhan	Clayey saline	107 m	N: 28°52.232′	E: 071°42.731′
16	Chananpir	Inter-dune sandy	108 m	N: 28°56.832′	E: 071°40.057′
17	Baylawala	Inter-dune sandy	125 m	N: 29°23.466′	E: 071°39.563′
18	Derawar fort	Sand-dune	102 m	N: 28°49.208′	E: 071°28.129′
19	Derawar fort	Inter-dune sandy	105 m	N: 29°23.465′	E: 071°39.560′
20	Chasma Dhar	Clayey saline	098 m	N: 28°39.864′	E: 071°15.632′

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DOI: http://dx.doi.org/10.15666/aeer/1806_78137827

Analytic phase (Botanical survey) was concerned with acquisition of all relative vegetation data, present in the sites. Phytosociological parameters consisting of frequency, density and plant cover are considered necessary for complete analysis of vegetation. To calculate the quantitative vegetation parameters, Line Transect and Quadrate methods were used (Mueller-Dumbois and Ellenberg, 1974; Chul and Moody, 1983) as shown in the layout *Figure 2*. The importance Value (IV) for each species was calculated by direct summation of relative density, relative frequency, and relative cover. The specific site position (latitude, longitude, and altitude) was determined by a GPS (Global Positioning System) named Garmin eTrex.

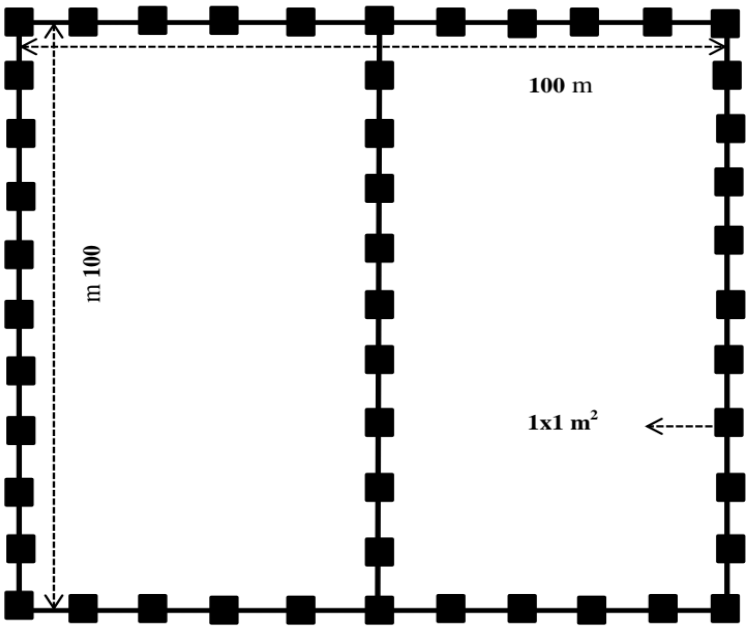

Figure 2. *Layout of data collection method at each site (05 transect of each 100 m length with quadrat of 1 × 1 m² on 10 m interval)*

Synthetic phase (Data analysis) follows the classification of data to obtain groupings of communities based on floristic and structural similarities. The arrangement of surveyed data and mathematical classification was obtained by using the multivariate statistical program MVSP Ver. 3.2 (Kovach 1985-2002). The vegetation classification was made using IVI based data and name of communities were given after two or three dominant species with higher synoptic values. The ordination techniques (DCA, CCA) were also applied to dataset to provide evidence for possible gradients in and between communities and to detect habitat gradients that may be associated with vegetation analyses, and to identify environment plant interactions.

Soil analysis

For soil analysis, soil sample of 1 kg was collected along each transect from the depth of 0-30 cm. Five soil samples are collected from each site and these samples were pooled together to form one composite sample, air-dried, thoroughly mixed, and passed through 2 mm sieve to liberate it from gravel and boulders. The collected samples were stored in polythene bags and labeled for physical and chemical analysis in soil laboratory (Head, 2006).

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Results

Classification

According to phytosociological survey twenty types of browse communities were identified based on Importance value index of each species, on three different landforms in Cholistan rangelands. Cluster analysis was used to classify the vegetation of Cholistan rangelands into relatively homogeneous groups of range sites by using importance value index of each species. Here results have been presented in form of dendrogram (*Fig. 3*) which has delineated three vegetation associations inhabiting the sand dunes, inter-dune sandy patch, and clayey saline flats. Pictogram images of three desert habitats have been shown in *Figure 4*.

In Sand-dune association (Cluster A) six sites were included i.e., 1, 5, 7, 9, 12, and 18, as shown in *Figure 3*. This association was comprised of six browse communities while the habitat of these sites was sand dune. Total 21 plant species were recorded in this association out of which there were six browse species. In sand-dune association dominant browse species were *Calligonum polygonoides* (IV = 60.78) and *Haloxylon salicornicum* (IV = 59.85) while *Aerva javanica* (IV = 14.43) *Crotalaria burhia* (IV = 12.79) and *Leptadenia pyrotechnica* (IV = 10.75) were associated species (*Table 2*). Physicochemical analysis of soil showed that texture of this association was sandy with pH 8, electrical conductivity (EC) 1.63, soil moisture 0.39% and organic matter was 0.32%. The concentration of sodium (Na), potassium (K), phosphorus (P), was calculated as 18.15 ppm, 29.54 ppm and 3.93 ppm respectively (*Table 3*).

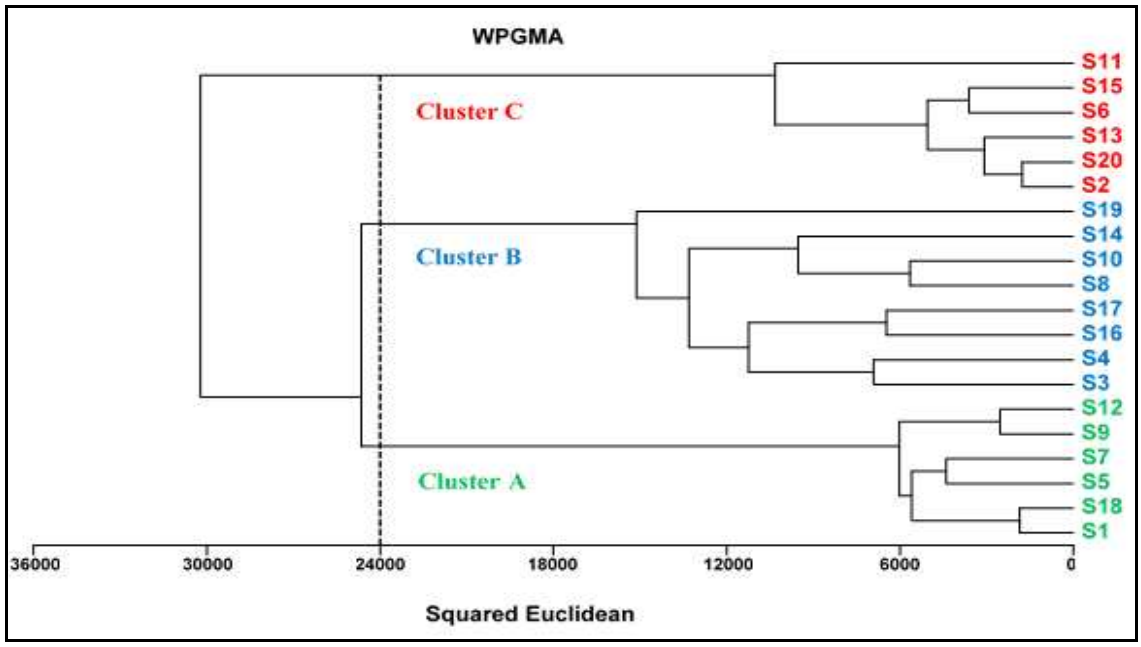

Figure 3. *Dendrogram from cluster analysis of 20 range sites representing A = sand-dune association, B = inter-dune sandy association, C = clayey saline association*

Inter-dune sandy association (Cluster B) was consisting of eight sites including 3, 4, 8, 10, 14, 16, 17, and 19 (*Fig. 3*). The habitat of these sites was inter-dune sandy. In this cluster, total 38 plant species were present out of which 13 species were browses. This association was comprised of eight browse communities, whereas most dominant

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browse species was *Leptadenia pyrotechnica* (IV = 46.40) followed by *Aerva javanica* (IV = 42.14) Crotalaria burhia (IV = 41.33) and *Salsola baryosma* (IV = 37.82) respectively. However, *Haloxylon salicornicum* (IV = 26.36) *Capparis deciduas* (IV = 22.85) *Calotropis procera* (IV = 20.81) *Calligonum polygonoides* (IV = 20.68) were considered as associated species (*Table 2*). Soil texture of this association was sandy loam with pH 08.25 and EC 03.61. The contents of Na, K, P, were calculated as 32.22 ppm, 56.60 ppm and 5.97 ppm respectively, whereas soil moisture was 0.63% and organic matter was 0.74% (*Table 3*).

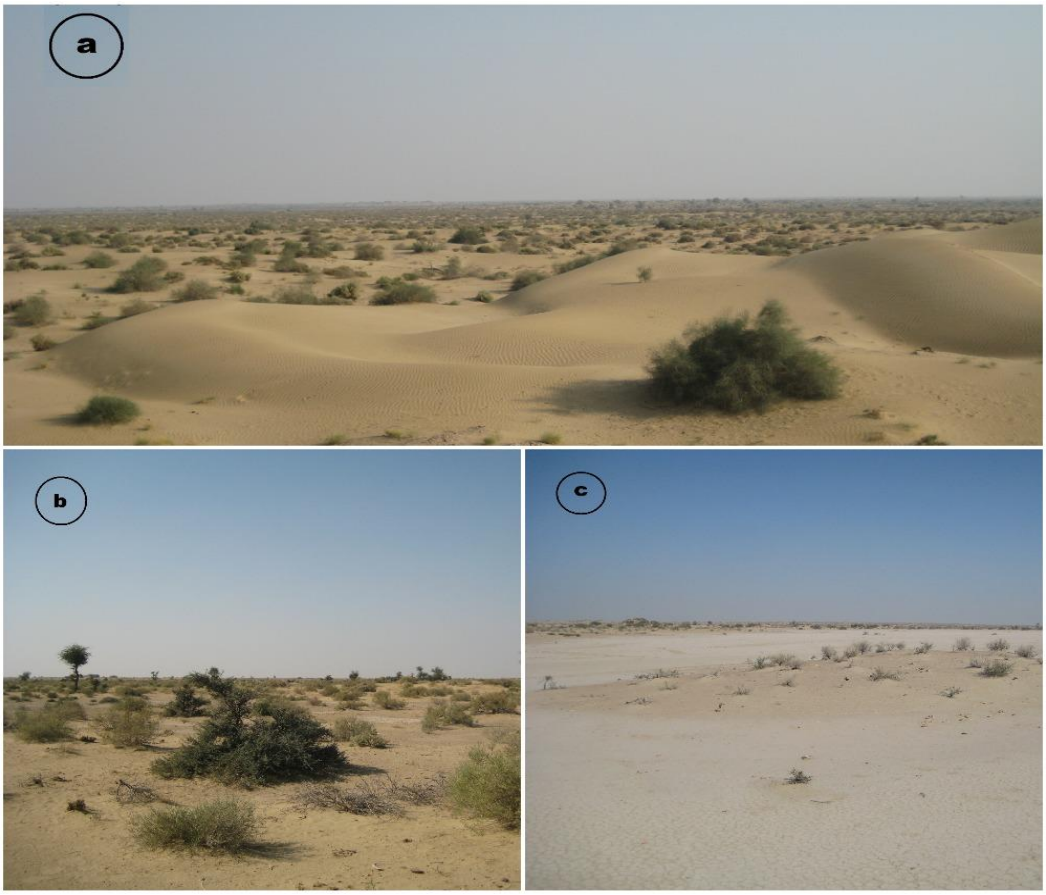

Figure 4. *Pictorial image of a = sand-dune association, b = inter-dune sandy association, c = clayey saline association*

Clayey saline association (Cluster C) was consisting of six sites i.e. 2, 6, 11, 13, 15, 20, as shown in *Figure 3*. This association was comprised of six browse communities and habitat of these stands was clayey saline. Total 23 plant species were observed in this association in which 11 species were browses, whereas dominant browse species were *Suaeda fruticosa* (IV = 79.02) and *Haloxylon recurvum* (IV = 75.25) while *Salsola baryosma* (IV = 39.38) *Calotropis procera* (IV = 20.68) *Pulicaria rajputanae* (IV = 15.60), *Abutilon muticum* (IV = 13.79) *Tephrosia uniflora* (IV = 13.24) were associated species (*Table 2*). Based on soil analysis of these sites, soil texture was clayey with pH 8.46 and EC 10.98. The concentration of Na, K, P, was 77.29 ppm, 22.61 ppm and 2.49 ppm respectively, whereas soil moisture was 0.84% and organic matter was 0.20% (*Table 3*).

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Table 2. *Mean importance values of plant species in three associations*

Sr. No.	Plant species	A	B	C	Habit
1	*Abutilon muticum* (Del. ex. DC.) Sweet.	0	12.52	13.79	Shrub
2	*Acacia nilotica* (Linn.) Del	0	2.74	0	Tree
3	*Aeluropus lagopoides* (Linn.) Trin. ex Thw.	0	0	28.26	Grass
4	*Aerva javanica* (Burm. f.) Merill	14.43	42.14	0	Shrub
5	*Aristida adscensionis* Linn.	0	5.20	0	Grass
6	*Calligonum polygonoides* Linn.	60.78	20.68	0	Shrub
7	*Calotropis procera* (Aiton.) Aiton.	0	20.81	20.68	Shrub
8	*Capparis decidua* (Forsskal.) Edgew.	0	22.85	10.97	Shrub
9	*Cenchrus biflorus* Roxb.	5.99	0	0	Grass
10	*Cenchrus ciliaris* Linn.	28.84	10.14	0	Grass
11	*Cenchrus setigerus* Vahl.	0	7.89	0	Grass
12	*Chrozophora plicata* (Vahl) A. Juss. ex Spreng.	4.00	3.12	0	Herb
13	*Citrulus colocynthis* (Linn.) Schrad.	3.19	2.23	0	Herb
14	*Convolvulus microphyllus* Sieb. ex Spreng.	0	0	3.13	Herb
15	*Corchorus depressus* (Linn.) Stocks	0	2.32	4.55	Herb
16	*Crotalaria burhia* Buch. Ham. ex Benth.	12.79	41.33	0	Shrub
17	*Cymbopogon jwarancusa* (Jones) Schult.	0	29.67	40.89	Grass
18	*Dipterygium glaucum* Decne.	62.74	44.41	0	Herb
19	*Euphorbia prostrata* Ait.	2.03	1.84	2.50	Herb
20	*Fagonia cretica* Linn.	0	7.94	10.90	Herb
21	*Farsetia hamiltonii* Royle	15.15	1.54	0	Herb
22	*Gisekia pharnaceoides* Linn.	0	1.87	0	Herb
23	*Glinus lotoides* Linn.	0	0	1.68	Herb
24	*Haloxylon recurvum* Bunge. ex. Boiss.	0	0	75.25	Shrub
25	*Haloxylon salicornicum* (Moq.) Bunge.	59.85	26.36	0	Shrub
26	*Heliotropium crispum* Desf.	0	11.18	10.34	Herb
27	*Lasiurus scindicus* Henr.	29.22	19.91	0	Grass
28	*Leptadenia pyrotechnica* (Forssakal.) Decne.	10.75	46.40	0	Shrub
29	*Limeum indicum* Stocks. ex. T. Anderson	10.14	3.32	0	Herb
30	*Mollugu cerviana* (L.) Seringe	2.16	1.13	0	Herb
31	*Ochthochloa compressa* (Forssk.) Hilu	0	41.03	9.25	Grass
32	*Pennisetum devisum* (Gmel.) Henr.	0	25.64	0	Grass
33	*Panicum turgidum* Forssk.	44.14	30.67	0	Grass
34	*Polygala erioptera* DC.	1.36	1.34	0	Herb
35	*Prosopis cineraria* (Linn.) Druce.	1.47	2.91	0	Tree
36	*Pulicaria rajputanae* Blatt. & Hollb	0	0	15.60	Herb
37	*Salsola baryosma* (Roem. et. Scult.) Dany.	0	37.82	39.38	Shrub
38	*Salvadora oleoides* Decne.	0	0	2.59	Tree
39	*Sesuvium sesuvioides* (Fenzl.) Verdc.	2.62	2.22	0	Herb
40	*Solanum surattense* Burm. f.	0	2.07	4.16	Herb
41	*Sporobolus iocladus* (Nees ex Trin.) Nees	0	31.51	41.26	Grass
42	*Stipagrostis plumose* (Linn.) Munro ex T. Anderss.	10.13	11.20	0	Grass
43	*Suaeda fruticose* (Linn.) Farsskal.	0	2.14	79.02	Shrub
44	*Tamarix aphylla* (Linn.) Karst.	0	0	2.62	Tree
45	*Tephrosia uniflora* (Linn.) Pers.	0	2.21	13.24	Shrub
46	*Tribulus longipetalus* Viv. subsp. macropterus (Boiss.)	1.81	2.45	0	Herb
47	*Zaleya pentendra* Linn.	0	0	2.47	Herb
48	*Zizyphus mauritiana* Lam.	0	0	2.54	Tree

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Table 3. *Summary statistics of environmental variables (soil) at three associations (mean ± SD)*

Assoc.	Depth cm	pH	EC ds/m	Na ppm	K ppm	P ppm	Soil moisture %	Organic matter %	Saturation %	Texture
A	0-30	8.00±0.09	1.63±0.47	18.15±2.82	29.54±2.27	3.92±0.28	0.39±0.07	0.32±0.05	17.67±1.21	Sandy
B	0-30	8.25±0.08	3.61±0.86	32.22±3.79	56.60±11.59	5.97±1.09	0.63±0.07	0.74±0.15	34.87±9.80	Sandy loam
C	0-30	8.46±0.08	10.98±1.0	77.29±10.04	22.61±2.23	2.49±0.38	0.84±0.07	0.20±0.02	61.83±0.98	Clayey

A = sand-dune association, B = inter-dune sandy association, C = clayey saline association, EC = electrical conductivity, Na = sodium, K = potassium, P = phosphorus, SD standard deviation

Ordination

In ordination, (correspondence analysis) DCA ordination (indirect gradient analysis) and CCA ordination (direct gradient analysis) procedures of multivariate statistical methods were used.

Detrended correspondence analysis (DCA)

The distribution of 20 sites along first axis and second axis of detrended correspondence analysis is represented in *Figure 5*. DCA analysis of sites has maintained the coherency with vegetation groups identified by cluster analysis (CA). However, scatter graph is easily interpretable in ecological terms. Stands situated to the left of diagram were representing the sand-dune association (A) and stands situated more to the right of diagram were showing the clayey saline association (C). While the stands in-between them were showing the inter-dune sandy association (B). This graph illustrates a gradient along ordination axes 01 which could be related to high pH, EC, Na and soil moisture in right side at association C and low in left side with association A. Summary of detrended correspondence analysis is given in *Table 4*.

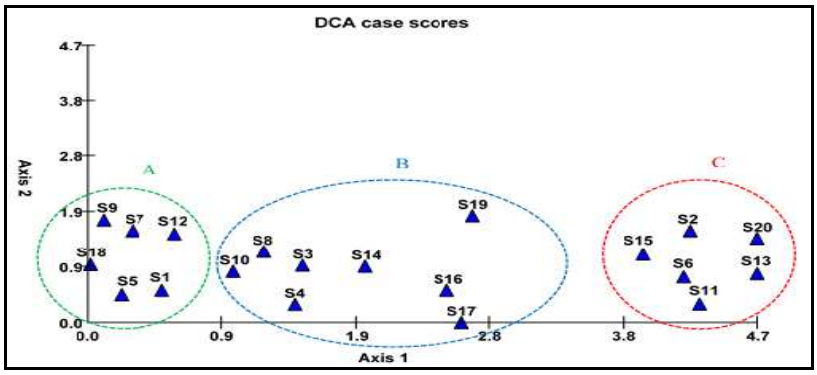

Figure 5. *Detrended correspondence analysis (DCA) of range sites showing A = sand-dune association, B = inter-dune sandy association, C = clayey saline association*

Table 4. *Summary of detrended correspondence analysis (DCA)*

Axis	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.823	0.157	0.084	0.052
Percentage	34.193	6.526	3.482	2.152
Cum. percentage	34.193	40.719	44.201	46.353

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Canonical correspondence analysis (CCA)

To determine the overall pattern of plant species and sites distribution based on environmental factors, CCA ordination was performed on a medium containing importance values of species (n = 48 species) in 20 sites. In biplot (*Fig. 6*), points were representing individual stands and arrow representing soil variables. The length and direction of an arrow representing a given environmental factor provide a sign of importance and direction of gradient of environmental change for that factor. Long arrow was more closely correlated in ordination than those with short arrow and was much significant in influencing the variations in community. Perpendicular vegetation associations near to or beyond the tip of arrows will be strongly correlated and influenced by an arrow while those at opposite end will be less affected.

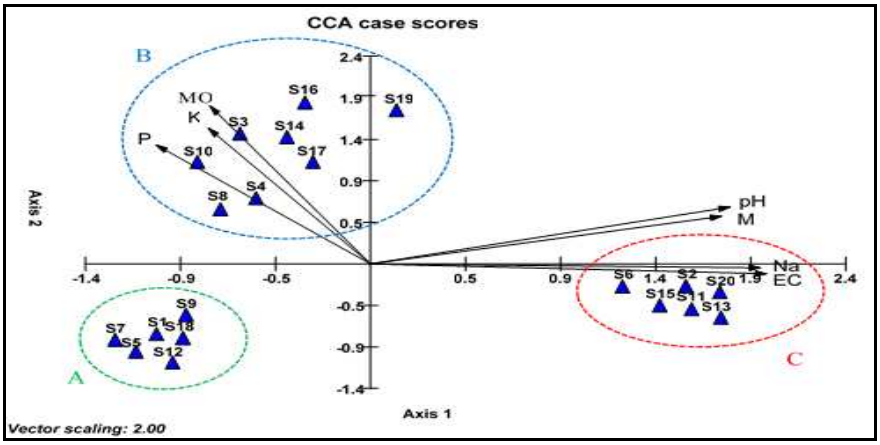

Figure 6. *CCA biplot of range sites with environmental (soil) variables showing A = sand-dune association, B = inter-dune sandy association, C = clayey saline association. (EC = electrical conductivity, Na = sodium, K = potassium, P = phosphorus, OM = organic matter, M = moisture)*

The angle between an arrow and each axis reflects its degree of correlation with axis. The eigenvalues of CCA axis are given in *Table 5*. The first two axes are most important in elucidating the variations in floristic data because these explain 100% variation. As first two axes are standards for determining the variation in data, hence variables that strongly correlate with these axes were considered as most important environmental factors. The continuous decrease of the eigenvalues along the CCA axes has stated a well-structured data set. In CCA of range sites EC, Na, OM, P, K, were most important variables influencing the sites distribution. While going through the axis 01 and 02, some stands were assembled on negative side of axis 01 away from the origin of the axis under the effect of EC and Na. Whereas some stands were scattered on positive side of axis 02 under the influence of OM, P, and K. It was observed, electrical conductivity and sodium were more towards the long arrow having strong correlation and portraying some significant role in grouping of sites.

Discussion

Multivariate procedures are often used to analyze the species composition and their relationships with environmental factors both quantitatively and qualitatively (Kargar

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Chigani et al., 2012). This study has quantitatively classified and ordinated the browsing vegetation with environmental factors (soil) in Cholistan Rangelands of Pakistan. The browse community structure was relatively simple with drought and salt-tolerant plant species. Though there was relatively low biodiversity in study area, variation in the species diversity may be explained by the spatial heterogeneity of habitat and environment (Zhang et al., 2005). Effects of environmental factors on plant communities have been the subject of many ecological studies. It has long been recognized that relationship between plant communities and soil characteristics is a key issue for the ecological restoration of Rangelands (Liu. et al., 2018).

Table 5. *Summary of canonical correspondence analysis (CCA)*

Axis	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5
Eigenvalues	0.77	0.355	0.08	0.069	0.065
Percentage	31.958	14.734	3.324	2.846	2.695
Cum. percentage	31.958	46.693	50.016	52.862	55.557
Cum. constr. percentage	54.266	79.284	84.928	89.76	94.336
Spec.-env. correlations	0.974	0.918	0.883	0.768	0.788

The assessment of vegetation in Cholistan rangelands has documented total 25 browse species, not all identified species were used in the classification of plant communities. There were total twenty browse communities recognized in the twenty range sites of Cholistan desert (Abdullah et al., 2013b). In the studied area, woody and perennial species almost remained same whereas shape of community changed due to the prevalence of annuals species during monsoon, which shows seasonal aspect. Bredenkamp and Brown (2003) agreed that differences in floristically defined plant communities were mostly linked with habitat variables such as topography (landform, aspect, and slope), geology, and altitude, although soil texture, and depth are also necessary components.

According to results, cluster analysis has classified the twenty range sites into three different vegetation clusters. Cluster A was consisting of sand-dune association, cluster B was consisting of inter-dune sandy association and cluster C was consisting of clayey saline association. Generally, these clusters were representing the three ecological habitats i.e. sand-dune, inter-dune and clayey saline in Cholistan rangelands. Results showed that vegetation diversity was poor at sand-dune habitat because soil texture was sandy and mostly consisted of unstabilized sand dunes. Sand-dune association was comprising of six browse communities i.e. *Haloxylon salicornicum-Calligonum polygonoides-Leptadenia pyrotechnica*, *Calligonum polygonoides-Haloxylon salicornicum-Crotalaria burhia*, *Haloxylon salicornicum-Calligonum polygonoides-Aerva javanica*, *Calligonum polygonoides-Haloxylon salicornicum-Aerva javanica*, *Calligonum polygonoides-Haloxylon salicornicum-Leptadenia pyrotechnica* and *Haloxylon salicornicum-Calligonum polygonoides-Crotalaria burhia*. Earlie Arshad and Akbar (2002) have reported related results who have studied the benchmark plant communities of Cholistan desert.

Inter-dune habitat was consisting of small hummocks of sand and vegetation cover was better. This association was comprised of eight browse communities i.e. *Leptadenia pyrotechnica-Aerva javanica-Crotalaria burhia*, *Crotalaria burhia-Leptadenia pyrotechnica-Haloxylon salicornicum*, *Crotalaria burhia-Aerva javanica-Haloxylon*

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salicornicum, *Aerva javanica-Crotalaria burhia-Calligonum polygonoides*, *Salsola baryosma-Crotalaria burhia-Haloxylon salicornicum*, *Salsola baryosma-Leptadenia pyrotechnica-Capparis deciduas*, *Leptadenia pyrotechnica-Salsola baryosma-Haloxylon salicornicum* and *Aerva javanica-Salsola baryosma-Calotropis procera*. Whereas clayey saline association was consisting of six browse communities i.e. *Haloxylon recurvum-Suaeda fruticosa-Pulicaria rajputanae*, *Suaeda fruticosa-Haloxylon recurvum-Salsola baryosma*, *Suaeda fruticosa-Salsola baryosma-Haloxylon recurvum*, *Haloxylon recurvum-Suaeda fruticosa-Tephrosia uniflora*, *Haloxylon recurvum-Suaeda fruticosa-Calotropis procera* and *Suaeda fruticosa-Haloxylon recurvum-Abutilon muticum*. In this association, vegetation diversity was low due salinity and poor organic contents in soil. Similar results have been reported by Akhter and Arshad (2006) who has studied the rangelands plant communities in Cholistan desert.

The results of cluster analysis were verified by detrended correspondence analysis. DCA of sites data has produced the similar vegetation groupings along the axis as identified by the cluster analysis (CA). The three habitats were clearly discernible in ordination diagram along the two axes. Results showed that group of range sites situated to the left of the diagram was representing the sand-dune association and sites situated more to the right of the diagram were showing with clayey saline association whereas sites in-between them were representing the inter-dune association. The DCA graph has illustrated a gradient along ordination axes 1 that could be related to high pH, EC, Na, and soil moisture in right side at clayey saline association (C) and low in left side at sand-dune association (A).

Subsequently the effect of environmental variables on three vegetation associations was carried out by canonical correspondence analysis. Ordination technique CCA has described the overall pattern of plant species and sites distribution based on soil variables. In CCA biplot the most effective soil variables, which have significant correlation with the distribution of vegetation associations were organic matter, phosphorus, potassium, electrical conductivity, and sodium. Result showed that inter-dune association (B) was strongly correlated with organic matter, potassium, and phosphorus whereas clayey saline association (C) was under the strong impact of EC and Na. It was observed that, sand-dune association (A) was located at opposite end of the high EC, Na, pH, and soil moisture, indicating the poor effect of these environmental variables. Arshad et al. (2008) has also reported that edaphic factors has strong influence in distribution plant communities in Cholistan desert.

Physiochemical analysis of soil has revealed that concentration of organic matter, potassium and phosphorus was better at association (B), therefor vegetation was also healthier at inter-dune sandy habitat. However, soil nutrient level was poor both at sand-dune (A) and clayey saline associations, further poor vegetation in these associations was be due to moving sand dunes problem and high salinity level, respectively. Overall, this multivariate analysis has sketched out the vegetation of Cholistan rangelands into three distinct associations: sand-dune, inter-dune sandy and clayey saline along with their specific soil features. To study the vegetation of Cholistan rangelands, much stress was paid on plant communities. The vegetation of Cholistan desert has not been studied properly however, our work confirms the findings of some earlier researchers in this area such as Arshad and Akbar (2002), Akhter and Arshad (2006) and Arshad et al. (2008).

Analyzing the ecological data by using multivariate methods makes simple understanding of complex relationship between plants and environmental gradients (McGranahan et al., 2013). Various studies have investigated the relationship between

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environmental factors and vegetation such as Zare et al. (2011), Mohtashamnia et al. (2011), Wang et al. (2012), McGranahan et al. (2013), Kargar-Chigani et al. (2017), Li et al. (2018) and Eghdami et al. (2019) have showed that soil strongly influence on vegetation formations. Distribution pattern of desert plants and their relationship to soil in Cholistan rangelands by using classification and ordination resulted in a clear display of spatial variability of plant communities. Our results provided the theoretical basis for vegetation conservation, which had laid a foundation for the sustainable development of research area. These results have important implications for management and conservation of rangelands in the context of ongoing climate change. Finally, the use of long-term monitoring which integrate field surveys, climate data, remote sensing, and multivariate context could be a useful tool for range managers with early signs for the onset of desertification (Davis et al., 2020).

Conclusion

This assessment has created a detail map about the status of browsing vegetation in relation to soil variables in Cholistan rangelands. Understanding the indicator of environmental factors of a given site leads us to recommend highly adapted species for improvement of that site. Previously no comprehensive study was reported therefore, present research provided a valuable baseline data on browse species of this arid ecosystem. Disturbed study sites require an immediate protection and species with low importance value index (IVI) needs conservation and those with high IVIs needs monitoring. Actions should be taken to address the problems faced by those species having low importance value index. This data should be incorporated into the current management plan which should serve as a valuable tool in the planning, conservation, and management of these rangelands. All factors considered, it was concluded that browsing species of Cholistan were under stress and they need proper rehabilitation through ecological approaches. Future studies should be focused on determining biological and physical properties of soil, collection of climatic data and protection of endangered species. Consequently, when managing the desert like environment in Pakistan or in world, consideration of appropriate soil factors that ensure biodiversity and ecosystem functions are very important.

Acknowledgments. This work was part of Ph.D. thesis of Mr. Muhammad Abdullah. Authors thankfully acknowledge support from Higher Education Commission (HEC) Pakistan under indigenous scholarship program. Authors are also grateful to Late Dr. Muhammad Arshad (Ex. Director Cholistan Institute of Desert Studies) for his valuable supervision during whole work.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78137827

SEX RATIO, LENGTH-WEIGHT RELATIONSHIPS AND MATURITY STAGES OF SALEMA (*SARPA SALPA* (LINNAEUS, 1758)) FROM THE CENTRAL ALGERIAN COAST (SOUTHWESTERN MEDITERRANEAN SEA)

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(Received 29th Feb 2020; accepted 2nd Jul 2020)

Abstract. The reproductive biology of Salema (*Sarpa salpa* (Linnaeus, 1758)) was investigated along the central Algerian coast (Southwestern Mediterranean Sea) from January to December 2015. A total of 712 individuals were collected and analysed from artisanal fisheries. The length frequency distribution indicated that males were frequent in the smaller size classes from 13.4 to 32.4 cm, while the females in the larger size classes from 24.5 to 48.8 cm. Characterized a protandrous and hermaphrodite species, the sex changes between 17.9 and 36 cm TL. The highest weights recorded were 446.18 and 1 170.6 g for males and females, respectively. The sex ratio (females: males) of the whole sample showed a homogeneous distribution for both sexes. The length-weight relationships indicated an isometric growth of *Sarpa salpa*. The length at 50% maturity was 21.31 cm and 33.15 cm for males and females, respectively. Mature males were found between 16.5 and 29.5 cm and females in length classes of 29.5 and 40.5 cm. Gonadosomatic index clearly indicated two spawning periods (spring and autumn) for both sexes.
Keywords: *Sarpa salpa, sex distribution, reproductive indices, spawning period, Algeria*

Introduction

The sparid *Sarpa salpa* (Linnaeus, 1758), commonly called Salema, is a benthopelagic gregarious fish, found upwards of 70 meters, from shallow waters, near algae or seagrass covered rocks, such as *Posidonia oceanica* and *Cymodocea nodosa*, as well as sandy bottoms (Harmelin-Vivien et al., 1995; Francour, 1997; Guidetti, 2000). This species is the main herbivorous demersal fish of the west Mediterranean Sea (Verlaque, 1990). It feeds on algae, diatoms and macrophytes (Havelange et al., 1997).

The *Sarpa salpa* is widely distributed along the European and African coasts (Eastern Atlantic), from the Bay of Biscay to South Africa (Bauchot and Hureau, 1986; Walt and Mann, 1998). In addition, it is present around Madeira, the Canary Islands, and Cape Verde (Bauchot and Hureau, 1986; Paiva et al., 2016). *Sarpa salpa* is also widely distributed throughout the Mediterranean Sea including Western, central and Eastern regions, and in the Black Sea (Bauchot and Hureau, 1986; Jadot et al., 2006; Pashkov and Reshetnikov, 2012). On the other hand, this species also been recorded in the Western Indian Ocean along the South African coast (Walt and Mann, 1998).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

Much information on several aspects of this species was reported; mainly on food and feeding habits (Christensen, 1978; Anato and Ktari, 1983a; Verlaque, 1985, 1990; Antolic et al., 1994; Havelange et al., 1997). In the Atlantic waters, studies were conducted on age and growth near the Canary Islands (Mendez-Villamil et al., 2001) and in the eastern waters of South Africa (Walt and Beckley, 1997) while age, growth, and reproduction of Salema have been reported on Portuguese coasts (Paiva et al., 2016).

In the Mediterranean Sea, growth, bimometric relationships, some aspects on the maturity and the reproductive biology of *Sarpa salpa* has been described (Sellami and Bruslé, 1975; Anato and Ktari, 1983b; Criscoli et al., 2006; Pallaoro et al., 2008; Acarli et al., 2014; Bayhan and Kara, 2015; El-Etreby et al., 2015). Recently, a study was conducted on *Sarpa salpa* in eastern Algeria (Groud and Kara, 2019) on sex ratio, growth and mortality, but no study on the reproductive biology has been mentioned so far on this species in Algeria. However, *Sarpa salpa* is known to be as a protandrous hermaphrodite (Mendez-Villamil et al., 2002; Paiva et al., 2014; Groud and Kara, 2019).

In Algeria, *Sarpa salpa* represents a potential source for local fishermen. Due to the lack of biological information on this species on the Algerian central coast, scientists are particularly interested in improving the regulation of its fisheries and proposing rational management measures for this species.

The purpose of this work is to acquire knowledge on the biology of reproduction of the *Sarpa salpa* by evaluating, for the first time on the central Algerian coast, its distribution of length and weight, sex ratio, length-weight relationships, maturity stages and the reproductive indices to ensure its sustainability.

Materials and methods

The specimens of *Sarpa salpa* were caught from artisanal fisheries using the trammel nets and the cast nets in the central region of Algeria, mainly at different ports of Cap Djinet, Zemmouri, Ain Taya, Bou Ismail and Cherchell (*Fig. 1*) from January to December 2015. A total of 712 individuals were analysed immediately in the laboratory.

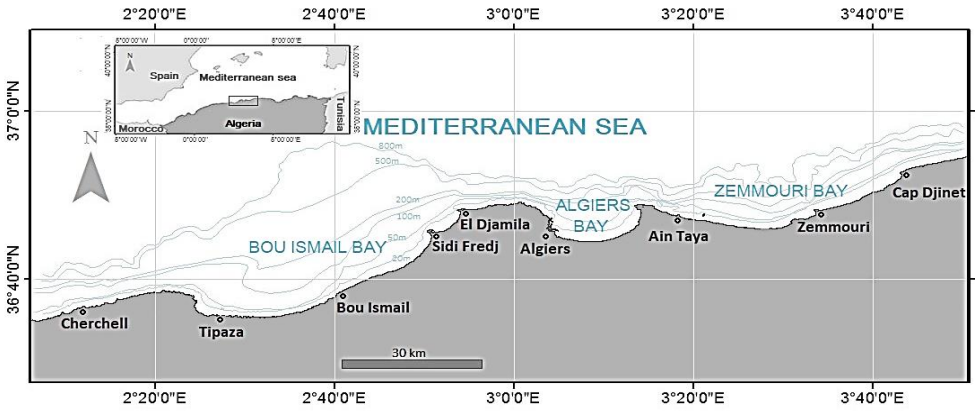

Figure 1. *Location of the study area between Cherchell and Cap Djinet*

For each specimen, the parameter of total length (TL) was taken accuracy 0.1 cm. The total weight (TW) and gonad weight (Wg) were recorded accuracy 0.01 g. Sex

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DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

(males, females, hermaphrodites and undetermined) and maturity stages were analysed macroscopically according to the maturity scale for partial spawners to classify the maturation stage (Holden and Raitt, 1974) (*Table 1*). It was difficult to distinguish stages I and II; this is why they were grouped and considered as the immature stage.

Table 1. *Maturity scale for partial spawners (Holden and Raitt, 1974)*

Stage	State	Description
I	Immature	Ovary and testis about 1/3rd length of body cavity. Ovaries pinkish, translucent; testis whitish. Eggs not visible to naked eye.
II	Maturing virgin and recovering spent	Ovary and testis about 1/2 length of body cavity. Ovary pinkish, translucent; testis whitish, more or less symmetrical. Eggs not visible to naked eye.
III	Ripening	Ovary and testis are about 2/3rds length of body cavity. Ovary pinkish-yellow color with granular appearance, testis whitish to creamy. No transparent or translucent eggs visible.
IV	Ripe	Ovary and testis from 2/3rds to full length of body cavity. Ovary orange-pink in color with conspicuous superficial blood vessels. Large transparent, ripe eggs visible. Testis whitish- creamy, soft.
V	Spent	Ovary and testis shrunken to about 1/2 length of body cavity. Walls loose. Ovary may contain remnants of disintegrating opaque and ripe eggs, darkened or translucent. Testis bloodshot and flabby.

The gonads for each sex, at different stages of maturity, were fixed in 5% formaldehyde and subsequently were performed using standard histological techniques. Sections were cut at 3 µm thickness and stained with Masson's trichrome.

Length-weight relationships were calculated for males and females and for the whole sample using the equation:

$$TW = a\,TL^{b} \qquad \text{(Eq.1)}$$

The isometric hypothesis growth (b=3) was tested using the student's *t*-test. The slopes were tested to verify significant differences between sexes by analysis of covariance (ANCOVA). The analysis of one-way ANOVA was used to verify the possible difference in the total length between males, females and hermaphrodites, performed by the Tukey's test. The sex ratio (females: males) was calculated monthly considering length and season. Significant differences in the ratio of 1:1 were tested by the Chi-square (χ^2).

The total length at which 50% of the fish were sexual mature ($TL_{50\%}$) was estimated for males and females during the spawning period (spring and autumn) using the following logistic function (Ghorbel et al., 1996):

$$p = \frac{1}{1 + e^{-(a+bTL)}} \qquad \text{(Eq.2)}$$

where P was the percentage of mature individuals in the length class. a and b are constants:

$$TL50\% = -\frac{a}{b} \qquad \text{(Eq.3)}$$

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Parameters a and b were determined using least squares regression (Dagnelie, 1973), after converting the data into a logarithmic expression:

$$\mathrm{Ln(TW)} = \mathrm{Ln(a)} + \mathrm{b} * \mathrm{Ln(TL)} \quad \text{(Eq.4)}$$

The monthly evolution of the gonadosomatic index (GSI) (Bougis, 1952) was calculated in order to locate the spawning period. For both sexes, only specimens having reached the size of maturity were considered in the calculation (GSI):

$$\mathrm{GSI} = \frac{\mathrm{Wg}}{\mathrm{TW}} * 100 \quad \text{(Eq.5)}$$

Results

Length and Weight frequency distribution

Of the 712 specimens analysed, 197 (27.69%) were males, 175 (24.58%) were females, 294 (41.29%) were hermaphrodites and only 46 (6.46%) were undetermined (*Fig. 2*).

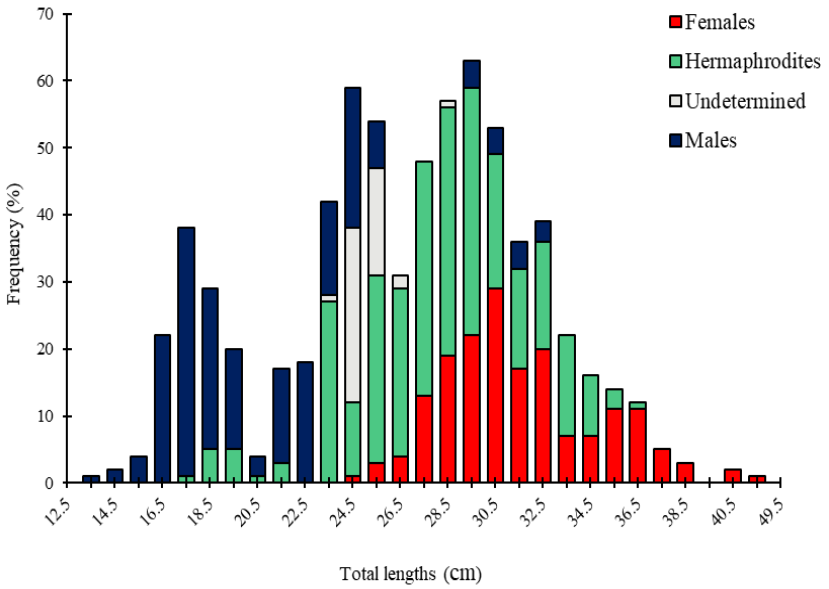

Figure 2. *Length frequency distribution for males, females, hermaphrodites and undetermined from the central Algerian coast*

The length frequency distribution indicated that males were frequent in the smaller size classes from 13.4 to 32.4 cm with an average of 20.81 ± 4.15 cm, while the females in the larger size classes from 24.5 to 48.8 cm with an average of 31.51 ± 3.45 cm. The size range of hermaphrodites individuals extended from 17.9 and 36 cm TL with an average of 27.99 ± 3.55 cm. The highest weights recorded were 446.18 and 1 170.6 g for males and females, respectively. For all sexes, the means, maximum, minimum and standard deviations for total length and total weight were presented in *Table 2*.

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Table 2. *Minimum, maximum, means, and standard deviations values of total length and total weight for females, males, hermaphrodites and undetermined from the central Algerian coast*

	Length (cm)			Weight (g)				Post-hoc test
	Minimum	Maximum	Mean (±SD)	Minimum	Maximum	Mean (±SD)	Number	S
Females	24.5	48.8	31.51 ± 3.45	213.35	1 170.6	440.75± 169.26	175	***
Males	13.4	32.4	20.81 ± 4.15	35.44	446.18	139.90 ± 91.14	197	***
Hermaphrodites	17.9	36	27.99 ± 3.55	76.38	685.78	321.08 ±122.91	294	***
Undetermined	23	28	24.83 ± 0.84	173.18	344.21	211.27 ± 29.19	46	***

S: significant level (*p < 0.05; **p < 0.01; ***p < 0.001; ns: not significant)

The ANOVA test showed that the difference in the total length was highly significant between males, females and hermaphrodites (F= 411.67, P< 0.001). The post-hoc analysis performed by Tukey's multiple comparisons showed that these sizes were highly different (P< 0.001).

Sex ratio

The sex ratio (females : males) of the whole sample showed a homogeneous distribution for both sexes. During all seasons, the sex ratio showed a significant difference between males and females (p< 0.05) with a clear dominance of females in winter and spring, contrariwise males dominated in summer and autumn. However, the monthly distribution of sexes showed that the sex ratio was homogeneous at several months. Furthermore, no sex was observed in January; while in October no female was registered (*Table 3*).

Table 3. *Sex ratio by season, month and total sample from central Algerian coast*

Seasons/Months	Nf	Nm	Ntot	% Females	% Males	Sex ratio	χ2	S
Winter	19	8	27	70.37	29.63	2.38	4.48	*
Spring	115	75	190	60.53	39.47	1.53	8.42	**
Summer	19	74	93	20.43	79.57	0.26	32.53	***
Autumn	22	40	62	35.48	64.52	0.55	5.22	*
January	-	-	-	-	-	-	-	-
February	7	7	14	50	50	1	-	ns
March	79	56	135	58.52	41.48	1.41	3.92	*
April	27	10	37	72.97	27.03	2.70	7.81	**
May	9	9	18	50	50	1	0	ns
June	6	4	10	60	40	1.50	0.4	ns
July	11	28	39	28.21	71.79	0.39	7.41	**
August	2	42	44	4.55	95.45	0.05	36.36	***
September	6	6	12	50	50	1	-	ns
October	-	26	26	-	100	-	-	-
November	16	8	24	66.67	33.33	2	2.67	ns
December	12	1	13	92.31	7.69	12	9.30	**
Total sample	**175**	**197**	**372**	**47.04**	**52.96**	**0.89**	**1.30**	**ns**

Nf: number of females, Nm: number of males, Ntot: total number of individuals, χ2: chi square test, S: significant level (*p < 0.05; **p < 0.01; ***p < 0.001; ns: not significant)

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In the length range where the two sexes overlap (24-33 cm), the sex ratio showed a significant difference ($p< 0.05$). As well at the length classes (25-26 cm), the sex ratio showed a homogeneous distribution of both sexes. Otherwise, the females were largely dominant at lengths range (29-33 cm) while males dominated at the size class (24-25 cm). However, no male was observed in the length range (26-29 cm) and beyond the size class (33-34 cm) (*Table 4*).

Table 4. *Sex ratio by length from the central Algerian coast*

Length class	Nm	Male frequency%	Nf	Female frequency%	Sex ratio	χ^2	S
13-14	1	100					
14-15	2	100					
15-16	4	100					
16-17	22	100					
17-18	37	100					
18-19	24	100					
19-20	15	100					
20-21	3	100					
21-22	14	100					
22-23	18	100					
23-24	14	100					
24-25	21	95.45	1	04.55	0.05	18.18	***
25-26	7	70	3	30	0.43	1.60	ns
26-27			4	100			
27-28			13	100			
28-29			19	100			
29-30	4	15.38	22	84.62	5.50	12.46	***
30-31	4	12.12	29	87.88	7.25	18.94	***
31-32	4	19.05	17	80.95	4.25	8.05	**
32-33	3	13.04	20	86.96	6.67	12.56	***
33-34			7	100			
34-35			7	100			
35-36			11	100			
36-37			11	100			
37-38			5	100			
38-39			3	100			
39-40							
40-41			2	100			
>41			1	100			

Nm: number of males, Nf: number of females, $\chi 2$: chi square test, S: significant level (*$p < 0.05$; **$p < 0.01$;***$p < 0.001$; ns: not significant)

Length-weight relationships

The length-weight relationships were studied for the overall sample; either for males, females and hermaphrodite sexes separately (*Table 5*). The student's *t*-test showed that the value of the slope (b) of the length-weight relationships was not statistically different from 3, which indicated an isometric growth of *Sarpa salpa*.

Furthermore, the slopes comparison (ANCOVA test) between males and females showed a high difference in the length-weight relationship ($F= 1.383$, $P< 0.001$). The same result was obtained ($P< 0.001$) when slopes where compared between hermaphrodite – males and hermaphrodite – females, respectively.

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DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

Table 5. *Parameters of length-weight relationships for males, females, hermaphrodites and total sample*

Sex	a	b	R^2	S
Males	0.0163	2.947	0.98	ns
Females	0.0117	3.042	0.87	ns
Hermaphrodites	0.0117	3.05	0.95	ns
Total	0.015	2.973	0.98	ns

R^2: determination coefficient, a and b: parameters, S: statistical significance of b compared to isometry at 3 (ns: not significant)

Length at first sexual maturity

The total length at 50% sexual maturity ($TL_{50\%}$) was 21.31 cm and 33.15 cm for males and females, respectively (*Fig. 3*).

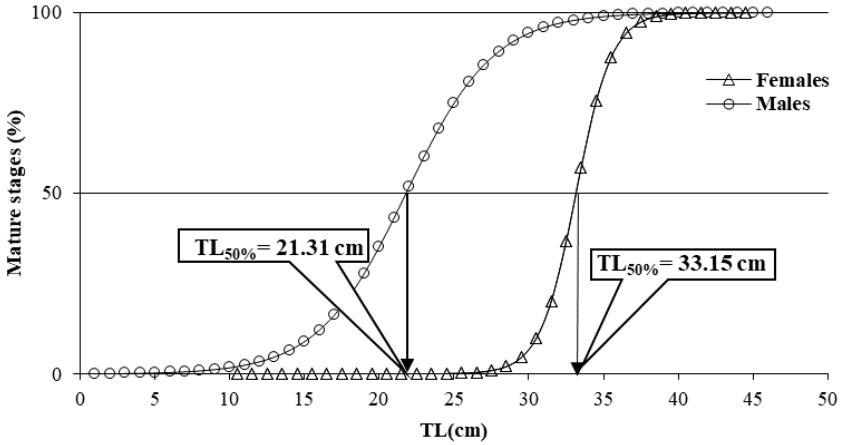

Figure 3. *Sigmoid curves with percentage of sexually mature individuals by length indicating $TL_{50\%}$ for males and females from the central Algerian coast*

The smallest mature male individual was found in the 16-17 cm length interval and measured 16.5 cm with 61.53 g for total weight. For females, the first mature individual was observed in 29-30 cm length interval with 29.5 cm of length and 371.06 g for total weight (*Table 6*).

Maturity stages and reproductive indices

In the whole sample, 28% of females and 16.25% of males were mature. The mature males were found between 16.5 and 29.5 cm, and females 29.5 and 40.5 cm (except for a female 48.5 cm) (*Table 6*). For both sexes, mature individuals appear from March to July and from September to November. However, in February, August and September all males and females were immature. GSI showed two highest spawning periods values in females (1.28 and 2.05) than in males (0.76 and 0.5) at spring and autumn, respectively. Throughout the year, the stage of maturity (IV) was not observed for both sexes (*Fig. 4A,B*).

The inversion of sex occurs between 17.5 and 36.5 cm total length and the examination of hermaphrodite gonads revealed that the juvenile gonad consists on an ovotestis in which, the tissues of males and females were completely separated with a clear domination of the male part (*Fig. 5*).

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Table 6. *Number (N) and percentage (%) of maturity stages for males and females*

Sexes	Males				Females			
	Immature		Mature		Immature		Mature	
Length	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
13-14	1	0.51						
14-15	2	1.02						
15-16	4	2.03						
16-17	19	86.36	3	13.64				
17-18	35	94.59	2	5.41				
18-19	23	95.83	1	4.17				
19-20	9	60.00	6	40				
20-21	3	60.00	2	40				
21-22	12	85.71	2	14.29				
22-23	16	94.12	1	5.88				
23-24	13	76.47	4	23.53				
24-25	17	85	3	15	1	100		
25-26	4	66.67	2	33.33	3	100		
29-30	2	66.67	1	33.33	4	80	1	20
30-31	3	42.86	4	57.14	13	92.86	1	7.14
32-33	2	66.67	1	33.33	19	82.61	4	17.39
33-34					21	77.78	6	22.22
34-35					28	90.32	3	9.68
35-36					13	72.22	5	27.78
36-37					14	60.87	9	39.13
37-38					4	30.77	9	69.23
38-39					2	28.57	5	71.43
39-40					2	40	3	60
40-41					2	50	2	50
>41							1	100
Total	165		32	**16.25%**	126		49	**28%**

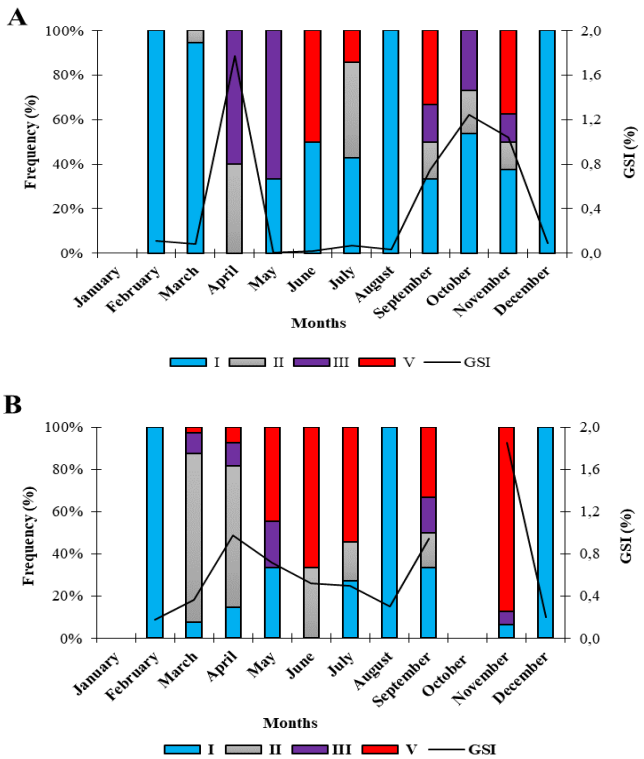

Figure 4. *Monthly variation of the maturity phases and gonadosomatic index (GSI) for Sarpa salpa. Males (A) and females (B). I, immature; II, maturing; III, mature; v, spent*

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DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

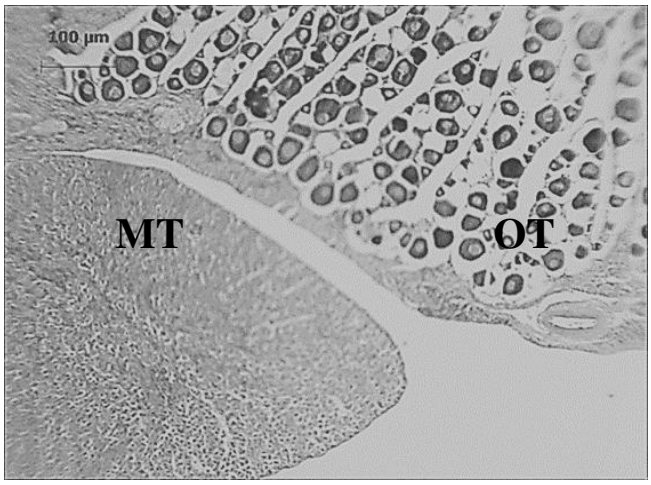

Figure 5. *Cross-section of juvenile hermaphrodite gonad (TL =23.9 cm, W=179.59 g). MT, male tissue; OT, ovarian tissue*

Discussion

The fishery management of hermaphrodite species, like most fish populations, requires knowledge of the lengths of the captured individuals and the specific reproductive pattern of each species. The length frequency distribution of *Sarpa salpa* indicated that males were frequent in the smaller length while the females in the larger length and the hermaphrodites size range was intermediate. This distinction in the average sizes of the three sexes in the sense of males, hermaphrodites and females, confirmed the gradual change of sex process and protandry of Salema. Lissia-Frau and Casu (1968), Walt and Mann (1998), Mendez-Villamil et al. (2002), Criscoli et al. (2006), Paiva et al. (2016) and Groud and Kara (2019) observed the same protandrous hermaphrodism of this species. In other sparid species, such as *Pagellus bogaraveo* and *Pagellus acarne*, the reproductive modalities are of the protandric type (Lechekhab et al., 2010; Boufersaoui and Harchouche, 2015), while they are protogynous in *Boops boops* (Amira et al., 2019). Indeed, Mellinger (2002), observed a wide variety of modalities of sexual change in Sparids.

In our work, the sex ratio (females: males) of the whole sample showed a homogeneous distribution for both sexes. By seasons, it showed a dominance of males in summer and autumn, unlike in winter and spring where the sex ratio was in favor of females that dominated large size classes. The differences in the sex ratio of males and females between the seasons could be explained by the differences in their behavior but the sex of the fish did not change.

However, the results obtained in the central-east Atlantic (Canarian Archipelago) (Mendez-Villamil et al., 2002) and along the eastern coast of Algeria (Groud and Kara, 2019) highlighted the dominance of male. Our findings are also in disagreement with those of Pollock (1985), claiming that the sex ratio of protandric sparids might be skewed towards males. Such differences in sex ratio with *Sarpa salpa* of the central Algerian coasts would be related to the selective fishing nets used for the landing of this species in the study area (the trammel nets and the cast nets).

Length-weight relationships revealed an isometric growth of *Sarpa salpa,* the value of the b parameters for males, females, hermaphrodites and total sample were not significantly different from 3. The same conclusion was reached by Mendez-Villamil et

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DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

al. (2002) for the population of Canarian Archipelago and Criscoli et al. (2006) along the Italian Mediterranean coast.

Allometric growth was shown for females and an isometric growth for males (Pallaoro et al., 2008) while Matic´-Skoko et al. (2004) and Acarli et al. (2014) have observed a positive allometric growth in the Kornati Archipelago and in Izmir Bay (Turkey), respectively. These authors suggested that the difference of the length-weight relationship between males and females was probably due to the difference in length distribution of both sexes as a consequence of the sexual pattern. Additionally, Froese (2006) has reported that data from different areas, not obtained in the same season / year influence the length-weight relationship.

In the overall sample, 28% of females and 16.25% of males were mature. this small ratio of mature fish could be explained by the small number sampled during the two spawning periods (March-June and September-November), as well as the total absence of males in October.

Monthly evolution of percentages of maturity and high GSI values of males and females of Salema during the total period suggested that autumn and spring were the two periods of intense reproductive activity. In the same species, Corbera et al. (1998) and Criscoli et al. (2006) reported a similar spawning periods in the Western Mediterranean Sea. In addition, the breeding activity of this species is spread over a single period while heading east of the Mediterranean Sea. Indeed, Anato and Ktari (1983b), Pallaoro et al. (2008) and El-Etreby et al. (2015) reported a single period in autumn, while Mouneimne (1978) observed maximum breeding in winter. In the northeast Atlantic, this activity also appears to follow a gradient from Cape Verde to Portugal (Mendez-Villamil et al., 2002; Russell et al., 2014; Paiva et al., 2016). Nonetheless, Van der Waltand Mann (1998) observed that the period of the reproductive activity of Salema extended from March to September with peak of maturation from April to August in the east coast of South Africa. All these changes in the reproductive period may be the result of several factors. Wootton (1990) suggested that this difference was due to environmental factors and that temperature appears to be the most important one. Likewise, Sarkar and Upadhyay (2011) suggested that the changes were due to the biotic and environmental factors or the combination of both (Falcón et al., 2003).

In this study, length at first sexual maturity was 21.31 cm and 33.15 cm for males and females, respectively. In the Mediterranean Sea, the determination of the $TL_{50\%}$ of males by different authors was all close to our result with 19.5 cm in central western coasts of Italy (Criscoli et al., 2006), 20.6 cm in the middle-eastern Adriatic (Pallaoro et al., 2008) and 21.1 cm in the Libyan coast (El-Etreby et al., 2015). In Atlantic waters, values of $TL_{50\%}$ for males were significantly higher and in the Portuguese waters 24.5 cm (Paiva et al., 2016) and Canarian Archipelago 26.6 cm (Mendez-Villamil et al., 2002).

In the Algerian coast, the smallest mature female observed was 29.9 cm. Close values have been observed in the Atlantic. Indeed, Paiva et al. (2016) in the Portuguese waters and Mendez-Villamil et al. (2002) in Canarian Archipelago enregistred the smallest mature females with the respective values of 28.6 cm and 29.4 cm.

Conclusion

This study provides knowledge on the sex ratio, length-weight relationships and maturity stages of *Sarpa salpa* (Linnaeus, 1758) from the Central Algerian coast. The length frequency distribution indicated that males were frequent in the smaller size

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DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

classes, while the females in the larger size classes. Characterized a protandrous and hermaphrodite species, hermaphrodites appear between 17.9 and 36 cm TL. The sex ratio (females : males) of the whole sample showed a homogeneous distribution for both sexes. On the other hand, the length-weight relationship indicated an isometric growth of *Sarpa salpa*. The length at 50 % maturity was 21.31 cm and 33.15 cm for males and females, respectively. The smallest mature male individual was found measured 16.5 cm with 61.53 g for total weight. For females, the first mature individual was observed with 29.5 cm of length and 371.06 g for total weight. Gonadosomatic index indicated two spawning periods (spring and autumn) for both sexes.

Future studies should attempt to determine the influence of the protandrous biology of *Sarpa salpa* at different age of its growth. It also very important to study the fecundity process for a better management of stocks in the Algerian coasts.

Acknowledgments. We would like to thanks to Mrs. AINOUCHE N and Mrs. LAMOUTI S, fishery researcher in the National Research Center for Development of aquaculture and fishery and Mrs KHEMILI A for their invaluable help in carrying out this work.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78297841

EFFECT OF SOWING TIME ON GROWTH, PRODUCTIVITY AND NET RETURNS OF ADVANCED COTTON (*GOSSYPIUM HIRSUTUM* L.) CULTIVARS UNDER THE AGROCLIMATIC CONDITIONS OF SOUTHERN PUNJAB, PAKISTAN

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(Received 12th Mar 2020; accepted 11th Aug 2020)

Abstract. Cotton (*Gossypium hirsutum* L.) as an important fiber crop is considered the back bone of Pakistan's economy. Climatic variations causing shift in cropping systems in Pakistan and worldwide. So, it is high time to readjust sowing times. Nutrient deficiency in cotton crops along with delayed sowing has made this situation worse. For this purpose, a two-year field study was carried out at the Central Cotton Research Institute (CCRI) Multan, Punjab, Pakistan during 2016 and 2017. The experiment was laid out in Randomized Complete Block Design (RCBD) with split plot arrangements with three replications. Experimental treatments comprised of five sowing dates *viz.* S_1 = 15th April, S_2 = 01st May, S_3 = 15th May, S_4 = 01st June and S_5 = 15th June and three cultivars *viz.* G_1 = CIM-620, G_2 = Cyto-120 and G_3 = CIM-608. Sowing time significantly influenced the performance of different cotton cultivars. Each delay in sowing dates significantly reduced the seed cotton yield as the lowest yield (1387 kg ha^{-1}) and (1264 kg ha^{-1}) was produced with late sown cotton (June 15th) while the highest seed cotton yield (2759 kg ha^{-1} and 2569 kg ha^{-1}) was produced when crop was sown early on 15th April during both study years 2016 and 2017, respectively. Moreover, cultivars also significantly differ under different sowing dates. Cultivar (CIM-620) produced significantly more seed cotton yield (2456 kg ha^{-1} and 2257 kg ha^{-1}) than (CIM-608) that produced 1971 kg ha^{-1} and 1773 kg ha^{-1} of seed cotton yield during 2016 and 2017 respectively. In conclusion; the cultivar (CIM-620) results in increased seed cotton yield (24.61 and 27.30%) and lint percentage with more fiber brightness. Similarly, the performance of crop sown earlier on 15th May produced higher number of fruiting points, more intact fruits and higher number of bolls. It is directed to the cotton growers not to adopt late sowing with any cultivar particularly CIM-620 for which 15th May is a fairly good suggestion to improve its seed yield with optimum net returns.
Keywords: *climate, cropping patterns, seed cotton yield*

Introduction

Cotton crop plays a major role in the economy of Pakistan and serves as a white gold. It occupies a prominent position in our textile as well as edible oil industry (Government of Pakistan, 2017-18;). Cotton represents the source of basic inputs for the textile industry (Killi et al., 2005), oil expelling and spindle units around the world (Ahmed et al., 2009; Ali and Hameed, 2011). Pakistan is facing continuously lower yield of cotton as compared to other countries. Poor management of soil fertility like improper and unbalanced use of fertilizers is a major reason of low cotton yield in Pakistan (Ali et al., 2009).

Ideal sowing time for a cultivar is thought to be the most vital factor in cotton (Bachubhai et al., 2018; Bozbek et al., 2006). Early sowing seems to cause higher yield potential and a few reports have demonstrated that early sowing of cotton results in extensive tallness of plants with higher number of branches, bolls and seed cotton yield

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78437852

(Ali et al., 2009; and Farid et al., 2017). Early sowing produces 10% more blooms, 23% more open bolls and 18% more seed cotton yield than late sowing. Late planting of cotton indicates vegetative development and hard to oversee bringing about lower seed cotton yield (Farid et al., 2017). Impact of sowing dates and capability of nitrogen fertilizer are necessary factors to check the response of cotton cultivar for its growth and performance under semi-arid conditions (Liu et al., 2015). Cotton fiber quality is for the most part impacted by cultivars yet agronomic practices and ecological conditions are the secondary elements affecting fiber quality (Subhan et al., 2001). Earlier planting of cotton produced more yield and yield components like sympodial branches, average boll weight and ginning out turn than late planting (Arshad et al., 2007; and Farid et al., 2017).

Cultivars vary in their genetic makeup and respond different to various biotic as well as abiotic stresses and climatic conditions. So, cultivar selection and proper sowing time are keys to enhance seed cotton yield under different agro-ecological zones (Bange and Milroy, 2004; Farid et al., 2017). Different cultivars have their own genetic makeup to develop canopy, those having slow growth rate have less leaf area index resulting reduced efficiency of converting radiant light to photosynthates (Iqbal et al., 2012).

Keeping in view the importance of cotton crop and climatic variations of this region, this comprehensive study was planned to assess the reaction of various cotton cultivars under different sowing time, to characterize the ideal sowing time and its impact on yield contributing characteristics and to evaluate the response of various cultivars under agro-environmental conditions of Multan, Pakistan.

Materials and methods

Experimental site and design

The experiment was carried out at Central Cotton Research Institute Multan, Pakistan in 2016 and 2017. The climate of this section is subtropical and semi-arid with very hot summers and cold winters. The temperature varies from summer to winter as the area is situated in subtropical region. The latitude and longitude of Multan is 30.26°N and 71.51°E, respectively. The experiment was planned under (RCBD) split plot arrangements with a net plot size of 7.5 m × 9 m.

Soil sampling and analytical methods

Soil samples were taken up to the depth of 30 cm with a sequence of 0-15 cm and 15-30 cm was taken from the experimental area. Three cores were taken from each depth, air dried, and ground, finally passed through a sieve 2 mm in diameter and all the samples were then mixed to get a composite sample and analyzed chemically. Physical and chemical characteristics of the soil were determined by using standard procedures. Soil sampling and chemical analysis of soil were repeated after harvest of each crop.

Crop husbandry

For seed bed preparation, initial irrigation with 4 cm depth was applied to make the soil in appropriate wattar condition which was followed by three cultivations and planking for sowing of cotton crop. Each experimental unit was sown on ridges with a tractor mounted ridger. The sowing was done by using single row hand drill at 2 cm depth vertically with R × R distance of 20 cm. Thinning was done to maintain the required plant population 20-25 days after sowing. Recommended doses of phosphorus

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and potassium fertilizers i.e. 60 kg ha^{-1} and 60 kg ha^{-1} were used as per standard practices respectively.

Weeding and recommended plant protection measures were used for the control of insect and pests. Amidachloprid (Sun Crop Pesticide Ltd.) was used to control the attack of sucking insects and to protect the plant at vegetative stage, after that biphenthrin (KanzoAgri-group) was applied when chewing insects were observed at the flowering stage of crop plants. Chloroperephos (Four brothers Ltd.) was used to lessen the intensity of termites that was further helpful to reduce the crop yield. The picking of the cotton crop was done during the 2nd week of November of both the study years.

Observations recorded

For measuring the plant height, number of nodes $plant^{-1}$ and internodal distance, ten random plants were initially selected and properly tagged for measuring the crop data. After that their average was taken and then statistically analyzed.

Leaf area index of the crop was measured with the ratio between leaf area and ground area of the cotton crop as the formula given by Watson et al. (1952). The crop growth rate of the plants was calculated during the whole crop growth season by using the formula as suggested by Hunt et al. (1978):

$$CGR\ (g\ m^{-2}\ day^{-1}) = (W_2\text{-}W_1) / (T_2\text{-}\ T_1)$$

Ten different plants of cotton from each experimental unit were selected to find out the total fruiting points and Number of bolls $plant^{-1}$. The total number of bolls $plant^{-1}$ were calculated in each experimental unit on per meter square basis and their average was calculated. The average was taken after calculating the total number of plants and their weight in grams were taken by using weighing balance. Before final harvest, picking of the bolls was done to calculate the seed cotton yield for each experimental unit. After picking, their staple length in mm was measured by adopting standard method. The strength of the fiber was measured in grams per tax.

Net income

Net income was calculated by using the following formula, as given:

$$\text{Net Income} = \text{Gross income} - \text{Total cost}$$

Benefit cost ratio

Benefit cost ratio was calculated by using the formula:

$$BCR = \frac{\text{Gross income } (Rs.ha^{-1})}{\text{Total cost } (Rs.ha^{-1})}$$

Statistical analysis

For the evaluation of every experiments in statistical manner, specific statistical design was used. to assess the effect of different dependent and independent variables Fisher's analysis of variance technique was applied and differences among treatment

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means was statistically calculated and compared through least significant difference test (LSD) at 0.05% probability level.

Results

Results showed that there were significant differences among different treatments i.e. sowing dates and cultivars (*Table 1*). Crop which was sown early on the 15th April, gave the tallest plants (129 cm) and the crop sown late on the 15th June produced significantly the shortest plants (74.7 cm). However, crop sown on the 1st June tended to produce shorter plants (108 and 104 cm) than treatments (75 and 75 cm) on the 15th May and statistically significant differences were observed among these treatments during both years of experiment. Among different cultivars, CIM-620 produced significantly taller plants than Cyto-120 and CIM-608 cultivars on each sowing dates. Although CIM-608 cultivar produced shorter plants than CIM-620 which were statistically different from each other. During both study years, it was observed that the interactions of sowing dates with cultivars were found to be non-significant. These non-significant differences might have occurred due to the cultivar CIM-608 which produced taller plants in early sown crops (15th April and 1st May) which might have been due to the genetic difference and more tolerance to late sowing. In *Table 1,* results clearly depicted that with each delay in sowing of the crop showed a decrease in number of nodes plant^{-1} but this decrease was statistically significant to all other treatments (*Table 1*). Crop sown early on the 15th April gave the maximum number of nodes plant^{-1}, and crop sown late on the 15th June produced significantly the minimum number of nodes plant^{-1} (17, 16) in both study years. Among all the cultivar treatments statistically significant differences were observed. Cultivar CIM-620 produced significantly greater number of nodes plant^{-1} than Cyto-120 and CIM-608 cultivars on each sowing dates. It is evident from the results that with each delay in sowing time the plants were shorter which results in the decrease of number of nodes plant^{-1}

Data (*Table 1*) showed that inter-nodal distance of the cotton cultivars was influenced by different sowing dates and cultivars. Both the cultivars Cyto-120 and CIM-608 did not show any significant difference in inter-nodal distance. However, delay in sowing dates showed decrease in inter-nodal distance and significantly the highest distance (3.6 cm) was observed in crop sown early on the 15th April, while the lowest (3.0 cm) inter- nodal distance was observed in crop on the 15th June.

Results showed that Leaf area index (LAI) was significantly influenced by different treatments (*Fig. 1*). It was observed that late sown cotton has showed promoted effects on LAI values especially at the final harvest (100 DAS) than early sown cotton growth stages. Early sowing on the 15th April produced significantly lesser leaf area index than sowing on the 15th June. While among the three different cultivars CIM-608 gave higher values of LAI than Cyto-120 and CIM-620 at (100 DAS). It is evident from the results that interactions between different treatments of sowing dates and cultivars were found to be non-significant (*Table 1; Fig. 2*). Cultivar CIM-620 increased significantly ($P \leq 0.05$) CGR at early growth stage (50 DAS), while later on Cyto-120 gave significantly higher CGR throughout the growing period when sown early on the 15th April. Cultivars sown on the 1st June 01 and on the 15th June showed statistically similar results until 100 DAS like that of sown early but at the final harvest CIM-620 gave higher growth rate. It is also clear from the results that each delay in sowing date significantly ($P \leq 0.05$) increased growth rate during early growth stages (50 and 100

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DOI: http://dx.doi.org/10.15666/aeer/1806_78437852

DAS) while, at the final harvest each delay in sowing date significantly ($P \leq 0.05$) reduced the growth rate by all the three cultivars.

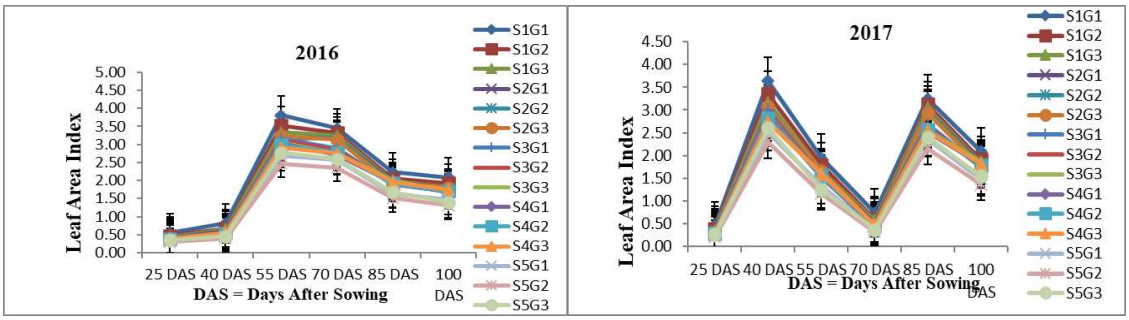

Figure 1. *Relationship between LAI and yield*

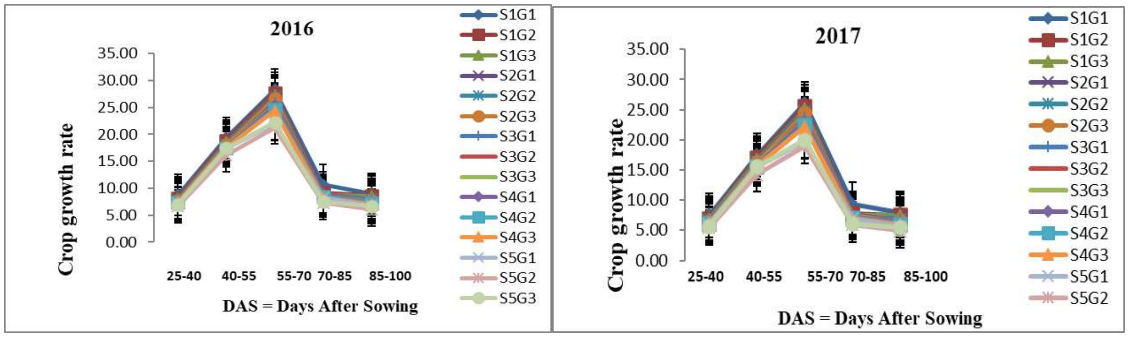

Figure 2. *Relationship between CGR and yield*

Table 1. *Effect of different sowing time and cultivars on the yield and yield contributing factors of cotton during 2016 and 2017*

Treatments	Plant height (cm)		No. of nodes per plant	Nodal distance (cm)			Leaf area index		Crop growth rate		Total fruiting points	
Sowing dates (main plot)	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**
S_1 = 15th April	131 a	127 a	32 a	29 a	3.6 a	3.5 a	2.4 e	2.2 e	12.6 a	12.3 a	406 a	390 a
S_2 = 01th May	114 b	115 b	29 b	27 b	3.3 b	3.2 b	2.5 d	2.4 d	12.1 b	11.8 b	389 b	356 b
S_3 = 15th May	108 c	104 c	27 c	25 c	3.2 c	3.1 c	2.6 c	2.5 c	10.8 c	11.6 c	377 c	328 c
S_4 = 01th June	96 d	91 d	20 d	18 d	3.1 d	3.0 d	2.8 b	2.7 b	10.1 d	9.6 d	354 d	296 d
S_5 = 15 th June	75 e	75 e	17 e	16 e	3.0 e	2.9 e	2.9 a	2.8 a	8.5 e	8.1 e	258 e	247 e
LSD ($P \leq 0.05$)	**1.2**	**1.8**	**0.9**	**0.9**	**0.07**	**0.06**	**0.04**	**0.05**	**0.4**	**0.4**	**7.4**	**8.2**
Cultivars (C) (subplot)												
C_1 = CIM-620	109 a	106 a	28 a	26 a	3.3 a	3.3 a	2.6 c	2.3 c	11.7 a	11.4 a	374 a	355 a
C_2 = Cyto-120	105 b	102 b	25 b	23 b	3.2 b	3.2 b	2.7 b	2.4 b	10.6 b	10.4 b	357 b	319 b
C_3 = CIM-608	101 c	98 c	23 c	21 c	3.2 b	3.1 b	2.8 a	2.6 a	10.1 b	9.7 c	340 c	298 c
LSD ($P \leq 0.05$)	**1.9**	**1.3**	**0.9**	**0.8**	**0.07**	**0.06**	**0.04**	**0.04**	**0.2**	**0.2**	**6.9**	**7.5**
(S*C)	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**

Mean values having similar letters were not different from each other $p \leq 0.05$; NS = Non-significant

The interactive effect between different sowing dates and cultivars were found to be non-significant during both study years (*Table 1*). The results indicated that different

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sowing dates also significantly influenced the fruiting points of the cultivars as each delay in sowing significantly ($P \leq 0.05$) reduced the number of fruiting points. The highest number 406 and 390 (m^{-2}) were observed in crops sown on the 15th April, while the lowest fruiting parts were produced in late sown crop on the 15th June during both the study years (*Table 1*).

Among the cultivars, CIM-620 significantly ($P \leq 0.05$) produced more number of fruiting points than other cultivars. The highest fruiting point 364 m^{-2} was produced in CIM-620 while the lowest (319 m^{-2}) was observed in cultivar CIM-608 However, the interaction between sowing dates and cultivars were found to be non-significant.

The results of *Table 2* showed that there were significant differences among different treatments i.e. sowing dates and cultivars and interactions between sowing dates and cultivars were found to be significant for boll weight and seed cotton yield (*Table 2*). The maximum (138 m^{-2}) number of bolls were obtained by the combination of CIM-620 with early sowing of crop while the lowest (46 m^{-2}) number of bolls were observed with CIM-608 sown on the 15th June. This significant effect might have occurred after maintaining the optimum plant population through thinning and gap filling at early stages of the crop in early sowing. Results relating to boll weight revealed that there were significant differences among different treatments of sowing dates and cultivars (*Table 2*). The crop sown on the 15th June produced the highest average boll weight of (2.9 g) while the lowest average boll weight of (2.3 g) was observed with sowing of cotton on the 15th April. Accordingly, crop sown on the 15th April gave lower boll weight than those sown on the 1st May that produced significantly higher average boll weight of (2.5 g) during both years of experimentation. Similarly, crop sown on the 1st June, also gave significantly higher boll weight than crop sown on the 15th May. In case of various cultivars, the cultivar CIM-608 gave significantly ($P \leq 0.05$) higher boll weight than CIM-620.

Table 2. *Effect of different sowing time and cultivars on the yield and quality parameters of cotton during 2016 and 2017*

Treatments	**No of bolls plant^{-1}**		**Boll weight (g)**		**Seed cotton yield (kg ha^{-1})**		**Staple length (mm)**		**Fiber strength**	
Sowing dates (main plot)	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**
S_1 = 15th April	128 a	119 a	2.4 e	2.2 e	2759 a	2569 a	28.4 a	27.9 a	94.8 a	94.3 a
S_2 = 01th May	116 b	105 b	2.5 d	2.4 d	2528 b	2281 b	27.4 b	26.6 b	93.1 b	93.1 b
S_3 = 15th May	96 c	83 c	2.6 c	2.5 c	2352 c	2050 c	26.8 c	26.1 c	92.5 c	91.9 c
S_4 = 01th June	82 d	71 d	2.8 b	2.7 b	1891 d	1783 d	26.3 d	25.4 d	91.9 d	91.3 d
S_5 = 15th June	65 e	51 e	2.9 a	2.8 a	1387 e	1264 e	25.7 e	25.0 e	90.7 e	90.0 e
LSD ($P \leq 0.05$)	**4.8**	**2.5**	**0.04**	**0.05**	**39.7**	**36.0**	**0.3**	**0.2**	**0.2**	**0.4**
Cultivars (sub plot)										
C_1 = CIM-620	111 a	99 a	2.6 c	2.3 c	2456 a	2257 a	27.6 a	26.8 a	93.4 a	92.7 a
C_2 = Cyto-120	95 b	84 b	2.7 b	2.4 b	2124 b	1939 b	26.9 b	26.2 b	92.5 b	92.2 b
C_3 = CIM-608	87 c	75 c	2.8 a	2.6 a	1971 c	1773 c	26.5 c	25.7 c	91.9 c	91.5 c
LSD ($P \leq 0.05$)	**3.6**	**2.6**	**0.04**	**0.04**	**46.4**	**38.8**	**0.2**	**2.6**	**0.2**	**0.2**
Interaction	**NS**	**NS**	*	*	*	*	**NS**	**NS**	**NS**	**NS**

Mean values having similar letters were not different from each other $p \leq 0.05$; NS = Non-significant

It is evident from the results that interactions between sowing dates and cultivar treatments were found to be non-significant. Sowing dates and cultivars had significant

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DOI: http://dx.doi.org/10.15666/aeer/1806_78437852

effect on seed cotton yield (*Table 2*). As each delay in sowing date reduced significantly the seed cotton yield and the highest yield of 2759 kg ha^{-1} and 2569 kg ha^{-1} was produced when crop was sown early on the 15th April and the lowest yield of 1387 kg ha^{-1} and 1264 kg ha^{-1} was produced with late sown crop on the 15th June during both years of the experimentation 2016 and 2017, respectively. Cultivar CIM-620 produced significantly more seed cotton yield (2456 kg ha^{-1} and 2257 kg ha^{-1}) than CIM-608 that gave lower yield of 1971 kg ha^{-1} and 1773 kg ha^{-1} during 2016 and 2017, respectively. It is clear from the results that each delay in sowing time significantly reduced seed cotton yield of all the cultivars (*Table 2*).

Sowing dates and cultivars had significant effect on staple length (*Table 2*). All the cultivars produced the highest staple length with early sowing treatment on the 15th April while the lowest staple length was recorded with late sown crop on the 15th June. The results in *Table 2* revealed that different sowing dates influenced significantly the fiber strength of the cultivars. Similarly, each delay in sowing produced significantly week fiber strength. The highest fiber strength of 94.8 and 94.3 (tppsi) was observed in early sown crop on the 15th April, while the lowest fiber strength of 90.7 and 90.0 (tppsi) was produced in late sown crop on the 15th June 15 during both study years 2016 and 2017, respectively (*Table 2*).

Among the cultivars, CIM-620 produced significantly higher fiber strength 93.1 (tppsi) while the lowest fiber strength (91.7 tppsi) was observed in CIM-608 cultivar. Moreover, the interaction between sowing dates and cultivars was found to be non-significant.

Economic analysis

Data regarding economic analysis is in *Table 3* showed that net income and benefit cost ratio were decreased linearly as the delay in sowing time during both years 2016 and 2017. The maximum net income of Rs. 155725 in 2016 and Rs. 142365 in 2017 was achieved with combination of (Sowing of cotton at 15thApril × CIM-620), while the minimum net income of Rs. 8285 in 2016 and Rs. 2125 in 2017 was achieved with combination of (sowing of the crop at 15th of May × CIM-608), respectively. The maximum benefit cost ratio (2.90 and 2.74) was achieved in treatment combination of (Sowing of cotton at 15thApril × CIM-620), in 2016 and 2017, respectively. While the minimum benefit cost ratio (1.10 and 0.97) was achieved in treatment combination of (sowing of the crop at 15th of May × CIM-608) in 2016 and 2017, respectively.

Discussion

Different planting dates significantly affected the growth, Leaf area index (LAI), leaf area duration (LAD), No. of sympodial branches $plant^{-1}$, total bolls $plant^{-1}$, average boll weight, total dry matter and seed cotton yield of cotton (*Table 1*). Likewise, cultivars also differed significantly for the growth, LAI, LAD and yield and yield contributing traits. The early sown cotton crop gets benefit of nutrients and more interrupted radiation due to increase in the growth period (Ali et al., 2009). Cultivars vary in their genetic makeup and respond differently to various biotic and abiotic stresses in addition to climatic conditions. So, cultivar selection and proper sowing time are key factors to enhance seed cotton yield under different agro-ecological zones (Bange and Milroy, 2014; Iqbal et al., 2012). Cotton growth and development is greatly impacted by sowing date, especially during flower initiation and development, resulting in delayed crop

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DOI: http://dx.doi.org/10.15666/aeer/1806_78437852

maturity (Wei et al., 2017). Late planting often delays flower initiation and extends the boll setting period relative to normal planted crop (Wei et al., 2017; Muharam et al., 2014; Zhao et al., 2012)., Delay in Sowing dates prolonged crop growth period due to low temperature (Bachubhai et al., 2016). The increase might be due to a longer cropping season which allows the crop to utilize available resources (e.g. light) and produce more fruit for an extended time period. The reduction in yield and yield contributors were mainly attributed to a shorter growing season and that poor light interception reduces leaf photosynthetic capacity and nutrient uptake (Khan et al., 2017). Late planted crop caused substantial reduction in yield due to low temperature and poor light interception at the end of the season (Liu et al., 2015; Cao et al., 2016). The late planting of cotton decreases the boll size and weight (Ali et al., 2009). The more seed cotton yield in planting date (15th May) might be due to longer growing period availability and good crop establishment under mild temperature of early season. The late sowing of cotton decreases the yield contributing traits and ultimately the seed cotton yield (Arshad et al., 2007; Ali et al., 2009; Iqbal et al., 2004; Farid et al., 2017).

Table 3. *Economic analysis as affected by different sowing time on the different cultivars of cotton during 2016 and 2017*

Treatments	Seed cotton yield (kg ha^{-1})		Value (Rs. ha^{-1})		Gross income (Rs. ha^{-1})		Total cost (Rs. ha^{-1})		Net return (Rs. ha^{-1})		Benefit cost ratio (Rs. ha^{-1})	
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
S_1C_1	2970	2803	237600	224240	237600	224240	81875	81875	155725	142365	2.90	2.74
S_1C_2	2854	2684	228320	214720	228320	214720	81875	81875	146445	132845	2.79	2.62
S_1C_3	2788	2640	223040	211200	223040	211200	81875	81875	141165	129325	2.72	2.58
S_2C_1	2640	2520	211200	201600	211200	201600	81875	81875	129325	119725	2.58	2.46
S_2C_2	2522	2412	201760	192960	201760	192960	81875	81875	119885	111085	2.46	2.36
S_2C_3	2476	2270	198080	181600	198080	181600	81875	81875	116205	99725	2.42	2.22
S_3C_1	2328	2102	186240	168160	186240	168160	81875	81875	104365	86285	2.27	2.05
S_3C_2	2264	2046	181120	163680	181120	163680	81875	81875	99245	81805	2.21	2.00
S_3C_3	2141	1872	171280	149760	171280	149760	81875	81875	89405	67885	2.09	1.83
S_4C_1	1857	1637	148560	130960	148560	130960	81875	81875	66685	49085	1.81	1.60
S_4C_2	1740	1462	139200	116960	139200	116960	81875	81875	57325	35085	1.70	1.43
S_4C_3	1682	1350	134560	108000	134560	108000	81875	81875	52685	26125	1.64	1.32
S_5C_1	1432	1287	114560	102960	114560	102960	81875	81875	32685	21085	1.40	1.26
S_5C_2	1320	1157	105600	92560	105600	92560	81875	81875	23725	10685	1.29	1.13
S_5C_3	1127	997	90160	79760	90160	79760	81875	81875	8285	2115	1.10	0.97

Conclusion

It is concluded from the present study that cultivar CIM-620 has a potential of bearing maximum No. of bolls and lower shedding percentage causes increased performance and output and lint percentage with more fiber brightness. The crop sown earlier on 15th May produced the higher number of fruiting points, highest fruit and boll numbers which causes maximum yield. Similarly, high nitrogen fertilized plots exhibited earliness, higher number of fruiting points, more boll numbers and higher seed cotton yield.

In the future, cotton crop should be cultivated on different locations to check its behavior under time and climatic conditions. It should be cultivated under different time interval in Wheat-cotton belt to minimize the time conflict between these two crops and

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DOI: http://dx.doi.org/10.15666/aeer/1806_78437852

their effect on the crop yield. Cultivars used in the trials should be evaluated under the green house and lab conditions for better recommendation to the cotton growers. To safeguard the environment, sustainable production of cotton with minimum utilization of resources as the aim of modern agriculture should be operated in Pakistan.

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APPENDIX

Economic analysis

Fixed cost

Input charges	Number of operations	Price per unit (Rs)	Total amount (Rs)
1) Preparation of seed bed			
Cultivations	4	1250/ha	5000
Planking	2	1250/ha	2500
Preparation of ridge	1	3000/ha	3000
2) Seeding expenditures			
Cotton seed	25 kg/ha	Rs. 300/kg	8125
Manual sowing	10 man days	250/man/day	2500
3) Weeds removal			
Interculture	1	3000/ha	3000
4) Plant protective measures			
Carbofuran	2.5 Packs	750/pack	1875
Charges of application	1 man/day	250/man/day	250
5) Irrigation			
Water rates (Abyana)	1 ha	375/ha	375
Water course cleaning	5 man days	250/man/day	1250
Charges of application	5 man days	250/man/day	1250
6) Cost of fertilizer	10-12 bags + application charges 2 man/day	18500/ha	18500
7) Cost of picking	10 man/day	300/man/day	3000
8) Rent of land	6 months	62500/ha/anum	31250
Expenses from 1-8	-	-	81875
Total Cost			**81875**

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78437852

FERTILIZATION MODEL FOR FLUE-CURED TOBACCO (*NICOTIANA TABACUM* L.) IN SOUTHWEST CHINA

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(Received 18th Mar 2020; accepted 20th Aug 2020)

Abstract. The fertilization model is an important breakthrough in the field of nutrient management for high quality crop production. However, there are few studies on the flue-cured tobacco fertilization model. In this study eight plots were selected, for use in a fertilization experiment to determine the optimal nitrogen, phosphorus and potassium fertilizer application amounts for flue-cured tobacco planting in the Southwest China. Based on parameters such as the nutrient requirements, fertilizer utilization rate, and soil nutrient correction coefficient of flue-cured tobacco, the curves of N, P_2O_5, K_2O and their corresponding nutrient correction coefficients were constructed. We fitted a non-linear curve model using soil parameters, i.e. yield per area, nutrient requirements and fertilizer utilization rate, etc. The result showed that the recommended fertilization models are $N = 0.091 \times Y - 149.505 \times S_N^{0.029}$; $P_2O_5 = 0.072 \times Y - 11.595 \times S_P^{0.749}$; $K_2O = 0.199 \times Y - 55.924 \times S_K^{0.351}$. The validation of the experiments showed that the yield of the flue-cured tobacco reached about 2,700 kg ha^{-1}. In addition, the recommended amount of fertilization was 30% less than the conventional fertilizer application. Therefore, the recommended amount will lower the production cost, reduce the environmental pollution caused by excess soil fertility, and promote the sustainable development of flue-cured tobacco planting.
Keywords: *soil nutrients, fertilizer utilization rate, yield, recommended fertilization, decision model*

Introduction

Plant nutrients are the major components of crop growth, of which nitrogen (N), phosphorus (P) and potassium (K) are considered the most basic elements for agricultural production. Thus, the maintenance of soil fertility is needed for crop productivity (Shen et al., 2010; Yavitt et al., 2011). The reasonable application of nitrogen phosphorus and potassium fertilizers provides the important contribution to increasing yield. To obtain the highest yield, farmers usually use excessive fertilization regardless of the environmental impact (Zhao et al., 2016; Williams et al., 2017). Excess fertilizer will be lost to the environment in various ways, including surface runoff, underground leaching and trace gas emission, causing considerable environmental problems (Pappa et al., 2011; Ahmed et al., 2017; Russo et al., 2017).

An increasing body of evidence based on long-term experiments have shown that environmental pollution from agricultural production caused by excessive fertilization use can be alleviated by changing tillage methods and crop rotation systems (Lehmeier et al., 2013; Sun et al., 2015; Guillaume et al., 2016), as well as by modifying the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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fertilization method and irrigation systems. Gilhespy et al. (2014) showed that different fertilization methods and fertilizer application amounts could change the nitrogen load of paddy fields. Hossain et al. (2018) noted that an appropriate fertilizer-water ratio can improve the physicochemical properties of the soil and increase cucumber yields.

Recently, scientists have conducted many targeted works on nitrogen phosphorus and potassium fertilizers (Subhashini, 2016; Kumaresan et al., 2019; Lisuma et al., 2020). Although most studies provide valuable perspectives on crop growth and development, the conclusions and recommendations obtained have some limitations. For example, the short length of the experimental period limited the possibility of obtaining robust conclusions, making difficult the establishment of appropriate cropping strategies with only few years of experimental data. Thus, strong recommendations for crop management would require a large number of experimental years with time and cost associated. Therefore, the combination of experimental work with the use of crop simulation models may help to overtake this limitation.

Flue-cured tobacco (*Nicotiana tobacum* L.) is an important cash crop in China. Many applications of flue-cured tobacco in drug resources and food development is being continuously discovered and utilized (Sheets et al., 2016; Crossthwaite et al., 2017). Therefore, high-quality flue-cured tobacco planting has broad prospects (Vann et al., 2013; Amankwa et al., 2014; Shen et al., 2017). The estimate of reasonable amounts of fertilization by constructing a flue-cured tobacco fertilization model through different fertilizing experiments is an important breakthrough in high-quality flue-cured tobacco production (Hu et al., 2018). At present, few studies have reported on flue-cured tobacco fertilization models, and most studies did not use factors such as flue-cured tobacco quality to verify the fertilization models in field experiments.

This study performed fertilization experiments on flue-cured tobacco in a mountainous area of Southwest China. And this study had two main objectives: (1) to determine a rational fertilization amount in flue-cured tobacco planting; (2) to establish fertilization model for flue-cured tobacco and to verify the model.

Materials and methods

Materials

Site description

From 2013 to 2017, eight experimental plots (1-8) in mountainous areas of southwest China were selected for field experiments. In 2018, three representative tobacco plots (I, II, III,) were selected to conduct validation experiments. These plots are located in the Experimental Station of the Yanbian County, Panzhihua City, Sichuan Province (27°05′N and 101°46′E, 2097 m above sea level) (*Figure 1*).

Our study area belongs to the subtropical dry-hot valley climate, and shows mean annual precipitation of 1065 mm, which is mainly concentrated between June and October. The annual average sunshine duration is 2307 h, and the annual average temperature is 19.2 °C (Li et al., 2018). The soils are Alumi-Ferric Alisols (FAO/UNESCO, 1988). Every experimental plot is flat and inside with uniform fertility. The basic physicochemical properties of the surface soil (0-20 cm) were measured before the experiment in 2013 (*Table 1*).

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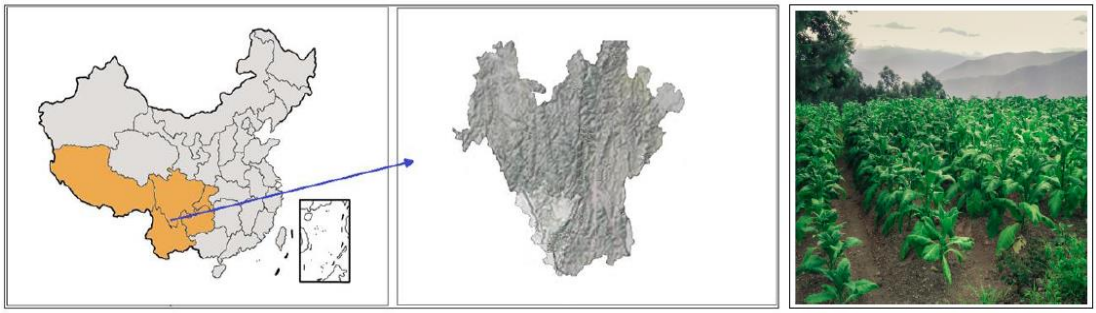

Figure 1. *Map of the experimental field in southwest China*

Table 1. *Initial soil properties of the experiment in Yanbian County during 2013, southwest China*

Experimental plots	pH	SOC g kg^{-1}	Total N g kg^{-1}	Alkali-N mg kg^{-1}	Olsen-P mg kg^{-1}	Ava-K mg kg^{-1}
1	6.15	28	1.33	83.5	2.2	142.5
2	5.85	31.8	1.62	115.35	8.35	135.62
3	6.51	9.58	0.85	86.7	8.53	105.28
4	6.48	25.85	1.25	95.36	2.05	138.85
5	6.52	14.35	0.92	78.35	10.38	102.35
6	5.72	23.35	1.38	125.32	11.24	125.18
7	6.65	13.25	1.32	92.35	12.55	123.75
8	6.25	25.55	1.68	118.34	8.48	135.65

SOC was soil organic carbon, Total N was total nitrogen, Alkali-N was alkali-hydrolyzale nitrogen, Olsen-P was Olsen phosphorus, Ava-K was available potassium

Experimental design

Seven treatments were conducted using a randomized block design with three replicates. All treatments were applied in every experimental plot (*Table 2*). The plot size was 77 m^2 (11 m x 7 m). The fertilizer used for the test was calcium ammonium nitrate for agricultural use (N content: 155 g kg^{-1}), superphosphate (P_2O_5 content: 160 g kg^{-1}) and potassium sulphate (K_2O content: 500 g kg^{-1}), of which 70% was basal fertilizer (applied at the transplanting period), and 30% was topdressing (applied 20 days after transplanting). The flue-cured tobacco variety used in the experiment was Yunyan87, which was provided by the China Tobacco Import and Export Sichuan Co. Ltd.

Table 2. *Application scheme of flue-cured tobacco fertilizer (kg ha^{-1})*

Treatments	N	P_2O_5	K_2O
1	0	0	0
2	0	135	270
3	90	0	270
4	90	135	0
5	60	90	180
6	90	135	270
7	120	240	360

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The experimental flue-cured tobacco was planted with floating seedlings that were transplanted on April 20th of each year, harvest and baking began on July 20, and the harvest ended on September 1st. The field growth period was 130 days, and the management methods for the field tillage were conducted according to the local conventional methods. The experimental plots were ploughed after 15 and 35 days of flue-cured tobacco transplanting, and irrigated when the soil moisture content is less than 60%. When the buds of flue-cured tobacco grow to 4-6 cm, remove the buds and the next 3 leaves. Tobacco leaves were harvested in different plots and uniformly baked before the yield was calculated. Central leaves (2 kg) with consistent maturity (at leaf positions 8, 9, 10 and 11) were selected from each treatment for the chemical composition analysis. The quality of flue-cured tobacco was evaluated by chemical composition content.

Evaluation criteria for high-quality flue-cured tobacco

Usually we use the content of chemical composition as the evaluation standard of flue-cured tobacco quality (White et al., 1979). A review of multiple sources of reference data showed that in high-quality flue-cured tobacco leaves, the nitrogen content is 2.5%; the phosphorus content is 0.6%; the higher the potassium content is, the better; the total sugar content is 20%; the reducing sugar content is 15%; the chlorine content is 0.5%; and the nicotine content is 2% (Sun et al., 2011).

Methods

The model was developed using Truog-Stanford equation of the nutrient balance method. The equation was that calculated the difference between the amount of fertilizer required by the crop and the amount of fertilizer provided by the soil, and then determined the amount of fertilizer applied to the crop. The core idea is that the nutrients plants need to grow are provided by both soil and fertilizer. Fertilizing is used to supplement the soil nutrient deficiency during crop growth. Only when the nutrient supply and demand balance is reached can the crop achieve the target yield. Therefore, the Truog-Stanford equation mainly involves parameters such as target yield, crops required fertilizer amount, soil fertilizer supply amount, and fertilizer utilization rate. First, the physicochemical properties of the soil had to be accurately measured. The required pattern for the crop fertilizer and the annual yield had to be determined. Then, field experiments were conducted to determine the target yield and determine the type, quantity and ratio of the fertilizer required for the target yield.

Determination of fertilizer utilization rate

The amount of N, P_2O_5, and K_2O uptake by flue-cured tobacco in yield per unit area was calculated based on analysis in the field experimental tests of eight experimental tobacco plots and laboratory tests from 2013 to 2017 (*Figure 2A*). The N, P_2O_5 and K_2O fertilizer utilization rates of the 8 experimental plots were calculated according to *Equation 1* (*Figure 2B*). It shows that the average N utilization rate was 38.21%, and its value varied from 31.95% to 45.33%; the average phosphorus (P_2O_5) utilization was 15.58%, with values ranging from 11.87% to 19.82%; and the average potassium (K_2O) utilization rate was 28.28%, with its value varying in the range of 20.65% to 34.53%.

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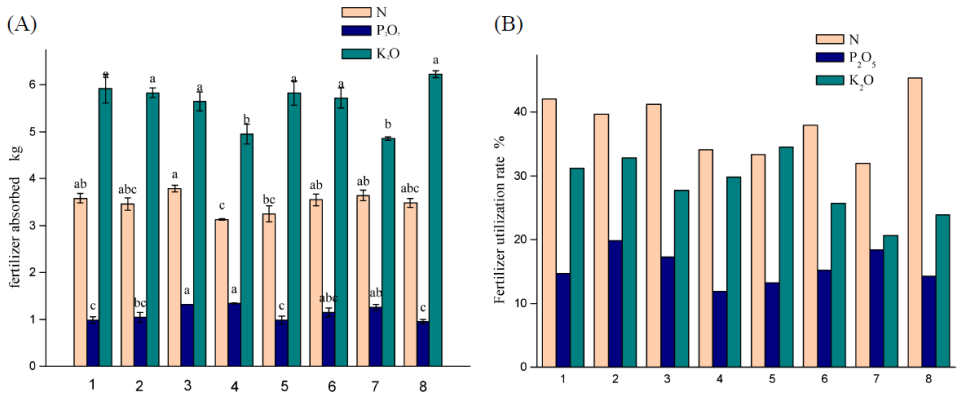

Figure 2. *The amount of N, P_2O_5 and K_2O fertilizer absorbed by per 100 kg flue-cured tobacco (A) and the fertilizer utilization rate of each experimental plot (B). Different letters within the same column indicated significant differences ($p < 0.05$)*

$$R = \frac{A_1 - A_0}{F} * 100\% \quad \text{(Eq.1)}$$

where, R represents the fertilizer utilization rate; A1 is the fertilizer absorption amount in fertilization area, and A0 is the fertilizer absorption amount in 0 fertilization area; F represents the fertilizer application amount in the experiment.

Calculate the soil fertility correction coefficient

The soil fertility correction coefficient for each experimental plot was calculated according to *Equation 2*. It shows that the correction coefficients of the soil Alkali-N, Olsen-P and Ava-K varied significantly, mainly relative to factors such as the soil available nutrient content, soil moisture status, soil types, climatic conditions and growth conditions of the flue-cured tobacco (*Figure 3*). The correction coefficients for the Alkali-N ranged from 22.25% to 37.68% with an average of 29.98%; the correction coefficients for the Olsen-P varied from 35.96% to 67.39% with an average of 50.57%; and the correction coefficient for the Ava-K ranged from 27.68% to 35.95%, with an average of 31.46%.

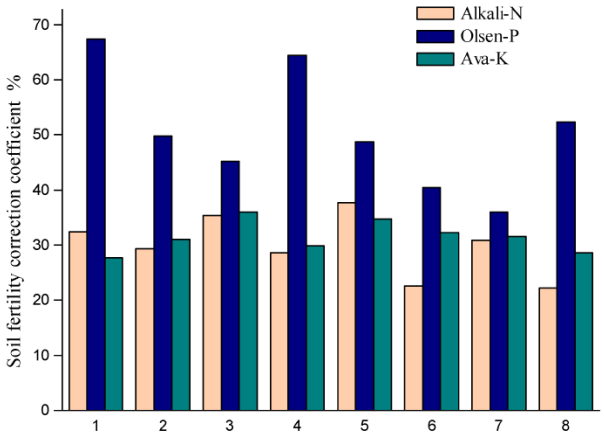

Figure 3. *Soil fertility correction coefficient (B) of each experimental plot*

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$$C = \frac{A_0}{S*0.15} * 100\% \quad \text{(Eq.2)}$$

where, C represents the soil fertility correction coefficient. S represents the soil fertility. 0.15 is the conversion factor of the measured value (mg kg^{-1}) of soil fertility to the potential fertilizer supply amount per hectare of arable soil.

Construction of the recommended fertilization model for flue-cured tobacco

In this study, we used the soil available fertility content (x, mg kg^{-1}) and the corresponding soil fertility correction coefficient (y) for regression analysis and model fitting to establish the regression equations (*Figure 4*). As can be seen in *Figure 4*, with the increase in the Alkali-N, Olsen-P and Ava-K concentrations in the experimental plots, the corresponding correction coefficients all showed a downward trend. The fitting was performed using a Curve model, and the F test showed that all regression relationships achieved significant levels ($p < 0.05$).

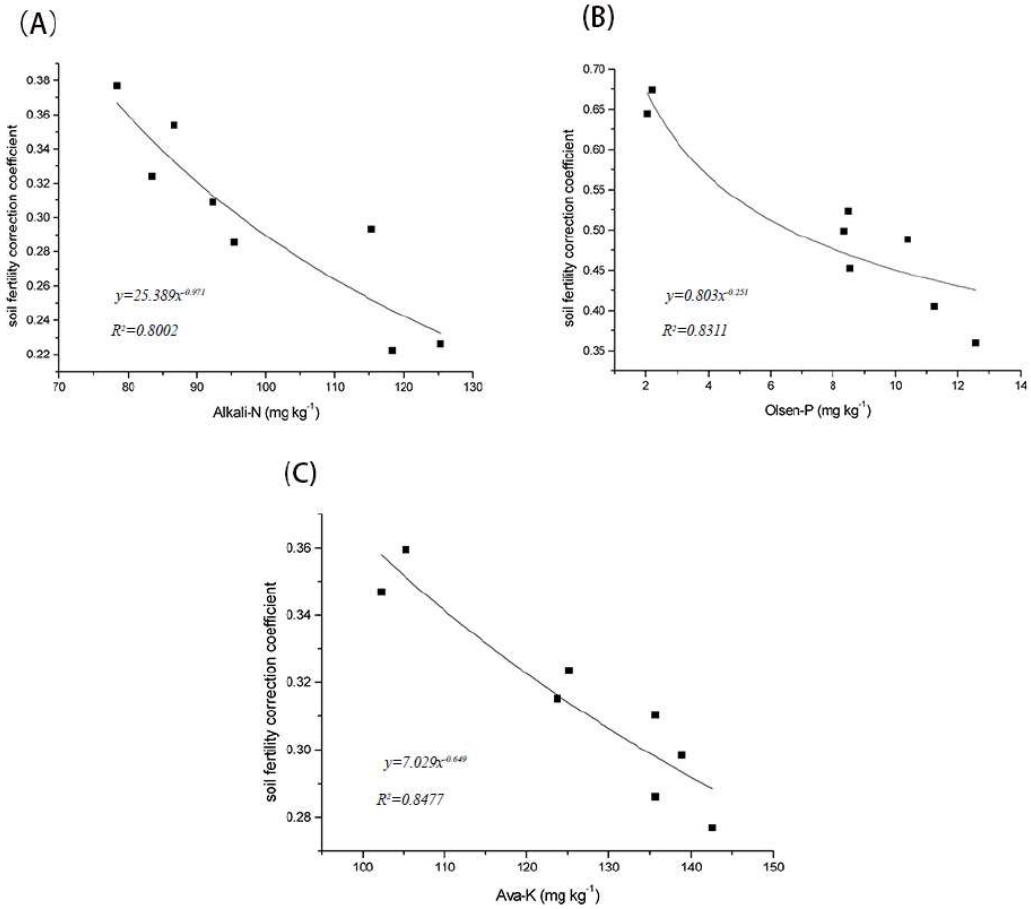

Figure 4. *Curve fitting model of Alkali-N (A), Olsen-P (B), Ava-K (C) and soil fertility correction coefficient*

The recommended fertilization model of flue-cured tobacco for nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) fertilizers with different target yields was obtained using the Truog-Stanford *Equation 3*.

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$$\text{Fertilizer application amount(pure nutrient)} = \frac{\text{Fertilizer requirement - Soil fertility supply}}{\text{Fertilizer utilization rate}} = \frac{Y * U - S * 0.15 * C}{R} \quad \text{(Eq.3)}$$

where: Y represents the target yield, and U stands for the required fertilizer in yield per unit area.

Based on the physicochemical properties of the soil, combined with the soil fertility correction coefficient, nutrient uptake by flue-cured tobacco, fertilizer utilization, etc., the fertilization models were as followed:

$$N = 0.091 \times Y - 149.505 \times S_N{}^{0.029}$$
$$P_2O_5 = 0.072 \times Y - 11.595 \times S_P{}^{0.749}$$
$$K_2O = 0.199 \times Y - 55.924 \times S_K{}^{0.351}$$

In the above three models, N, P_2O_5, and K_2O, were the recommended amounts of the nitrogen phosphorus and potassium (pure nutrients) fertilization. *Y* was the target yield, and *SN*, *SP* and *SK*, were the contents of the Alkali-N, Olsen-P, and Ava-K in the soil.

Result and Discussion

Validation the fertilization model

In 2018, the verification experiments were conducted in Yanbian County, Panzhihua City. According to the yield and quality conditions of flue-cured tobacco in Panzhihua City over the years, combined with relevant literature and consultation with relevant experts, the target yield of flue-cured tobacco was set at 2,700 kg ha^{-1}. Based on the studied fertilization models for recommended nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O) fertilization, the recommended fertilization amount at each verification plot was calculated (*Table 3*).

Table 3. *Fertilization verification experiment of flue-cured tobacco in 2018*

Verification plot	Original soil-fertility (mg kg^{-1})			The model recommended fertilizer application (kg ha^{-1})		
	Alkali-N	Olsen-P	Ava-K	N	P_2O_5	K_2O
I	125.83	9.31	128.06	73.69	132.74	230.18
II	72.95	16.27	82.75	76.39	100.73	273.82
III	118.37	2.53	185.5	74.00	171.16	187.52

In this study, we selected 2 kg of central leaves with consistent maturity from the three validation plots for each chemical composition analysis. The chemical composition and yield of flue-cured tobacco from the three verification plots are shown in *Table 4*. It shows the chemical component contents of the flue-cured tobacco from each plot were consistent with those of high-quality flue-cured tobacco, and the yield reached the expected target approximately 2,700 kg ha^{-1}. The recommended amount of fertilization was 30% less than that of the conventional fertilizer application. Therefore, in the verification plot, the application of N was 74.00-76.39 kg ha^{-1}, the application of P_2O_5 was 100.73-171.16 kg·ha^{-1}, and the application of K_2O was 187.52-273.82 kg ha^{-1}. The recommended fertilization model is reasonable and feasible for the experimental area.

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Table 4. *The chemical composition and yield of flue-cured tobacco from the verification plots*

Verification plot	Nitrogen %	Phosphorus %	Potassium %	Total sugar %	Reducing sugar %	Chlorine %	Nicotine %	Yield kg ha^{-1}
I	2.03	0.64	2.36	19.2	14.71	0.41	1.82	2805.5
II	2.11	0.52	2.28	18.87	14.15	0.53	1.95	2630.3
III	1.96	0.48	2.45	21.03	15.32	0.48	2.17	2735.5

Effect analysis of the fertilization model

Studies have shown that crop yield and quality vary with different apply of fertilization (Wang et al., 2015). In recent years, in order to obtain higher yield, farmers usually apply excessive fertilizer, and the phenomenon of blind overfertilization is quite common. For instance, the current N fertilization rate for paddy fields of China is approximately 300 kg ha^{-1}, which exceeds the actual demand of rice growth (Chen et al., 2011; Xia and Yan, 2012). In most areas of China, the application of nitrogen and phosphate were too high, which affects the absorption of other nutrients (Zhang et al., 2010). The excess fertilizers will be lost to the surroundings through different pathways, including surface runoff, subsurface leaching and trace gas emissions, causing considerable environmental issues (Zhao et al., 2016).

Scientists have been actively searching for the best fertilization plan to promote continuous improvement of crop yield and quality. The fertilization model is one of the main methods of precision fertilization. Currently, the construction of a fertilization model mainly adopts a regression function model (Thompson et al., 2015), comprehensive fertilization models (Basso et al., 2016), and artificial neural network models (Alvarez and Steinbach, 2017). Each of the above methods has advantages but also has limitations. The regression function method determines the functional relationship between fertilization and yield or quality and usually does not consider the effect of basic soil fertility. The comprehensive fertilization model uses orthogonal design and orthogonal trends to analyse the fertilizer effect while the largest experimental workload used, and the error is difficult to control. The artificial neural network model requires a relatively large database, and the modelling cost is relatively high.

This study is a fertilization model based on the Truog-Stanford equation of the nutrient balance method. The fertilization amount of flue-cured tobacco recommended by this model is simple and effective. The yield of flue-cured tobacco was estimated in advance, and the best amount of fertilizer was calculated according to the fertilizer utilization rate and soil fertility correction coefficient. Sharma and Singh (2000) suggested that fertilizer recommendations based on targeted yield concept were more balanced, profitable and helpful in controlling soil nutrient mining and was essential for sustainable crop production. The verification tests showed that the model can help improving the formulation, balanced fertilizer management strategies in flued-cured tobacco. Such fertilizer management strategy will be more advantageous compared to conventional blanket and imbalanced fertilizer recommendation.

In this study, the amount of fertilizer recommended by the model was 30% less than the amount of conventional fertilization. In flue-cured tobacco planting, the application amount of N, P_2O_5, and K_2O fertilizer recommended by the model can decrease the input of fertilizer, reduce the production cost, and farmers can harvest more tobacco

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with less input. In addition, reducing the application of N, P_2O_5, and K_2O fertilizer can alleviate the soil pollution caused by excessive fertilization, and maintain the sustainable development of agricultural production. Therefore, the fertilization model obtained in this study plays a promoting role in flue-cured tobacco planting and can keep the health of ecological environment.

Conclusion

In this study, eight representative tobacco plots were selected in the mountainous area of southwest China for field experiments. Based on the Truog-Stanford equation of the nutrient balance method, the recommended flue-cured tobacco fertilization model was constructed. The fertilization models are $N = 0.091 \times Y - 149.505 \times S_N^{0.029}$; $P_2O_5 = 0.072 \times Y - 11.595 \times S_P^{0.749}$; $K_2O = 0.199 \times Y - 55.924 \times S_K^{0.351}$. The recommended fertilizer amount by the model was 30% less than the amount of conventional fertilization, and the model was reasonably feasible in the experimental area. The model in this study was closely related to the varieties of flue-cured tobacco and the basic soil fertility of the experimental plot. The verification and improvement of the model in more areas will be the focus of future studies.

Acknowledgments. This work was supported by the National Natural Science Foundation of China (31571621); and the Science and Technology Planning Project in Sichuan Province (2014RZ0026). We are very grateful to the College of Agronomy, Northwest A & F University and School of Biological and Chemical Engineering, Panzhihua University for providing support. We also thank the editors and reviewers for their constructive comments during the preparation of the manuscript.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78537863

PHOSPHORUS RELEASE AND UPTAKE OF A DENITRIFYING PHOSPHORUS-ACCUMULATING BACTERIUM WITH DIFFERENT ELECTRON ACCEPTORS

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(Received 18th Mar 2020; accepted 20th Aug 2020)

Abstract. The phosphorus release (anaerobic) and phosphorus uptake (anoxic) capacity of one denitrifying phosphorus-accumulating bacterial strain, identified as *Acinetobacte*r by 16S rRNA sequence, and the influence of electron acceptors (NO_3^- and NO_2^-) were explored. Batch experiments with different environmental factors including pH. The types and levels of electron acceptors were performed. The results showed that anaerobic phosphorus release was greater in neutral and alkaline environments. Both nitrate and nitrite can act as electron acceptors to enable anoxic phosphorus uptake. In addition, the phosphorus uptake in the nitrate system was relatively higher than in the nitrite system in a neutral-alkaline environment (pH = 7 and 9), the average percentage of phosphorus uptake in the nitrate system reached 15.0 mg/L. For nitrite, the uptake percentage slowly increased with the increase of pH, from 9.5 mg/L (pH = 3) to 14.3 mg/L (pH = 7 and 9). The optimal stoichiometric concentration of electron acceptors and pH for phosphate removal in nitrate and nitrite systems appeared at ρ(nitrate) = 30 mg L^{-1}, pH = 9, and ρ(nitrite) = 5 mg L^{-1}, pH = 7, respectively. Our results indicated that *Acinetobacter* N-8 had a strong capacity for denitrifying phosphorus- accumulation and the utilization of oxygen, nitrate, and nitrite as electron acceptors, but the efficiency is significantly impacted by environmental pH.
Keywords: *activated sludge, Acinetobacter, pH, nitrate, nitrite*

Introduction

Phosphorous and nitrogen pollutants compounds are the primary causes of eutrophication in rivers, inland lakes and reservoirs (Chislock et al., 2014). In recent years, forty percent of reservoirs and lakes worldwide have faced varying degrees of eutrophication, approximately (Fu, 2016). Previous researches reported that nitrogen and phosphorus are the main factors affecting water quality improvement during the prevention and control of water pollution (Achieng et al., 2017; Yu et al., 2017). Therefore, the primary means to mitigate the problem is through efficient nitrogen and phosphorus removal. At present, the main conventional nitrogen and phosphorus removal technologies are physical, chemical (Fukahori et al., 2015; Srithep and Phattarapattamawong, 2017) and biological methods (Emara et al., 2014; Simsek et al., 2012). Recently, microbiologically based bioremediation technology is of increasing interest because of its advantages, including low cost, convenient operation, significant treatment effect, and low secondary pollution (Singh et al., 2019). The increasing number of wastewater treatment plants (WWTPs) removed nitrogen and phosphorus pollutions adopted biological methods for decades (Joshi et al., 2018; Zhang et al., 2018). Biological phosphorus removal from wastewater is based on the activity of phosphorus-accumulating bacteria, while biological nitrogen removal primarily relies on the nitrate

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78657880

reduction provided by denitrifying bacteria under anoxic conditions. However, denitrifying bacteria and phosphorus-accumulating bacteria compete for carbon sources.

The discovery of denitrifying phosphorus-accumulating bacteria (DPAB) and further research on the theory of denitrifying nitrogen and phosphorus removal provide new concepts and perspectives for the improvement and innovation of advanced treatment technology enabling simultaneous nitrogen and phosphorus removal. Denitrifying nitrogen and phosphorus removal technology overcomes the competition for carbon sources encountered in traditional treatment technologies and enables the full utilization of the carbon source (Lee et al., 2001). DPAB are regarded as one kind of facultative anaerobic bacteria that can simultaneously use O_2 and NO_3^--N or NO_2^--N as the final electron acceptors to assimilate phosphorus under anoxic or aerobic conditions (Li and Huang, 2013; Xie et al., 2016). Recently, a lot of previous researches has reported the progress of isolation and biological characteristics of DPAB (Ling et al., 2015; Miao et al., 2016), with the isolation results showed the DPAB mainly include *Acinetobacter* (Tsuneda et al., 2006), *Aeromonas* (Qiang et al., 2008) *Paracoccus*, *Planctomycetes* (Liu et al., 2013) and so on. Regarding biological characteristics, the influences on the growth and degradation of DPAB have also been studied to optimize the function of DPAB to removal efficiency of nitrogen and phosphorus pollutants via a biological denitrifying-phosphorus process. Xie et al. (2016) isolated one denitrifying phosphorus-accumulating strain from activated sludge, and the results show this DPAB strain has high removal capacity for both NO_3^--N and PO_4^{3-}-P (87% and 75%, respectively). Wan et al. (2017) also showed that DPAB have good performance in denitrification and phosphorus removal and can overcome the competition for the carbon source between anaerobic phosphorus release and denitrification.

For efficiency improvement purpose, many previous works have investigated the influencing factors of denitrifying phosphorus-accumulating bacteria. Researchers believed that pH (Li et al., 2018), temperature (Figdore et al., 2018), dissolved oxygen (Yuan and Oleszkiewicz, 2011), sludge retention time (Merzouki et al., 2001) and carbon source (Sun et al., 2016) could affect the metabolism of denitrifying phosphorus-accumulating microorganisms. Among them, pH is regarded as an important control index which indicates the potential of biological phosphorus removal, and it is a crucial factor which impacts the surface chargeability and permeability of microbial cell (Zafiriadis et al., 2011). However, few reports have investigated the effects of types of electron acceptors on the anaerobic phosphorus release and anoxic phosphorus uptake activity of DPAB. In the present study, the anaerobic phosphorus release and the effects of two key electron acceptors (NO_3^- and NO_2^-) on anoxic phosphorus uptake were investigated to obtain a better understanding of the metabolic behavior of DPAB by using a strain screened from activated sludge. In addition, the influences of environmental factors, including pH and electron acceptor types and levels, on the activity of removal efficiencies were studied.

Materials and methods

Media

All biochemical reagents were of analytical grade. The pH was adjusted using 0.1 mol L^{-1} HCl and 0.1 mol L^{-1} NaOH. Basically, phosphorus-accumulating organism isolation medium, beef-peptone medium, phosphorous-limited medium and phosphorous-rich medium used in this work were prepared as shown in *Table 1*.

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Table 1. *The constituents of the media adopted in this work*

Media	Constituents	Quantity	pH	Reference
Phosphorus-accumulating organisms isolation medium (1000 mL)	$CH_3COONa·3H_2O$	3.6 g	7.0	Xie et al. (2016)
	$MgSO_4·7H_2O$	0.132 g		
	$Na_2HPO_4·2H_2O$	0.0287 g		
	K_2SO_4	0.027 g		
	NH_4Cl	0.0573 g		
	$CaCl_2·2H_2O$	0.017 g		
	Agar	20 g		
	HEPES buffer	12 mL		
	Trace element solution	2 mL		
Trace element solution (1000 mL)	$FeCl_3·6H_2O$	1.5 g	7.0	Sun et al. (2015)
	H_3BO_3	0.15 g		
	$CuSO_4·5H_2O$	0.03 g		
	$Na_2MoO_4·2H_2O$	0.06 g		
	KI	0.03 g		
	$MnCl_2·4H_2O$	0.12 g		
	$ZnSO_4·7H_2O$	0.12 g		
	$CoCl_2·2H_2O$	0.12 g		
Beef-peptone medium (1000 mL)	Peptone	10 g	7.2	Xie et al. (2016)
	Beef	3 g		
	NaCl	5 g		
	Agar	20 g		
Phosphorous-limited medium (1000 mL)	CH_3COONa	3.32 g	7.0	Merzouki et al. (1999)
	$Na_2HPO_4·2H_2O$	0.023 g		
	$MgSO_4·7H_2O$	0.081 g		
	K_2SO_4	0.018 g		
	NH_4Cl	0.153 g		
	$CaCl_2·2H_2O$	0.011 g		
	HEPES buffer	7 mL		
	Trace element solution	2 mL		
Phosphorous-rich medium (1000 mL)	CH_3COONa	3.32 g	7.0	Xie et al. (2016)
	KH_2PO_4	0.025 g		
	$MgSO_4·7H_2O$	0.091 g		
	$CaCl_2·H_2O$	0.026 g		
	NH_4Cl	0.305 g		
	PIPES buffer	8.5 mL		
	Trace element solution	2 mL		
Nitrate reduction medium (1000 mL)	KNO_3	1 g	7.0	Sun et al. (2015)
	K_2HPO_4	2.42 g		
	$C_6H_{12}O_6$	1 g		
	Agar	1 g		
	Peptone	20 g		

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Analysis methods

The final data were presented in terms of mean ± standard deviation (SD) from all the replicates. Statistical analysis, such as correlation analysis and paired-samples T-test was carried out using Statistical Product and Service Solutions (SPSS, version 20.0). Bacterial growth was measured with a Finland Bioscreen Automatic Growth Curve Analyzer. The absorbance was represented by optical density (OD) at 600 nm. The pH value was measured with a METTLER FE20-FiveEasy Plus™ pH meter, and PO_4^{3-}-P was detected using Mo-Sb spectrophotometry. NO_3^--N and NO_2^--N concentrations were determined by using the phenoldisulfonic acid method and α-naphthylamine spectrophotometry, respectively. Each treatment was performed in triplicate.

Enrichment culture of DPAB

The activated sludge used in this study was inoculated in an operating activated sludge reactor located in Beijing Normal University, Beijing, China. Ten grams of active sludge, which had been cleaned three times with distilled water at 200 rpm for 3 min, was added to the beef-peptone medium (200 mL), followed by 2 days' incubation at 30 °C and 140 rpm min^{-1}. One milliliter of culture was subsequently added to the triangle bottle with 9 mL sterile water and thus formed the 10^{-1} diluent solution. Serial dilutions of bacterial suspensions from 10^{-1} to 10^{-8} were obtained using the same method, and the phosphate-accumulating organisms (PAO) isolation mediums were inoculated with 0.1 mL of each dilution in duplicate and cultured in a 30 °C incubator for 2-3 days. Single colonies were sub-cultured by picking and streaking three times to obtain isolated pure colonies (Sun et al., 2015).

Screening of strains

Screening for phosphate uptaker: single colonies were isolated and streaked on phosphorous-limited medium (100 mL) followed by 2-days incubation at 30 °C, 140 rpm min^{-1}. These cultures were centrifuged at 10^4 rpm for 2 min, after which the supernatant was removed. Then, the cultures were re-suspended in 200 mL of phosphorus-rich medium and incubated under the same conditions for 2 days to an OD_{600} of 0.1. The absorbance of PO_4^{3-}-P in the culture supernatant was measured via spectrometry as described. The phosphorus removal rates were calculated from the changes in absorbance. The following equation was used to determine phosphorus removal:

$$R=(C_0-Ct)/C_0\times 100\% \quad \text{(Eq.1)}$$

where C_0 represents the initial concentration, and C_t indicates that at time t.

The strains with higher phosphorus uptake rate (more than 50%) were chosen for the following Poly-P and poly-β-hydroxybutyrate (PHB) staining. Positive strains were chosen as DPAB. All the strains were preserved in beef-peptone medium slant cultures at 4 °C. Then screening for DPAB has been carried out on the denitrification medium with and without (Blank) KNO_3 as a substrate. Strains were inoculated on nitrate reduction medium and incubated at 30 °C for 7 days with observation every day.

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Identification of strains

Strain characteristics were determined according to previously described methods (Sun et al., 2015). Bacterial genomic DNA was used as a template to amplify 16S rRNA with a pair of universal primers: forward primer (F), CCTCCTACGGGNBGCASCAG, reverse primer (R), GACTACNVGGGTATCTAATCC. All PCR reactions were carried out in 30 μL reactions with 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μmol of forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s, with a final cycle at 72 °C for 5 min. The same volume of 1 × loading buffer (containing SYB green) was mixed with PCR products and electrophoresed on 2% agarose gel for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the combined PCR products were purified with the GeneJET Gel Extraction Kit (Thermo Scientific). As a template to build the library, a computer test was conducted.

The screened strain was placed in beef-peptone medium (200 mL) with the initial $OD_{600} \approx 0$ and cultured at 30 °C for 48 h. Then, the bacterial growth curve was drawn based on the measured OD_{600}.

Effects of different electron acceptors

Denitrifying phosphorus-accumulating bacteria has been enriched via alternative anaerobic and anoxic operations. The batch experiments of anaerobic phosphorus release and anoxic phosphorus uptake were performed with different environmental factors in order to examine the influence of the initial pH and the types and levels of electron acceptors on the efficiency of denitrifying-phosphorus removal.

The entire experiments were operated with a cycle of 7 h, consisting of a 4-h anaerobic stage and a 3-h anoxic stage. During the initial 4 h anaerobic stage, 160 μL of phosphorus-rich medium with different pH values and 40 μL of bacterial suspension ($OD_{600} = 0.4$) were fed into the 96-well plates, forming suspensions. During the anoxic stage, 50 μL of different concentrations of electron acceptor solution (nitrate or nitrite solution) was added. Specifically, the concentration levels of electron acceptors are set as follows: 10, 20, 30, 40 and 50 mg L^{-1} for nitrate and 1, 2, 3, 4 and 5 mg L^{-1} for nitrite. The environmental pH values were set as 3, 5, 7, 9 and 11. The initial phosphate concentration in the system was 25 mg L^{-1}. Each treatment was performed three times. The following equation was used to determine phosphorus release and uptake amount:

$$R_{releace}=(C_{4h}-C_0)\times 100\% \quad \text{(Eq.2)}$$

$$A_{uptake}=C_{4h}-C_{7h} \quad \text{(Eq.3)}$$

where $R_{release}$ is phosphorus release rate, A_{uptake} is phosphorus uptake amount, C_0 represents the original concentration of PO_4^{3-}-P, C_{4h} represents the concentration of PO_4^{3-}-P at the end of the anaerobic stage (4 h), and C_{7h} represents the concentration of PO_4^{3-}-P at the end of anoxic stage (7 h).

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Results and discussion

Strain enrichment, screening and identification

Strain enrichment and screening

The screening results show that 21 colonies with various morphologies were selected and sub-cultured from selected mediums of 10^{-6} culture. The isolation of single-colony was carried on with phosphorous-limited culture medium, and then phosphate uptakers were screened according the growing conditions.

Eleven colonies were selected because they exhibited phosphorus removal rates exceeding 50% (*Fig. A1* in the *Appendix*). Staining for Albert's metachromatic granules, staining for PHB granules and screening for denitrifiers among these 11 strains resulted in one all positive strain, as shown in *Table A1,* named N-8. Thus, we identified this strain as DPAB (Sun et al., 2015). In other words, N-8 could obtain denitrification and phosphorus removal, simultaneously. We then stored the strains in a refrigerator at 4 °C for the subsequent experiments.

Morphological characteristics and 16S rRNA sequence analysis

Round milk-white colonies with neat edges were been formed by N-8. The colonies were non-transparent, and had a smooth, moist surface. The gram staining experiment results showed that the bacteria were gram-negative. A scanning electron microscope image of N-8 is shown in *Figure 1*. These germ cells were catenary, and with dimensions of 1.0-1.5 μm × 1.0-1.2 μm. Granule staining results illustrated the accumulation of PHB and Poly-P particles present in these cells (*Fig. A3*).

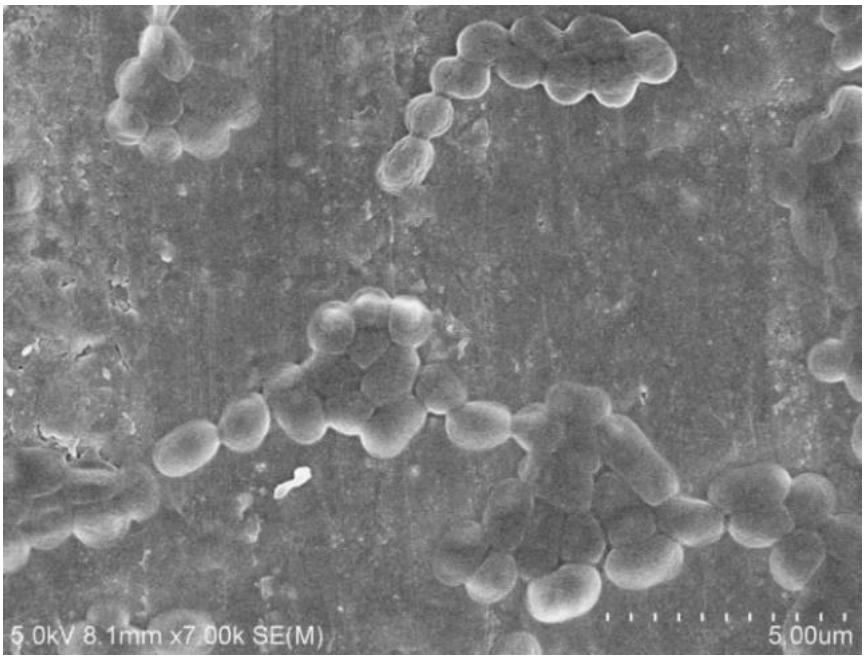

Figure 1. *Scanning electron microscope image of denitrifying phosphorus-accumulating bacteria strain N-8*

The 16S rDNA sequences of N-8 were submitted to the GenBank database and analyzed against known gene sequences using BLAST. The N-8 strain rDNA exhibited

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more than 90.15% identity with ID 219151. Through the comparison and annotation of the GreenGenes database, strain N-8 was determined to be *Acinetobacter*. The top 20 multi-sample operational taxonomic units (OTUs) and the phylogenetic trees are shown in *Figure 2,* including N1, N2, N3 from three parallel experiments. The results for 16S rDNA of DPAB in Sun's experiments showed that the strain rDNA exhibited 99.1% similarity to the known *Thauera linaloolentis* (Sun et al., 2015). Tsuneda et al. (2006) demonstrated that one strain of DPAB, *Acinetobacter*, could be found in a sequencing batch reactor and could have high average nitrogen and phosphorus removal efficiencies.

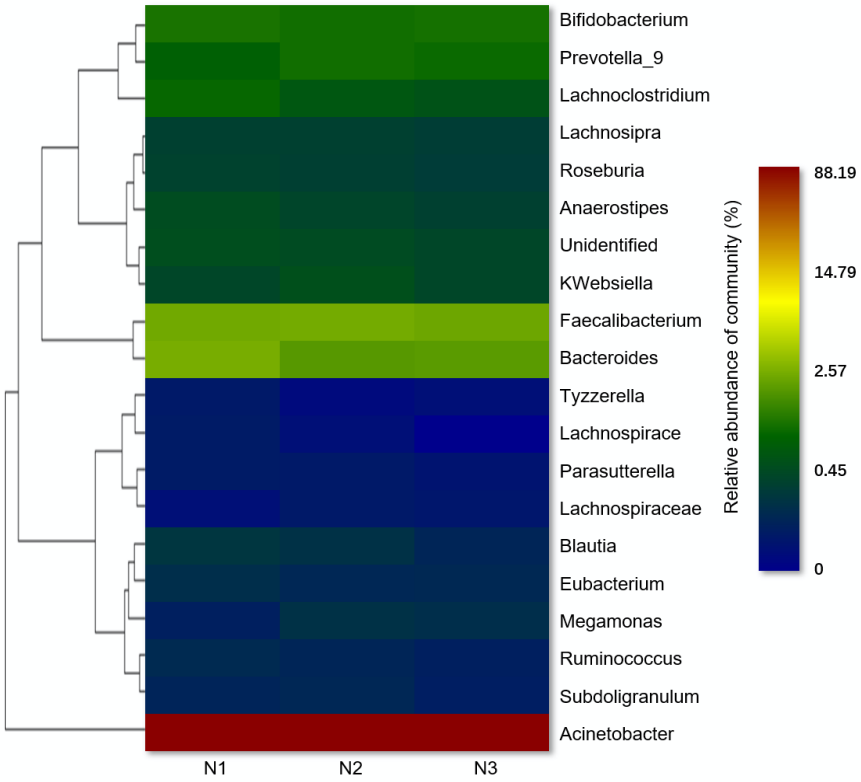

Figure 2. *The heatmap-phylogenetic trees of the top 20 multi-sample OTUs*

Growth curve of bacteria

Using the absorbance of bacteria at 600 nm to estimate bacterial growth was the principal method. The results of bacterial growth in beef-peptone medium are shown in *Figure 3*. Within the 6 h adaption period from inoculation, the population of the N-8 strain grew slowly. From 6 h to 25 h, logarithmic growth occurred, during which time N-8 grew rapidly, and the OD_{600} increased from 0.042 to 1.003. After 25 h, the growth rate of the N-8 strain starting to decreased, and the OD_{600} remained stable at 1.0, approximately. The results (*Fig. 3*) showed that the population of the N-8 strain varied with time during the logarithmic period, significantly.

Anaerobic phosphorus release

The anaerobic phosphorus release experiment was carried out with initial phosphate concentration was 25 mg L^{-1}. The phosphorus variation of the systems during the

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anaerobic period is shown in *Figure 4*. In this part, we used the phosphorus release rate follow *Equation 2* to characterize whether phosphorus release occurs. The $R_{release}$ greater than 1 indicated the occurrence of phosphorus release, whereas a value less than 1 indicated the absence of phosphorus release. The results show that phosphorus release will occur under anaerobic conditions and only in neutral and alkaline environments. *Figure 4* shows that the phosphorus contents in the neutral and alkaline environments (pH = 7, 9 and 11) were all higher than 1.0 and have the significantly different with that in the acid conditions (pH = 3 and 5) at $p < 0.05$ level based on the paired-samples T-test. The average values of phosphorus release rate at pH = 7, 9, 11 were 1.21 (±0.23), 1.19 (±0.20) and 1.17 (±0.18), respectively. In contrast, the contents under acidic conditions (pH = 3 and 5) were less than 1, meaning that no phosphorus release occurred. Thus, phosphorus release was more likely to occur in neutral and alkaline environments for DPAB, which may occur because the acidic conditions are not suitable for the growth of these microorganisms (Tsuneda et al., 2006). The phenomenon of phosphorus release may be caused by the microbes' poly-P degradation (Thwaites et al., 2018) to maintain their own activities in the absence of electronic receptors and HAc.

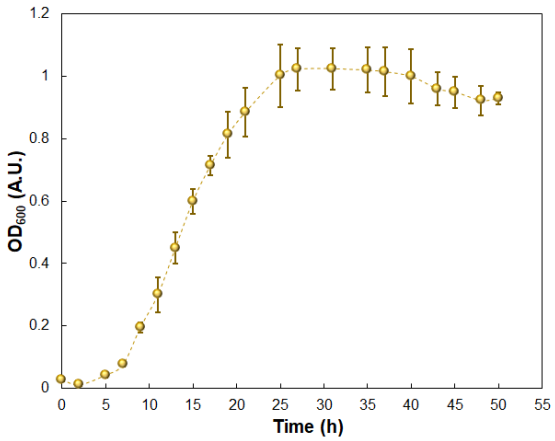

Figure 3. *Growth curve of denitrifying phosphorus-accumulating bacteria strain N-8 in 48 h*

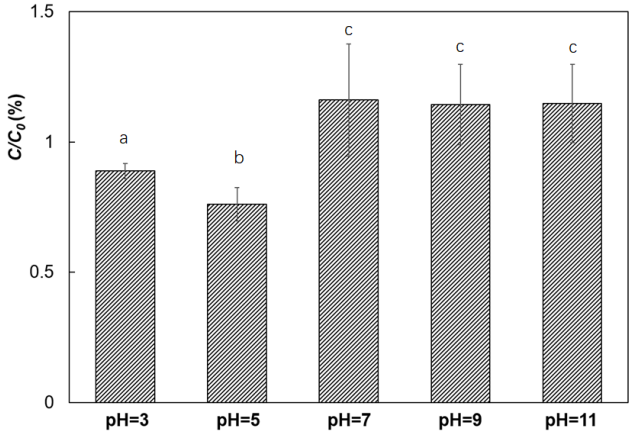

Figure 4. *The phosphorus release rate after the anaerobic period (4 h). Data are mean ± standard deviation and the maximum extent of their errors is shown as the error bar. Bars with the same letter are not significantly different at $p < 0.05$*

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Anoxic phosphorus uptake

Batch experiments in this part were performed to investigate the effect of different types and concentrations of electron acceptors (NO_3^- and NO_2^-) on the anoxic phosphorus uptake of DPAB under different pH conditions. The batch experiments were defined as A, B, C, D and E, with nitrate concentrations of 10, 20, 30, 40, and 50 mg L^{-1} or nitrite concentrations of 1, 2, 3, 4 and 5 mg L^{-1}, respectively. The pH values were 3, 5, 7, 9 and 11. At the beginning of the anoxic stage, electron acceptors (nitrate or nitrite) in different concentrations were added to start the phosphorus uptake of DPAB.

The concentrations of phosphate at the end of a cycle (C_{7h}) are shown in *Figure 5a*. According to *Equation 3,* the values and changes of phosphorus uptake with each sample are shown in *Figure 5b*. The results show that the phosphorus contents of all systems at the end of anoxic stage decreased. So, we can conclude anoxic phosphorus uptake did occur with both nitrite and nitrate as electron acceptors. And from *Figure 5b* we could speculate the average phosphorus uptake of nitrite system (12.5 ± 2.3 mg/L) was higher than nitrate (10.3 ± 4.8 mg/L, $p < 0.05$). But when pH = 7 and pH = 9, the amount of nitrate system (15.0 ± 2.7 mg/L) slightly higher than that of nitrite system (14.3 ± 1.7 mg/L) with no significant difference ($p > 0.05$). Zhou et al. (2010) demonstrated that anoxic phosphorus uptake could be achieved successfully by using nitrate or nitrite as the electron acceptor, and the relatively small amount of anoxic phosphorus was taken up by microorganisms with nitrite for one DPAB. There was a slight difference in activity between the two types of electron acceptor systems, and the amount of phosphorus uptake under anoxic conditions also had significant relationships with pH and the electron acceptor concentration.

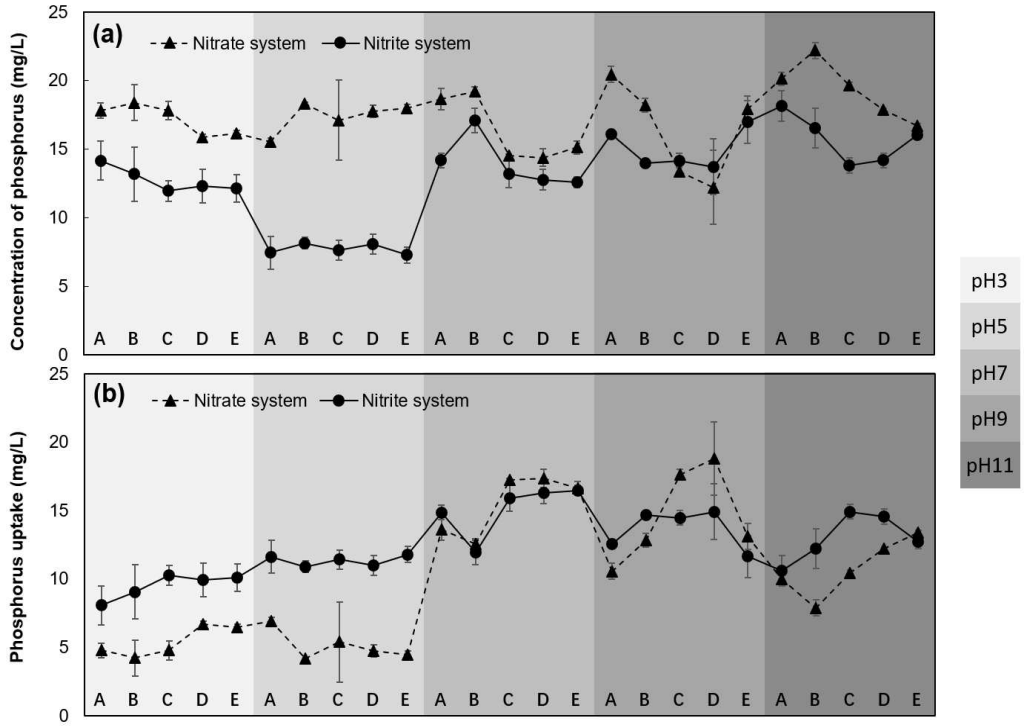

Figure 5. *Concentration of phosphate (a) at the end of the anoxic stage (C_{7h}) and the amount of phosphate uptake (b) during the anoxic stage. From A to E represent the concentration of nitrate (10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1}, respectively) and nitrite (1 mg L^{-1}, 2 mg L^{-1}, 3 mg L^{-1}, 4 mg L^{-1} and 5 mg L^{-1}, respectively)*

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Phosphorus uptake among different pH systems exhibited obvious differences, with better efficiency in neutral-alkaline environments. The changes in the nitrate system were more significant than those in the system with nitrite as the electron acceptor. For nitrate as the electron acceptor, the amount of uptake in neutral-alkaline environments was significantly higher than that in acidic environments, with average uptake amount of 13.6 ± 3.2 mg/L and 5.2 ± 1.0 mg/L, respectively. In contrast, with nitrite as the electron acceptor, the increased uptake with the increase of pH was from 9.5 ± 0.8 mg/L (pH = 3) to 15.1 ± 1.7 mg/L (pH = 7), stabilized at 13.6 ± 1.3 mg/L (pH = 9) and 13.0 ± 1.6 mg/L (pH = 11). The level of phosphorus uptake under acidic conditions (pH = 3 and 5) with nitrite as the electron acceptor was better than that in the nitrate system ($p < 0.01$). One reason for this phenomenon may be that the nitrite aqueous solution is alkaline (Mack and Bolton, 1999), and it can neutralize a portion of the hydrogen ions.

Phosphorus uptake had been influenced significantly by the initial concentration of nitrate or nitrite. According to paired-samples T-test, A level vs. C, D and E level, and B level vs. C, D, and E level showed a significant difference ($p < 0.05$), but C, D and E did not show that with each other. The highest average phosphorus uptake showed at C level whether for nitrate (15.5 mg/L) or nitrite (15.1 mg/L), then decreased with the increased or decrease of electron acceptor concentration. Therefore, within a certain range, a higher electron acceptor concentration could promote the activity of anoxic phosphorus uptake. However, if the concentration rose beyond the optimal value, it would be inhibitory to phosphorus removal. Zhou et al. (2010) showed that phosphorus uptake was impacted by the limitation of electron acceptor when limited concentration of nitrate present under anoxic conditions, but when the concentration out of the range of 60 to 120 mg L^{-1}, the activity was not further promoted. Thus, ρ(nitrate) = 30 mg L^{-1} and ρ(nitrite) = 3 mg L^{-1} are proved to be the optimal concentration of electron acceptor for the phosphorus uptake efficiency in this study.

Phosphorus removal efficiency

The profiles of denitrifying-phosphorus removal are shown in *Figure 6a*. The removal rate was evaluated as *Equation 1*. The phosphorus removal rates of the nitrite systems were higher than those of the nitrate systems ($p < 0.01$). The average rates of the nitrite systems were 49.2 ± 3.3% (pH = 3), 69.2 ± 1.4% (pH = 5), 44.2 ± 6.7% (pH = 7), 40.1 ± 5.2% (pH = 9) and 49.2 ± 3.3% (pH = 11), respectively, while for the nitrate systems, they were 31.2 ± 4.0% (pH = 3), 30.7 ± 4.0% (pH = 5), 34.6 ± 8.4% (pH = 7), 34.3 ± 12.5% (pH = 9) and 22.8 ± 7.6% (pH = 11), respectively. Similarly, Zeng et al. (2013) showed that phosphorus removal efficiency under a nitrite system was higher than that in a completely nitrifying environment. Moreover, the denitrifying-phosphorus removal rate increased with the increased initial concentration at low conditions of both electron acceptors, and high concentrations will inhibit the effect, such as pH = 9, ρ(nitrate) = 50 mg L^{-1}, ρ(nitrite) = 5 mg L^{-1} and pH = 11 ρ(nitrite) = 4 and 5 mg L^{-1}. The optimal stoichiometric concentration of electron acceptor and pH for the phosphate removal by microorganisms in the nitrate and nitrite systems appeared at ρ(nitrate) = 30 mg L^{-1} at pH = 9 and ρ(nitrite) = 5 mg L^{-1} at pH = 7, respectively.

The influence of pH on phosphorus removal was somewhat complicated (Li et al., 2018) and could be divided into two circumstances. The microorganism played a significant role on removal rates under neutral and alkaline conditions. However, the removal rate was mainly controlled by the physical and chemical processes under acidic conditions because the most suitable pH for DPAB was pH = 7~9 (Sun et al., 2016).

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Another reason for this phenomenon was the physical properties of nitrite, for which the aqueous solution was alkaline (Mack and Bolton, 1999). Under acidic environments, greater H^+ is consumed with the increased pH.

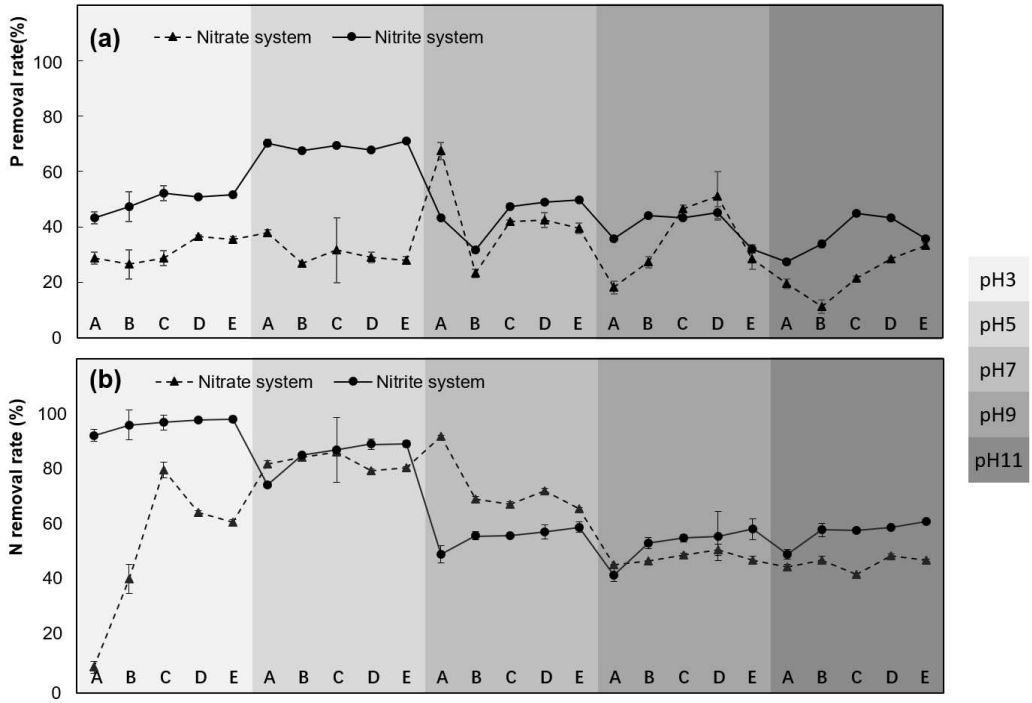

Figure 6. *Removal rate of phosphate (a) nitrogen (b) during the cycle. From A to E represent the concentration of nitrate (10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1}, respectively) and nitrite (1 mg L^{-1}, 2 mg L^{-1}, 3 mg L^{-1}, 4 mg L^{-1} and 5 mg L^{-1}, respectively)*

Nitrogen removal efficiency

The results of nitrogen removal in the two systems obtained from the tests are shown in *Figure 6b*. Regarding the variation of nitrogen removal, there was no significant difference between nitrate and nitrite systems with various environmental factors, except at pH = 3. The removal rates in the nitrite systems were obviously higher than those in the nitrate systems at pH = 3. The influence of pH on nitrogen removal was similar to its influence on phosphorus removal, with a higher removal rate in nitrite systems in acidic environments (pH = 3 and 5) and higher removal rates in nitrate systems in neutral-alkaline environments (pH = 7, 9 and 11). The highest nitrogen removal rates for nitrate and nitrite were observed around ρ(nitrate) = 10 mg L^{-1} at pH = 7 and ρ(nitrite) = 5 mg L^{-1} at pH = 3, respectively. Additionally, nitrogen removal in nitrite systems slowly increased with an increased electron acceptor concentration in neutral-alkaline environments, whereas no such changes were observed in nitrate systems, as shown in *Figure 6*. The higher nitrogen removal by microorganisms appeared at pH = 5, with removal rate of 82.69% (±2.66%) for nitrate and 85.36% (±6.13%) for nitrite.

Correlation and comparison

The correlation analysis of phosphate removal rate (R_P) and nitrate removal rate (R_N) of the entire operation was investigated, as shown in *Table 2*. Pearson's correlation

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analysis results revealed considerable differences between the two types of systems under different conditions. In the nitrate system, the Pearson's correlation coefficient (PCC) between R_P and R_N with all pH levels showed no obvious correlation, with PCC = 0.37 (P = 0.068). In the nitrite system, there was a significant positive correlation between the removal of PO_4^{3-} and the removal of NO_2^-, with PCC = 0.624 (P = 0.001). The removal of nitrogen-phosphate at all pH levels in the nitrite system appears to be more complicated than that in the nitrate system. Moreover, under neutral-alkaline conditions, the correlation coefficient in the nitrate system, PCC = 0.686 (P = 0.005), was much higher than that for nitrite.

The experimental results discussed above reveal that both nitrate and nitrite as electron acceptors were effective for DPAB in denitrifying-phosphorus removal. Phosphorus removal was mainly completed by denitrifying-phosphorus removal occurring under anoxic conditions. The level of phosphorus uptake by microorganisms in the nitrite system was higher than that with nitrate as the electron acceptor. Furthermore, the results demonstrated that there is a certain relationship in both nitrite and nitrate systems between the activities of anoxic phosphorus uptake and the initial concentration of electron acceptors. The optimal environmental factors of nitrate and nitrite for denitrifying-phosphorus removal were ρ(nitrate) = 30 mg L^{-1} at pH = 7 and ρ(nitrite) = 3 mg L^{-1} at pH = 7, respectively. Other stoichiometric studies on the denitrification processes showed that nitrite is, compare to nitrate, more active and sensitive electron acceptor for achieving nitrification (Zhou, 2010, 2001).

Table 2. *Pearson's correlation coefficients analysis of different electron acceptors*

Condition		R_P v.s. R_N	
		PPC	p
All pH	Nitrate	0.37	0.068
	Nitrite	0.624**	0.001
pH = 7.9 and 11	Nitrate	0.686**	0.005
	Nitrite	0.249	0.371

*Significant correlation at .05 level (bilateral)
**Significant correlation at .01 level (bilateral)

Conclusion and recommendations

To further improve the efficiency of sewage treatment and management, it is important to understand the metabolic behavior of denitrifying phosphorus-accumulating bacteria, which play a crucial role in the removal of nitrogen and phosphorus elements in wastewater treatment. In this work, the screened strain of DPAB, named N-8, Acinetobacter sp., could adopt both nitrate and nitrite as electron acceptors and has high synchronous nitrogen and phosphorus removal efficiency under anoxic condition. The types and concentrations of electron acceptors, as well as the initial pH of the systems, have significant effects on the removal of nitrogen and phosphorus. There were a stronger phosphorus and nitrogen removal efficiency by DPAB in the nitrite system than that in the nitrate system. Moreover, the effect of DPAB was greater in neutral and alkaline environments. The optimal stoichiometric concentrations of electron acceptor and pH for phosphate removal in the nitrate and nitrite systems were ρ(nitrate) = 30 mg L-1, pH = 9, and ρ(nitrite) = 5 mg L-1, pH = 7,

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respectively. Based on previous studies, almost all of DPAB are able to utilize oxygen (Hu et al., 2002; Tsuneda et al., 2006) as electron acceptors to remove phosphorus, we speculate that strain N-8 also can utilize oxygen with a different pattern from nitrate and nitrite, but the detail is not investigated in this work. Therefore, further research on the utilization patterns of DPAB for three types of electron acceptors, oxygen, nitrate and nitrite, is strongly recommended.

Acknowledgements. This research was supported by National Major Science and Technology Program for Water Pollution Control and Treatment (No. 2017ZX07103-004), Beijing Science and technology plan (Research and demonstration of water environment security technology based on reclaimed water ecological replenishment, Z181100005518012) and National Key R&D Program of China (No. 2019YFC0409201and No. 2018YFC1508705).

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APPENDIX

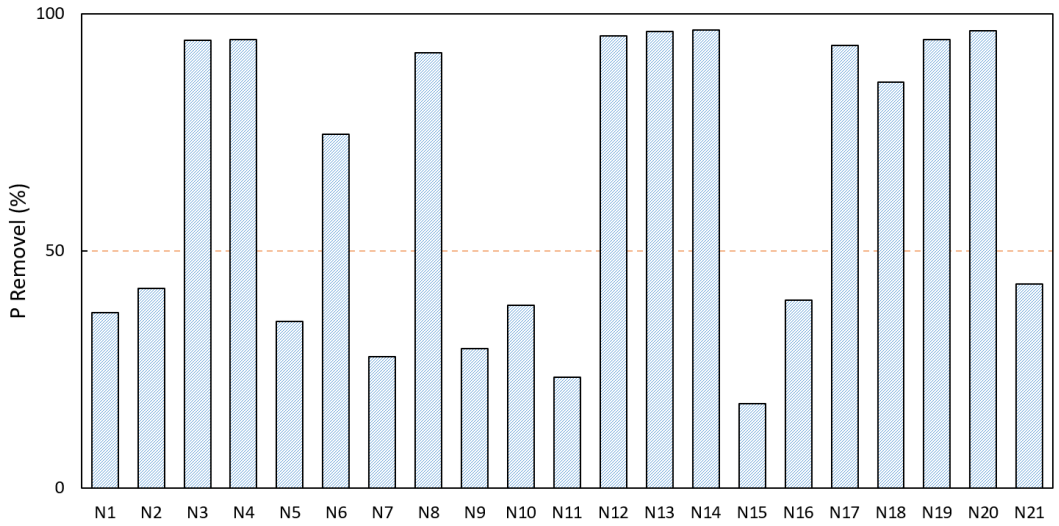

Figure A1. *Phosphorus removal rates of 21 colonies that can survive on the isolation medium. These colonies that showed a greater than 50% ratio will be further tested*

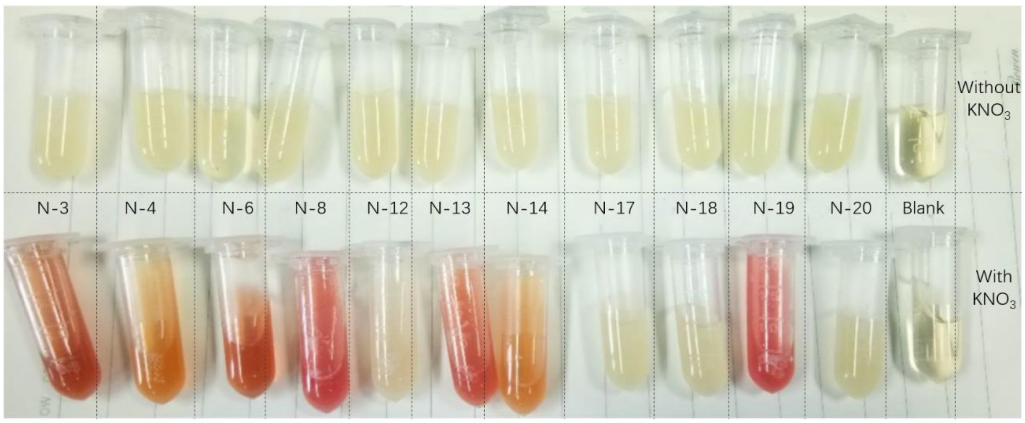

Figure A2. *Nitrate reduction test results of 11 colonies whose phosphorus removal rates exceeding 50%*

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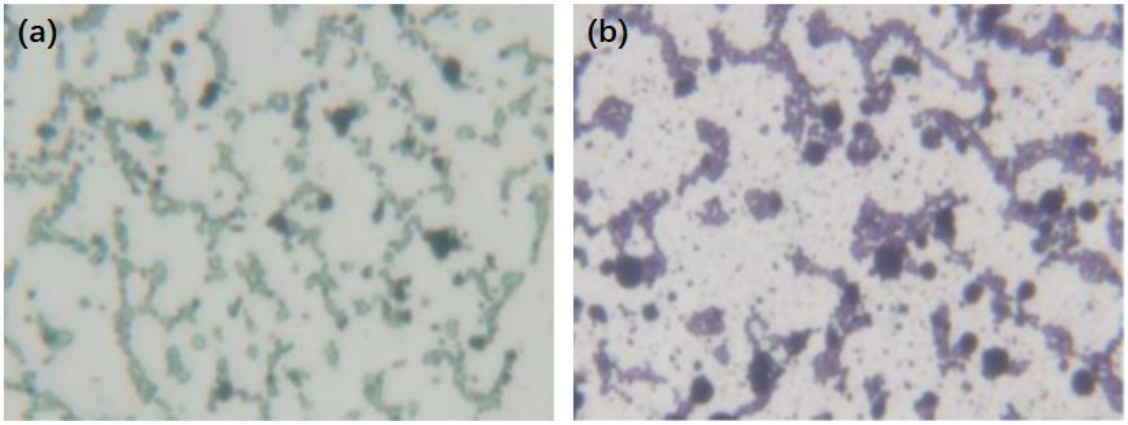

Figure A3. *Albert's metachromatic granules (a) and PHB granules (b) staining results for N-8*

Table A1. *Results of denitrifying phosphorus accumulating bacteria screening test*

	N-3	N-4	N-6	N-8	N-12	N-13	N-14	N-17	N-18	N-19	N-20
P-removel (%)	94.5	94.6	74.6	91.8	95.4	96.3	96.9	93.4	85.7	94.6	96.4
Albert Staining	+	+	-	+	+	+	-	+	+	-	+
PHB Staining	-	-	+	+	+	-	-	+	-	+	+
Nitrate reduction test	+	+	+	+	-	+	+	-	-	+	-

" + " indicates positive, "-" indicates negative

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DOI: http://dx.doi.org/10.15666/aeer/1806_78657880

ANALYSIS ON THE COORDINATED DEVELOPMENT OF URBANIZATION AND ECOLOGICAL ENVIRONMENT OF THE CITY CLUSTER ALONG THE MIDDLE REACHES OF THE YANGTZE RIVER IN CHINA

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(Received 18th Mar 2020; accepted 20th Aug 2020)

Abstract. This paper studies the evolution process of the urbanization and ecological environment level of 28 cities in the city cluster along the middle reaches of the Yangtze River in China from 2007 to 2017 on the scale of city cluster and discusses the coordination relationship between the two. The research found that: (1) During the research period, the urbanization level and the ecological environment level of 28 cities increased significantly. (2) The urbanization is generally at a low level and the spatial imbalance among cities is gradually expanding; the ecological environment is generally at a medium level and the spatial imbalance among cities is gradually decreasing. (3) The center transferring track and the center variation consistency of the urbanization level and the ecological environment level present a changing trend of "reverse direction - same direction", and the overall coordination of the two is gradually enhanced. (4) The spatial coordination between the urbanization level and the ecological environment level is constantly enhanced. The Getis-Ord Gi* index generally shows a spatial distribution feature of "high in the southeast area and low in the northwest area".

Keywords: *spatiotemporal differentiation, spatial equilibrium, kernel density estimation, exploratory spatial data analysis, PSR model, center of gravity coordination model*

Introduction

The city cluster area is the strategic core area of national economic development and the main area of national new urbanization which undertakes the historical responsibility of bearing the transfer of the world's economic centers (Fang, 2014). The development of the city cluster not only determines the future of China but also determines the development progress of urbanization in the world (Fang et al., 2015). As a huge engine to promote China's economic and social development, urban agglomerations focus too much on the speed of development in the process of urbanization, resulting in serious environmental problems such as environmental pollution, ecological damage and resource shortage. These problems in turn restrict the development process of urbanization. Coordinating the relationship between urbanization and ecological environment now is a worldwide strategic issue that is widely concerned by the academic circles and the government decision-making departments (Fang et al., 2016; Liang et al., 2019). Statistics show that, at present, China's urban agglomerations discharge more than 67% of the country's industrial

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DOI: http://dx.doi.org/10.15666/aeer/1806_78817900

waste water, waste gas, solid waste, etc. Although urban agglomerations concentrate more than 3/4 of the total economic output and economic output of the country, at the same time, more than 3/4 of the national pollution output is concentrated, and the environmental pollution problem is becoming increasingly prominent (Fang et al., 2016). According to the prediction of the Chinese Academy of Sciences, in the next 20 years from 2011 to 2030, it will additionally require 3.2 billion m^3 of urban water and 3460 km^2 of urban construction land, consume 210 million tons of standard coal and add 2.34% of ecological overload for every 1% increase of the urbanization level in China, which are respectively 1.88 times, 3.45 times, 2.89 times and 2.42 times more than those in the past 30 years (1980-2010). Whether these resource and environmental issues can be addressed will directly affect the bright future of China's urbanization development in the next few decades (Fang and Fang, 2013). According to China's urbanization development planning target, the urbanization rate is estimated to reach 65%-70% by 2030 (Hou et al., 2014), which means there will be about 400-500 million rural people entering the town in the future, and this process is bound to bring huge impacts on the ecological environment. Therefore, the research on the coordinated development of urbanization and ecological environment is not only the hot spot and frontier of international scientific research but also the strategic demand for sustainable development of the city cluster areas in China (Kates et al., 2001; Clark, 2007; Reid et al., 2010).

Objectively, there is an interactive coercing relationship between the urbanization and the ecological environment. With rapid development of the economy, the impact and pressure on the ecological environment brought by urbanization become more obvious, and the constraints and restrictions to the urbanization by ecological environment also become more prominent (Sun et al., 2017; Fang and Yang, 2006). The research on the interactive coupling effect between urbanization and ecological environment in mega-city cluster areas is the frontier and a high-priority theme of the scientific research of the earth system in the next 10 years (Fang et al., 2016). Currently, the research on the coordinated development of urbanization and ecological environment mainly focuses on the following aspects:

(1) Research of theoretical framework. Scholars paid attention to the issue of urban development and ecological environment at an earlier time, but systematic research on the relationship between urbanization and ecological environment mainly began from the middle of the 20th century (Liu et al., 2005a). In 1971, the United Nations Educational, Scientific and Cultural Organization conducted a comprehensive exploration of the relationship between humanity and the natural environment and prepared the "Man and the Biosphere" program, which caused people's widespread concern about environmental pollution. The book *Limits to Growth* by Meadows predicted "limited growth" about the world's urbanization prospects, which aroused widespread concern about the world's resources and environmental issues caused by urbanization (Sun et al., 2017). B. Ward commented the impact of economic development and environmental pollution on different countries in his book *Only One Earth* and called on countries to pay attention to the ecological environment and the problems faced by urbanization and industrialization (Wandesforde-Smith and Rosenbaum, 1986); in Goldsmith's *Blueprint of Life* and West's *Cities in Crisis*, it comprehensively analyzed and predicted the ecological crisis in the urbanization process and drove related research into diversified fields (Kjellstrom and Corvalan, 1995). Since then, related scholars and institutions have conducted deep research

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closely around topics such as ecological cities and sustainable cities, providing the direction for the study of complex problems in the urbanization process (Lin, 2006; Friedman, 2006; Zhang and Zhao, 2003). Ma and Wang (1984) proposed the idea of complex ecosystem, enriching the theoretical basis of urban ecological research. Wang and Liu (1988) proposed the use of methods like ecosystem cybernetics and general goal planning to study the internal structure and external environment of the ecosystem. Fang et al. (2016) created the theoretical framework of the near and remote interactive coupling effect based on the complex nonlinear coupling relationship between the two. Some scholars have carried out a series of explorations on sustainable development (Yan and Tang, 1984; Yang and Zhu, 1999).

(2) Analysis of the interactive coupling mechanism. Related research studies focus on aspects including key elements of the interactive coercing between urbanization and ecological environment, spatial-temporal evolution features, coupling mechanism and rules and risk diagnosis and evaluation. David J. Rapport and Tony Friend proposed the theoretical framework of "Pressure-State-Response" (PSR) (Berger and Hodge, 1998). Grossman and Krueger discovered the evolution rule of inverted "U"-type relationship between the two, namely the famous Environmental Kuznets Curve (EKC) (Caviglia-Harris et al., 2009). York et al. (2003) built a double logarithmic model to describe the relationship between the two. Gu (1994) proposed three models of domestic urbanization and ecological environment. Liu et al. (2005b) revealed the main controlling factors of the interactive coupling and the mutual restraining effect between the two. Huang and Fang (2003) deduced the double exponential curve between the two by fitting the Environmental Kuznets Curve and the logarithmic curve of urbanization, which further enriched the theoretical research.

(3) Technical methods and path research projects. Mainly based on GIS, integrated modeling and big data methods, an integrated spatial-temporal coupling dynamics model is constructed to quantitatively study the interactive coupling effect between the two (Fang et al., 2016). Sultan et al. (1999) used the 3 "S" technology to analyze the pressure of urbanization on the ecological environment of the Nile Delta. Varis and Fraboulet-Jussila (2002) used the neural network model to analyze that the urbanization process aggravated the water resource crisis in the Senegal River basin; Veziroglu and Macário (2014) used the system dynamics model to evaluate the water resource use efficiency under the background of rapid urbanization; Ducrot et al. (2004) simulated the water resource response in the process of urbanization based on the CA model; Saysel et al. (2002) used the gravity model to reveal the mechanism of agricultural land transformation and development under the background of rapid urbanization; Kok et al. (2000) analyzed the dynamic correlation mechanism between urbanization and water resource in Indonesia based on the fuzzy sets theory. Qian and Wang (1995) explored the relationship between the two by means of remote sensing and GIS technology; Shi and Chen (1996) used dynamic simulation system to explore the structure and layout of complex ecosystems; Qiao and Fang (2005) studied the path of coordinated development of them. In general, these studies gradually transformed from qualitative description to quantitative analysis and laid theoretical and practical foundation for the follow-up scholars' research (Sharma et al., 2013; Radhi et al., 2013; Fang and Wang, 2013; Xiong et al., 2012; Lenschow et al., 2016). As to the research contents, scholars mainly explored the relationship between the two under the framework of sustainable development theory, and qualitative analysis was more than quantitative analysis. Regarding the research areas, scholars were mainly concerned in arid areas and

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economically developed areas. The research areas included national, provincial and prefecture-level cities, but there were few research studies on the city cluster and its internal areas.

Based on this, this paper takes the city cluster along the middle reaches of the Yangtze River in China as the research object to build a comprehensive evaluation index system. Firstly, the entropy weight - TOPSIS model was used to evaluate the index value of urbanization and ecological environment system. Then, the kernel density estimation and the spatial barycenter transfer model were used to explore the spatial evolving characteristics and distribution patterns of each system. Finally, we use the coupling coordination model to determine the coordinated development pattern of each system, in order to provide reference for the coordinated sustainable development of urbanization and ecological environment system of urban agglomerations.

The main research objectives of this paper are as follows: (1) Evaluate the comprehensive level of the urbanization and the ecological environment of the city cluster along the middle reaches of the Yangtze River, and understand the development situation of the urbanization level and the ecological environment level. (2) Quantitatively analyze the coordination status of the urbanization and the ecological environment and their spatial distribution features using the coordination level model. (3) Based on the conclusions of the above theoretical discussion and empirical analysis, propose corresponding countermeasures and suggestions.

Study area, index selection and data sources

Overview of the study area

The city cluster along the middle reaches of the Yangtze River is a super large urban agglomeration with Hubei, Hunan and Jiangxi provinces as the main body (*Fig. 1*). It is located in the middle of the Yangtze River Basin in China, with a total of 31 cities. (In this study, Xiantao City, Qianjiang City and Tianmen City are excluded due to the lack of data. The research unit includes 28 cities.) In 2017, the city cluster along the middle reaches of the Yangtze River has a land area of 326100 km^2, a total population of 125 million people, and a GDP of 7.90 trillion yuan, ranking the fifth among the urban agglomerations in China. The per capita GDP was 63205 yuan, 3545 yuan higher than the national average. The level of urbanization is 59%, 2.5 percentage points higher than that of the whole country. The city cluster along the middle reaches of the Yangtze River accounts for 9.6% of the total economic volume with 3.4% land area and 9.0% population in China. The economic structure of the city cluster along the middle reaches of the Yangtze River is dominated by industry, and the heavy chemical industry is obviously dominant in the industrial system. In 2017, the industrial output value of iron and steel, non-ferrous metallurgy, petrochemical industry, coal mining and electric power, building materials and other energy raw materials accounted for 50% of the total industrial output value, and the output value of equipment manufacturing industry accounted for 32% of the total industrial output value. The industrial output value of the industries mentioned above were 82% of the total industrial output value. Most of the industries are high energy consuming industries, which are the main sources of industrial pollution in urban agglomerations. This urbanization process led by industry not only intensifies the demand for natural resources, but also causes a lot of environmental problems. Therefore, the research on the coordinated relationship

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between urbanization and ecological environment has certain practical significance for the healthy and harmonious development of the region.

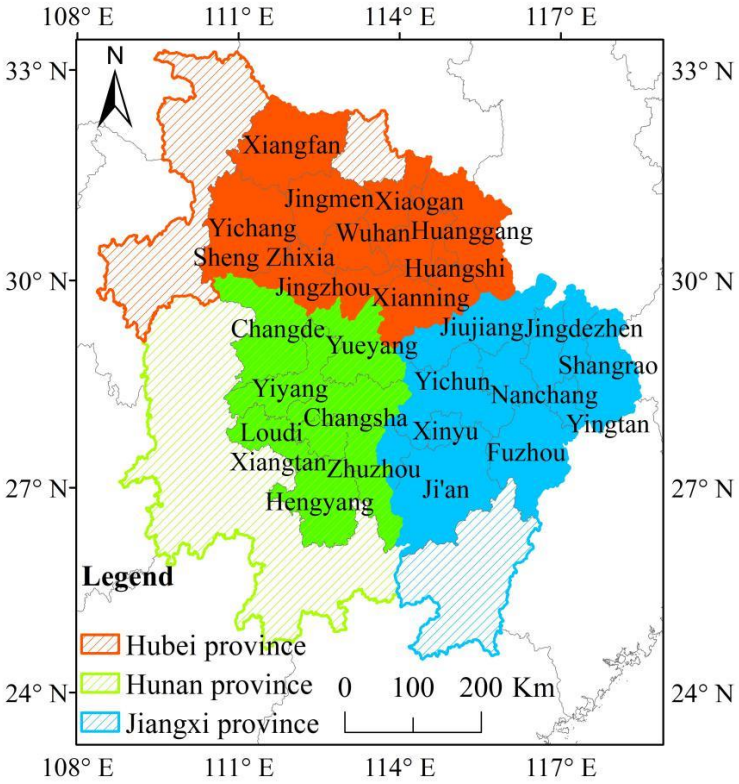

Figure 1. *Overview of the study area*

Index selection and data sources

Index selection

Based on the understanding of the meaning, features and functions of urbanization and ecological environment development, with reference to existing researches (Liang et al., 2019; Zhang et al., 2014; Xie et al., 2015), a comprehensive evaluation index system is constructed (*Table 1*).

Research objects and data sources

28 cities in the city cluster along the middle reaches of the Yangtze River (excluding the 3 cities of Tianmen, Xiantao and Qianjiang) are selected as the research objects. Three time sections in 2007, 2012 and 2017 are selected in this paper, and the data comes from *China Statistical Yearbook*, *China City Statistical Yearbook*, *the statistical yearbooks of Hubei Province*, *Jiangxi Province* and *Hunan Province*.

Research methods

Measurement of the urbanization level and the ecological environment level

The entropy weight - TOPSIS model is an improvement of the weight determination method in the traditional TOPSIS evaluation method by using the entropy weight

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method. It has the advantages of practicality and objectivity and has little demand for the sample but the result is reasonable (Ren et al., 2017). In this paper, existing research studies are used for reference (Ren et al., 2017), and the entropy weight - TOPSIS model is adopted to measure the urbanization level and the ecological environment level of the researched area.

Table 1. *Evaluation index system for urbanization level and ecological environment level*

System layer	Criterion layer	Index layer	Index nature
Evaluation index system for urbanization level	Basic process	Urban population/total population	+
		Non-agricultural employed population/total employed population	+
		Non-agricultural output value/GDP	+
		Total export-import volume/GDP	+
		Foreign direct investment/GDP	+
	Economic benefit	Per capita GDP	+
		Per capita fixed asset investment	+
		Per capita social consumption	+
		Per capita disposable income of residents	+
		Per capita local fiscal revenue	+
	Social development	Per capita urban park green land area	+
		Hot gas penetration rate	+
		Number of medical beds per 10,000 people	+
		Urban and rural insurance coverage rate	+
		Number of college students per 10,000 people	+
	Urban-rural integration	Urban per capita disposable income	+
		Rural per capita disposable income	+
		Urban-rural consumption ratio	-
		Urban-rural dual structure index	-
	Technology support	Number of patent grants per 10,000 people	+
		Per capita postal and telecom services	+
		Per capita science and technology investment	+
		Number of Internet broadband access users per 10,000 people	+
Evaluation index system for ecological environment level	Ecological environment pressure	Industrial wastewater discharge intensity	-
		Industrial sulfur dioxide discharge intensity	-
		Industrial smoke (dust) discharge intensity	-
		Per capita water consumption	-
		Per capita liquefied petroleum gas consumption	-
		Natural population growth rate	-
		Built-up area/administrative area	-
	Ecological environment situation	Per capita cultivated land area	+
		Per capita water resource	+
		Good or moderate air quality achievement rate in urban area	+
		Forest coverage rate	+
		Per capita park green land area	+
		Per capita industrial sulfur dioxide discharge	-
		Per capita industrial waste water discharge	-
		Per capita industrial solid waste discharge	-
	Ecological environment response	Green coverage rate in built-up areas	+
		Forest plantation area	+
		Comprehensive utilization rate of industrial solid waste	+
		Centralized treatment rate of sewage treatment plant	+
		Decontamination rate of domestic garbage	+
		Unit GDP energy consumption	-
		Number of patent grants per 10,000 people	+

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Measurement of imbalance

This paper uses the kernel density estimation method in Eviews 9.0 software to analyze the kernel density of the urbanization level and the ecological environment level of the researched area (Wang et al., 2018). The estimation formula of kernel density is:

$$f(x) = \frac{1}{nh}\sum_{i}^{n}\frac{K(x_i - x)}{h} \quad \text{(Eq.1)}$$

In the formula: $f(x)$ is a probability density function; n is the number of researched areas, $i = 1, 2, ..., n$; h is the bandwidth; $K(x_i - x)$ is the random kernel estimation function.

Measurement of coordination

(1) Measurement of overall coordination

In this paper, the center model and the spatial coupling situation of the center are used to reflect the overall coupling situation between the two. The center model is (Zhou et al., 2016):

$$G_I(x, y) = \frac{\sum_{j}^{n} I_j(Q(x_j, y_j))}{\sum_{j}^{n} I_j} \quad \text{(Eq.2)}$$

In the formula: G_I respectively represents the center of the urbanization level and the ecological environment level; n is the number of researched areas; I_j is the urbanization level and the ecological environment level of the number j city respectively; x_j and y_j are respectively the longitude and latitude coordinates of the number j city.

The spatial distribution overlapping and the center variation consistency are used to investigate the spatial coupling situation of the centers of them. Among them, the calculation formula for the spatial distribution overlapping is (Zhou et al., 2016):

$$S = \sqrt{(\Delta x_G)^2 + (\Delta y_G)^2} \quad \text{(Eq.3)}$$

In the formula: S represents the distance between the centers of urbanization level and ecological environment level. x_G and y_G represent the longitude and latitude of the center; The closer the distance is, the more the overlapping is.

The calculation formula for the center variation consistency is (Zhou et al., 2016):

$$C = \cos\theta = \frac{\Delta x_A \Delta x_B + \Delta y_A \Delta y_B}{(\Delta x_A{}^2 + \Delta y_A{}^2) \times (\Delta x_B{}^2 + \Delta y_B{}^2)} \quad \text{(Eq.4)}$$

In the formula: $\cos\theta$ represents the cosine of the vector intersection angle θ of the displacement of the centers of urbanization level and ecological environment level at this time compared with the centers at the previous time, and the value range is [-1, 1]. The larger the value is, the more consistent the variation is; Δx and Δy respectively represent the change value of the longitude and latitude of the center at this time compared with the center at the previous time.

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(2) Measurement of spatial coordination

A coupling coordination model is constructed in this paper to measure the coupling coordination level between the two (Zhang et al., 2014).

Coupling level. Coupling level is an index reflecting the level of interactive coercing between two (or more) systems. Its function is:

$$C = \sqrt{F(x) \times G(y) / \left((F(x) + G(y))/2\right)^2} \quad \text{(Eq.5)}$$

In the formula: C is coupling level with the value range of [0, 1]; $F(x)$ is the urbanization level. $G(y)$ is the ecological environment level; the larger C is, the better coupling level is.

Coordination level. The coordination level model in which both the development level of the two and the coupling relationship between the two are considered is introduced to measure the coordinated development between the two. Its function is:

$$T = \alpha F(x) + \beta G(y) \quad \text{(Eq.6)}$$

$$D = \sqrt{C \times T} \quad \text{(Eq.7)}$$

In the formula: D is coordination level; T is the development index. α respectively the contribution share of urbanization; β respectively the contribution share of ecological environment. Considering that the status of the two is equally important, take $\alpha = \beta = 0.5$.

Exploratory spatial data analysis

In order to reflect the spatial correlation pattern feature of the spatial coordination of the two, the Arcgis10.2 software is used in this paper. The global Moran's I statistics was used to analyze the overall spatial correlation level in the area and the Getis-Ord Gi* index was used to explore the distribution of local cold and hot spots in regional cities.

The global Moran's I index is calculated as follows:

$$I = \frac{\sum_{i=1}^{n}\sum_{j=1}^{n} W_{ij}(x_i - u)(x_j - u)}{S^2 \sum_{i=1}^{n}\sum_{j=1}^{n} W_{ij}}; S^2 = \frac{1}{n}\sum_{j=1}^{n}(x_i - u)^2 \quad \text{(Eq.8)}$$

In the formula: the Moran's I index has a value range of [-1, 1]. x_i and x_j are respectively the coordination level value of the city i and j; u is the average value of coordination level; W_{ij} is the spatial weight matrix. If the city i and j have a common boundary, W_{ij} equals to 1. If there is no common boundary between the two, W_{ij} equals to 0.

The Getis-Ord Gi* index is calculated as follows:

$$G_i^* = \frac{\sum_{i \neq j} W_{ij} x_j}{\sum_{i \neq j} x_j} \quad \text{(Eq.9)}$$

In the formula: x_j is the coordination level value of the city j; W_{ij} is the spatial weight matrix.

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Results

Analysis of the urbanization level and the ecological environment level

Analysis of the spatial-temporal pattern

The entropy weight - TOPSIS model are used to perform subjective and objective comprehensive weight determination for the index, and the comprehensive evaluation value of urbanization and ecological environment system was calculated (*Fig. 2*). In order to explore the difference of the development level of different cities, the natural breaks method is used in this paper to classify the comprehensive level of urbanization and ecological environment of the city cluster along the middle reaches of the Yangtze River in three time sections in 2007, 2012 and 2017 into five levels (*Table 2*).

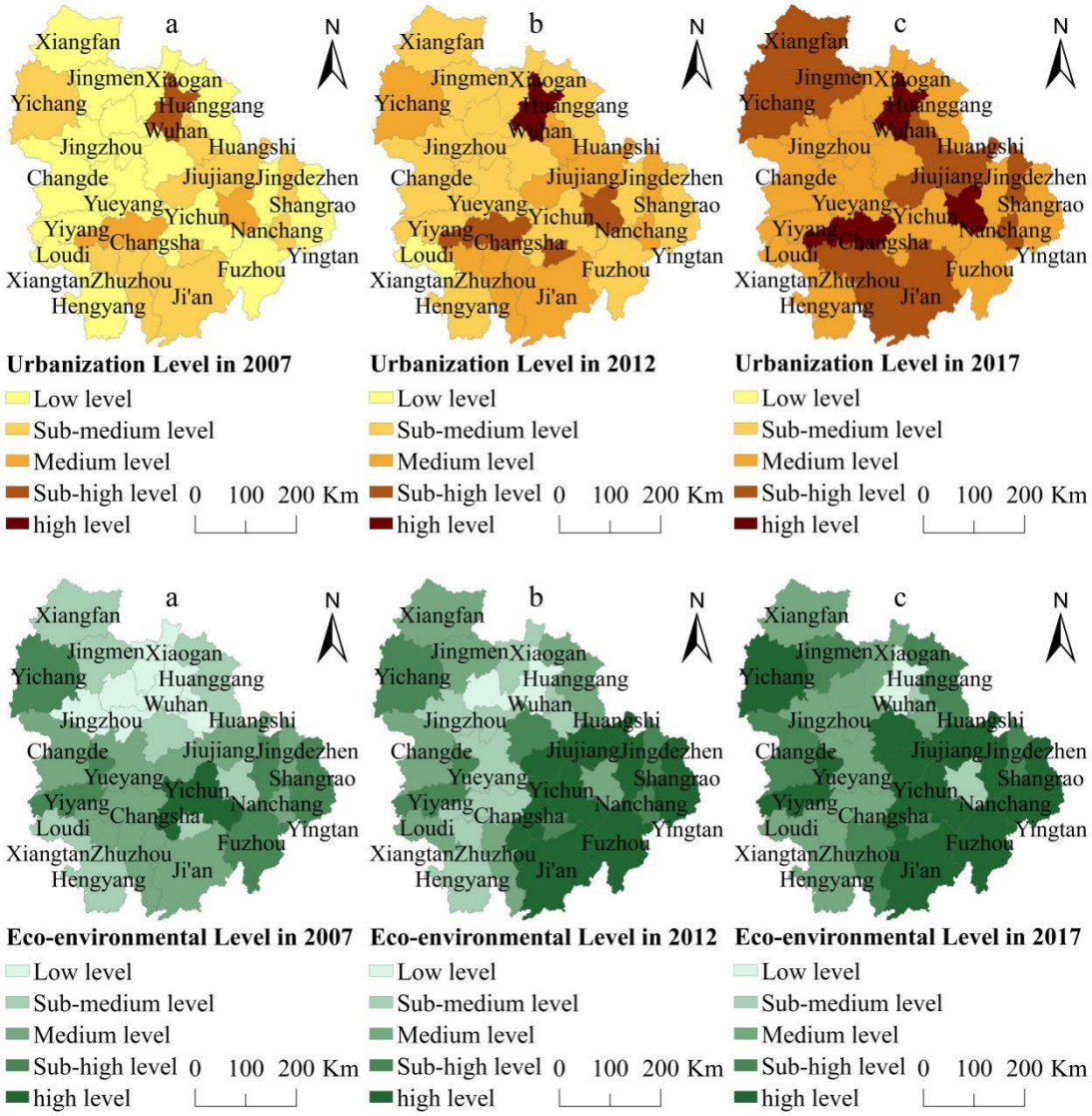

Figure 2. *Spatial-temporal evolution of urbanization and ecological environment from 2007 to 2017*

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Table 2. *Classification criteria for the comprehensive level of urbanization and ecological environment*

Horizontal level	Low level	Sub medium level	Medium level	Sub advanced level	Advanced level
Urbanization	< 0.169	0.169-0.228	0.228-0.309	0.309-0.444	0.444 <
Ecological environment	< 0.400	0.400-0.476	0.476-0.526	0.526-0.567	0.567 <

It can be seen from *Figure 2* that during the research period, the urbanization level of the city cluster along the middle reaches of the Yangtze River increases significantly. The average urbanization levels in 2007, 2012 and 2017 were respectively 0.178, 0.252, and 0.344, and there was no city with negative growth of urbanization level. The urbanization level generally shows the spatial pattern of "scattered distribution of high-value areas and grouped distribution of low-value areas", and the hierarchical structure is relatively stable. The cities with high value mainly include Wuhan, Changsha, Nanchang and Xinyu among which all cities are provincial capital cities except Xinyu. Because the provincial capital city has gathered a large number of economic, policy and talent resources, its urbanization level can continue to improve rapidly. Although the provincial capital city plays a leading role in the province, it also causes the slow development of other cities in the province. The cities with high value basically form three groups which are Yichang - Xiangfan - Jingmen group, Wuhan - Ezhou - Huangshi - Jiujiang - Nanchang group and Changsha - Xiangtan - Pingxiang - Zhuzhou - Xinyu - Ji'an group, and there are also some scattered distributed cities such as Jingdezhen and Yingtan. The cities with high value are basically distributed around the provincial capital cities. The urbanization of the cities with middle and low value is basically maintained at the same level, the distribution is strongly concentrated, mainly distributed at the junction of three provinces and the marginal areas of the provinces, and the number of cities with low level cities is gradually decreasing.

The stability of the ecological environment level is not as good as that of the urbanization. There is obvious volatility in most cities, but the volatility is within a reasonable range. In general, the ecological environment level is in an upward trend, and the spatial distribution presenting a distribution pattern of high in the south and east and low in the north and west. The average ecological environment level in 2007, 2012 and 2017 were 0.473, 0.516 and 0.536 respectively. During the research period, cities with high value are mainly distributed in some areas of Jiangxi Province and Hunan Province. The number of cities is gradually increasing, and cities are gathering in the southeast. There are also some scattered distributed cities such as Yichang and Yiyang. The cities at medium level have great variation and are mainly scattered around the cities with high value, especially Yueyang, Changsha and Xiangtan in which the ecological environment levels first decrease and then increase. The development of these cities is highly dependent on natural resources. However, with the continuous emphasis on environmental protection by the country, these cities begin to pay attention to protect the environment in the later development and improve the enterprises with "high pollution, high energy consumption and high water consumption", which makes the ecological environment recovered. The number of cities with low value is small and constantly decreasing, and they are almost distributed around the provincial capital cities of Wuhan and Changsha. In particular, the ecological environment level of the two capital cities of Wuhan and Nanchang is low, indicating that although the urbanization level in these cities is relatively high,

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the ecological environment is under great pressure and the ecological environment protection needs to be strengthened.

Analysis of imbalance

This paper uses the kernel density estimation method to enter the urbanization level and the ecological environment level of various cities into the Eviews 9.0 software, According to *Equation 1*, the kernel density distribution of urbanization level and ecological environment level was calculated in 2007, 2012 and 2017 and a kernel density distribution map was drawn (*Fig. 3*). From *Figure 3*, it can be seen that: (1) Based on the change trend of the urbanization level: the density curves of 2007, 2012 and 2017 all present a "single peak" distribution, and the level values at peak are 0.16, 0.23, and 0.32, respectively. The increase of the level value is within 0.1, indicating that the urbanization level increases a little and most cities are at low level. The density curves show a change trend from sharp peak to broad peak, the height of the peak decreases and the width increases, indicating that the difference among cities increases and the spatial imbalance is gradually expanding. (2) Based on the change trend of the ecological environment level: the density curve of the ecological environment level presents obvious "double peaks" distribution in 2007, and the level values at peak are 0.36 and 0.48 respectively, indicating that the ecological environment level in 2007 is polarized and there is obvious spatial imbalance. The density curves of 2012 and 2017 present "single peak" distribution, and the level values at peak are 0.50 and 0.57 respectively. The increase of the level value is within 0.1, indicating that the ecological environment level increases a little and most cities are at medium level. The density curves in 2007 and 2012 present a change trend from "double peaks" to "single peak", the height of the peak decreases and the width increases, indicating that the difference among cities decreases but the spatial imbalance problem is still outstanding. The height of the peak of the density curves in 2012 and 2017 increases and the width decreases, indicating that the ecological environment level of most cities is gradually increasing, and the spatial imbalance is gradually decreasing.

Analysis of the coordinated development pattern of urbanization and ecological environment

Analysis of overall coordination

(1) According to *Equation 2,* the center transferring track of the urbanization level and the ecological environment of cities is calculated (*Fig. 4*). From *Figure 4*, it can be seen that both the centers of the urbanization level and the ecological environment level are located in the western area of Jiujiang City. During the research period, the center of the urbanization level moved to the southwest by 2.32 km first, then moved to the northwest by 15.91 km. In general, it moved to the northwest by 15.38 km, with an average annual moving distance of 1.82 km. The center of the ecological environment first moved to the northeast by 7.03 km, then moved to the northwest by 5.79 km. In general, it moved to the north by 6.80 km, with an average annual moving distance of 1.28 km. Generally, the moving distance of the two centers is relatively short, indicating that the distribution of the two centers is relatively reasonable. The growth of the urbanization level is mainly concentrated in the western and northern parts, especially the rapid development of cities such as Yichang, Xiangfan and Jingmen, and the center generally moves to the northwest. The center of the ecological

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environment is affected by cities such as Yueyang, Changsha and Xiangtan and generally moves towards the north.

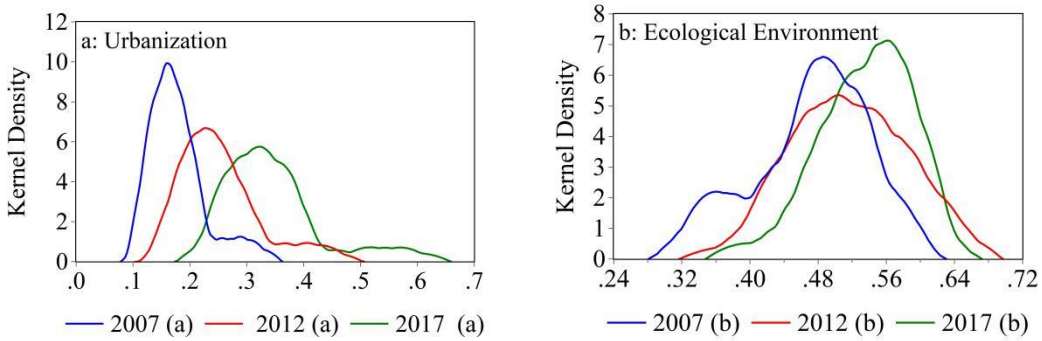

Figure 3. *Kernel density distribution of the urbanization level (a) and the ecological environment level (b)*

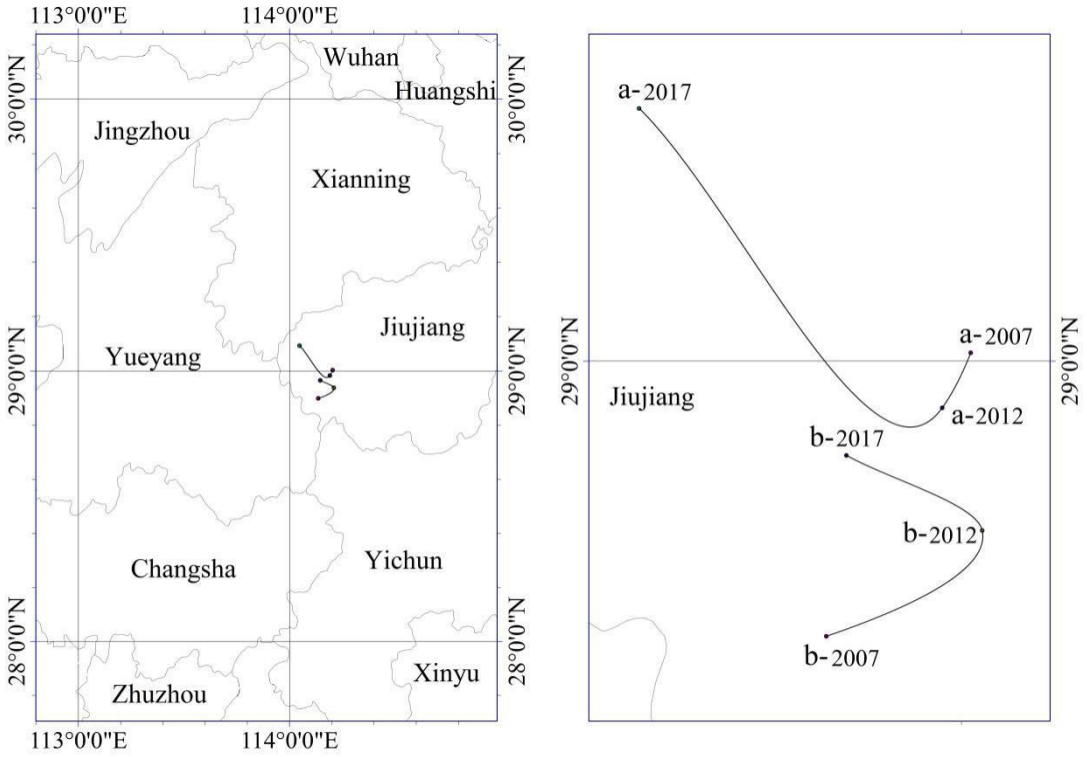

Figure 4. *Center transferring track of the urbanization level (a) and the ecological environment level (b)*

(2) According to *Equations 3* and *4*, the spatial overlapping (a) and the variation consistency (b) of the urbanization level and the ecological environment level of cities are calculated (*Fig. 5*). From *Figure 5*, it can be seen that the distance between the centers of the two presents a change trend of decreasing first and then increasing. It decreased from 11.89 km in 2007 to 4.82 km in 2012 and then increased to 15.11 km in

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2017, with an average of 10.61 km. In general, the coordination between the two shows a downward trend. The variation consistency of the centers of the two presents an alternate change of "reverse direction - same direction". The variation consistency index increases from -0.88 to 0.96, which is 0.08 in general, indicating that the movements of the centers of the two are in the same direction and the distance between the centers is in an upward trend.

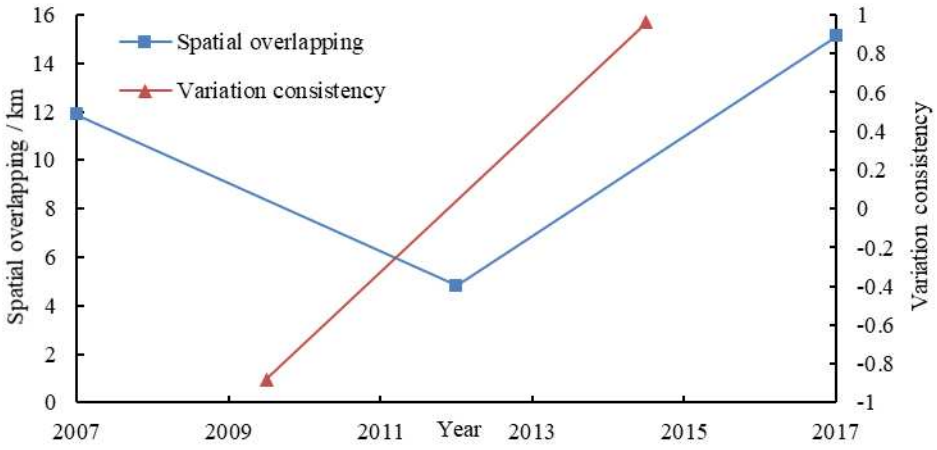

Figure 5. *Spatial overlapping and variation consistency of the centers of urbanization level and ecological environment level*

Analysis of spatial coordination

The coordination level between the two is calculated according to *Equations 5, 6* and *7* (*Fig. 6*). According to related researches (Liang et al., 2019), the coordination level is divided into 5 types - serious imbalance ($0 < D \leq 0.2$), moderate imbalance ($0.2 < D \leq 0.4$), on the brink of imbalance ($0.4 < D \leq 0.6$), moderate coordination ($0.6 < D \leq 0.8$), high coordination ($0.8 < D \leq 1$). As can be seen from *Figure 6*, in 2007, only Changsha City was of moderate coordination type, accounting for 3.57%. Other cities were on the brink of imbalance. In 2012, 12 cities were of moderate coordination type, accounting for 42.86%. In 2017, except for Loudi City, other cities were of moderate coordination type, accounting for 96.43%. On 2007 to 2017, the average growth rate of the coordination level of cities was 22.12%. The growth rates of 9 cities of Changde, Ezhou, Huanggang, Jingmen, Jingzhou, Xianning, Xiangyang, Xiaogan and Xinyu were above the average, accounting for 32.14% of the number of researched cities.

In order to further analyze the spatial agglomeration features of the coordination level, the global autocorrelation index of the coordination level is calculated based on *Equation 8*. The global Moran's *I* indices of the coordination level in 2007, 2012 and 2017 were 0.278, 0.303 and 0.338 respectively. The normal statistic z values of the Moran's *I* in the corresponding year were 3.89, 4.31 and 4.44 respectively. All were greater than the confidence level threshold (1.92), and the P value was less than 0.05, passing the significance test, which indicates that there is a relatively strong spatial correlation of the coordination level of cities and the spatial autocorrelation is constantly increasing.

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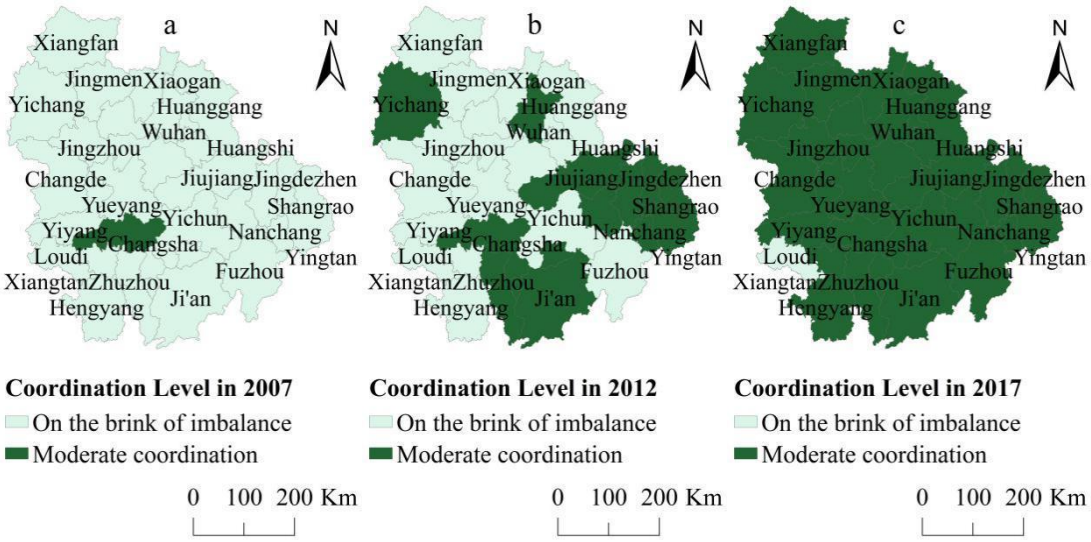

Figure 6. *Spatial-temporal evolution of the coordination level on 2007 to 2017*

The Getis-Ord Gi* values of the coordination level in 2007, 2012 and 2017 are calculated based on *Equation 9*, and it is divided into five types with the Jenks classification method: hot spot area, sub hotspot area, transition area, sub coldspot area and cold spot area (*Fig. 7*). Generally, the spatial evolution features of the cold and hot spot areas of the coordination level are showed in the following aspects: (1) The Getis-Ord Gi* index of the coordination level generally presents the spatial distribution feature of "high in the southeast area and low in the northwest area". (2) The number of hot spot areas and sub hotspot areas is in the trend of fluctuated rise, the agglomeration scope of high-value areas expands and the agglomeration situation of high-value centers tends to be more prominent, forming a hot spot area with Wuhu City, Xuancheng City and Pingxiang City as the core. (3) During the research period, the number of cold spot areas and sub coldspot areas gradually decreases, the significance level of cold spot areas becomes weak, but the distribution is more concentrated, forming a cold spot area with Chengde and Loudi as the core. (4) During the research period, the hot spot areas present a trend of expansion from the southeast to the northwest of the city cluster, and the cold spot areas present a trend of shrinking to the southwest of the city cluster.

Discussion

How to prepare a planning program for the coordinated development of urbanization and ecological environment protection is an important issue that needs to be solved for the current new urbanization in China (Fang and Fang, 2013; Liu et al., 2005a). In the rapid urbanization process of the city cluster along the middle reaches of the Yangtze River, the extensive economic development model has triggered a series of ecological and environmental problems. Research on the development model of urbanization and ecological environment system and their coupling effect has become an urgent need of the academic circles and the political circles (Liang et al., 2019; Sun et al., 2017). Taking the city cluster along the middle reaches of the Yangtze River as an example, this paper evaluates the coordination degree and its dynamic spatial-temporal evolution of urbanization and ecological environment by building a coordination degree model.

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The research results can better reflect the actual situation of the region and provide the basis for the formulation of regional development policies. From the research results, we can see that there are two types of change trend of coordination degree between urbanization and ecological environment in the middle reaches of the Yangtze River. One is that with the continuous advancement of urbanization level, the ecological environment will be further damaged, and then collapse, so the overall eco-economic system will also face extinction accordingly; the other is that with the continuous improvement of urbanization level and the increase of the economic aggregate, the city becomes more capable of making environmental protection investment, which will alleviate the ecological pressure to a certain extent, and ultimately help to achieve the coordinated development of the two. This method can also provide reference for the study of the relationship between urbanization and ecological environment in other regions in China.

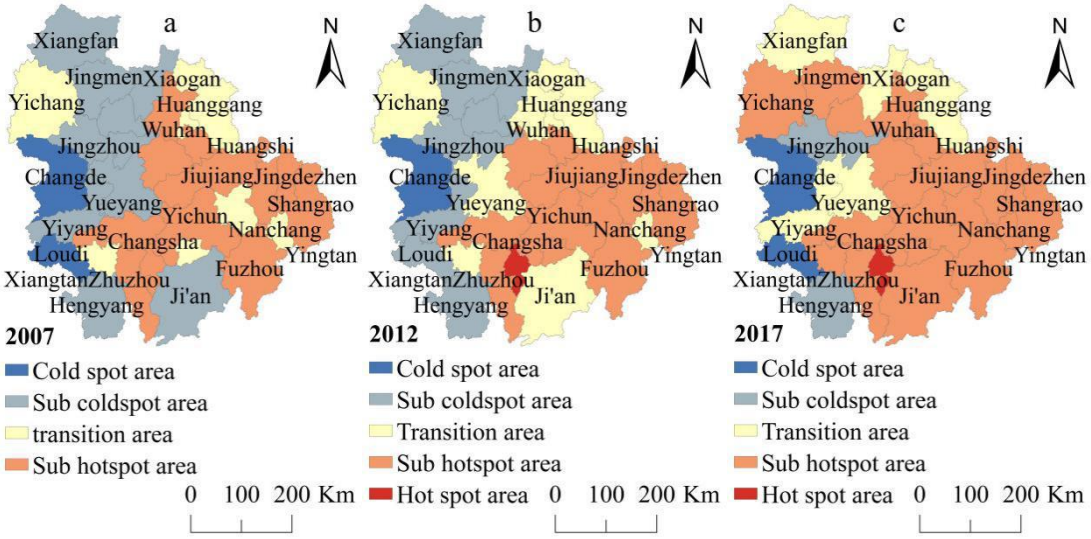

Figure 7. *Evolution of hot spots of the coordination level on 2007 to 2017*

The major research values of this paper are as follows:

(1) Researching the development level of urbanization and ecological environment and their spatial distribution features of 28 cities in the city cluster along the middle reaches of the Yangtze River has important practical significance for revealing the internal development rules of the interaction between the urbanization and the ecological environment and promoting coordinated development of the two.

(2) The 28 cities in the city cluster along the middle reaches of the Yangtze River belong to three different provinces in administration, and administrative barriers restrict the integration of cities (Sun et al., 2017). Researching the coordination relationship between urbanization and ecological environment can provide some reference for government departments to establish cross-regional coordination organizations at the level of city cluster and to promote integrated development among the regions.

(3) As the strategic core area of national economic development and the main area of national new urbanization, the city cluster area determines the development progress of urbanization in the world (Fang et al., 2016). At present, there is still a lack of research on the coordinated development of urbanization and ecological environment of the city

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78817900

cluster along the middle reaches of the Yangtze River. Through empirical analysis in this paper, it provides a reference for the government and related departments to solve the problem of imbalance between urbanization development and ecological environment and enriches the related theoretical research contents.

The shortcomings and prospects are explained in details around the following aspects:

(1) The index system needs to be further supplemented and improved. The contents of the two systems of urbanization and ecological environment are extremely complicated, and there has not yet been a uniform, scientific and comprehensive standard for the establishment of the index system. Therefore, the selection of multisource data (such as the combination of statistical data, remote sensing data and big data, etc.) will be the focus of future research.

(2) In this paper, only three time node data of 10 years are selected. From the perspective of urban development history, the research period is short, which leads to insufficient discussion on the dynamic evolution characteristics and laws of the coordination between urbanization and ecological environment. In the future, with the support of effective data, it is still necessary to conduct further research in a longer period of time, so as to provide more scientific decision-making basis for comprehensive regulation and sustainable development of cities.

(3) In this paper, the factors which affect the coordination of urbanization and ecological environment is not explored and studied, which is a shortcoming of this paper and also it is a problem to be studied and solved in the future.

Conclusion

This paper studies the evolution process of urbanization level and ecological environment system level of the city cluster along the middle reaches of the Yangtze River from 2007 to 2017 on the scale of city cluster and discusses the coordination relationship between the two. It is found in the research that:

(1) During the research period, the urbanization level and the ecological environment level of the city cluster along the middle reaches of the Yangtze River increased significantly. The urbanization level generally shows the spatial pattern of “scattered distribution of high-value areas and grouped distribution of low-value areas”. The spatial distribution of the ecological environment level presenting a distribution pattern of high in the south and east and low in the north and west.

(2) The analysis of imbalance shows that the urbanization level of most cities is relatively low, the difference among cities is increasing, and the spatial imbalance is gradually expanding. The ecological environment of most cities is at a medium level, the difference among cities is decreasing, and the spatial imbalance is gradually decreasing.

(3) The center transferring track and the center variation consistency of the urbanization level and the ecological environment level show a change trend of “reverse direction - same direction”. The distance between the centers of the two decreases first and then rises, and the overall coordination gradually increases.

(4) The coordination of the urbanization level and the ecological environment level of the 28 cities is continuously increasing. The Getis-Ord Gi* index of the coordination generally presents a spatial distribution feature of “high in the southeast area and low in

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DOI: http://dx.doi.org/10.15666/aeer/1806_78817900

the northwest area". The hot spot areas expand from the southeast to the northwest of the city cluster, and the cold spot areas shrink to the southwest of the city cluster.

Based on the current situation of the coordinated development of urbanization and ecological environment in the middle reaches of the Yangtze River, and according to the construction requirements of "ecological civilization city", this paper puts forward the following suggestions on how to realize the coordinated development:

(1) Accelerate the development of urbanization, and make full use of ecological environment resources, and gradually realize the synchronous development of the two. On the one hand, timely adjust the less advanced industries, actively adopt energy-saving and environment-friendly technologies to transform traditional industries, give priority to the development of low-carbon environment-friendly industries, continuously improve the use rate of clean energy, and promote green urbanization (Liu et al., 2005b; Huang and Fang, 2003). On the other hand, vigorously exploit the connection role of "Internet+" in urban construction, make full use of information technology such as Internet of Things, cloud computing and big data, promote information sharing and business coordination, and improve urban management level and efficiency to make the living environment better (Ren et al., 2017).

(2) These 28 cities need to take different development paths according to their own urban development characteristics. Cities with lagging ecological environment need to reduce pollution emissions and avoid the destruction of the original ecosystem. Cities with lagging urbanization level need to develop high-quality urbanization development path from population, economy, society and space on the premise of ensuring ecological environment security.

(3) The coordinated development of urbanization and ecological environment is the result jointly promoted by mechanisms including population agglomeration and resource utilization, economic operation and structural transformation, urban civilization and technological innovation and policy regulation and guarantee (Fang et al., 2016). On the one hand, mobilize all the people to participate in the construction of ecological civilization, cultivate residents to take a diligent and economical, green and low-carbon lifestyle, encourage the people to actively exercise the right to supervise, report and sue against behaviors which cause environmental pollution, and speed up the construction of ecological civilization (Wang et al., 2018). On the other hand, strengthen the government's comprehensive coordination function in guiding urban development and ecological protection. Learn from the successful experience of developed countries and promote urbanization construction with a global perspective to form an important support for the benign interaction between urbanization and ecological environment (Fang et al., 2016).

Acknowledgments. The authors would like to thank the editors and the anonymous reviewers for their constructive comments and suggestions, which greatly helped to improve the quality of the manuscript.

Author contributions. Pengchao Zhou and Yongfang Yang conceived and designed the research, Pengchao Zhou, Hongmin Zhang analyzed the data; Yongfang Yang and Yating Li revised the manuscript of this study; and Pengchao Zhou wrote the paper. All authors read and approved the final manuscript.

Funding. This research was funded by the National Natural Science Foundation of China, grant number 41601120; Humanity and Social Science Youth foundation of Ministry of Education of China (15YJC790049); Soft Science Project of Henan Science and Technology Development Plan, grant number 182400410202; Key Scientific Research Projects of Higher Education Institutions in Henan

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78817900

Province, grant number 18B170002 and Research Topics of Henan Federation of Social Sciences, grant number SKL-2019-3023.

Conflict of interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_78817900

OCCURRENCE OF CHLOROPHENOL COMPOUNDS IN AQUATIC ENVIRONMENTS OF CHINA AND EFFECT OF SUSPENDED PARTICLES ON TOXICITY OF THESE CHEMICALS TO AQUATIC ORGANISMS: A REVIEW

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(Received 18th Mar 2020; accepted 20th Aug 2020)

Abstract. Chlorophenol compounds have received widespread attention because of their sources, applications and characteristics. As an important environmental medium, suspended particles are ubiquitously distributed in aquatic ecosystems. In order to investigate the occurrence of chlorophenol compounds in aquatic environments of China and their toxicity in the case of suspended particles, literature retrieval methods were used to analyze their presence in waters, sediments and organisms in China and the effect of suspended particles on their toxicity to aquatic organisms was also investigated. The results showed that pentachlorophenol (PCP) or NaPCP present ubiquitous pollution and distributions in various aquatic environments. Meanwhile, in order to better protect human health and aquatic organisms, considering the effects of suspended particles on toxicity of these chemicals towards aquatic organisms, appropriate standards for chlorophenols were needed.
Keywords: *pollution, distribution, chemical toxicity, water quality criteria, environmental protection*

Introduction

The occurrence and ecotoxicity of the persistent organic pollutants (POPs) are particular issues in environmental governance. As a group of aromatic compounds with high toxicity and carcinogenic characteristics, chlorophenol compounds are one of the typical POPs and can be persistently present in the environment (Garba et al., 2019). The entrance of chlorophenol compounds into the aquatic environments mainly results from industrial, domestic and agricultural activities (Czaplicka, 2004; Vlastos et al., 2016), particularly the industrial wastewater discharge from pesticide plants, refineries, wood and paper mills (Tarighian et al., 2003; Olaniran and Igbinosa, 2011). These compounds have been intensively studied especially 2, 4-dichlorophenol(2, 4-DCP), 2,4,6-trichlorophenol(2,4,6-TCP) and pentachlorophenol (PCP) (Jin et al., 2010), and the other compounds such as 2-chlorophenol(2-CP), 2, 6-dichlorophenol(2, 6-DCP), 2,3,5-trichlorophenol(2,3,5-TCP), which were also detected in some water environment (Wu et al., 2012a, b, c). PCP has been evaluated as an environmental endocrine disruptor according to the existing research (Zha et al., 2006), and also has been listed as a priority contaminant by the U.S. Environmental Protection Agency due to the toxicity and associated risks.

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DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

Chlorophenol compounds have received intensive concern due to their widespread distribution and toxicity on humans and organisms that are exposure to chemical compounds in the environment, particularly in aquatic environments. Because of their strong toxicity, endocrine interference effect, mutagenicity, carcinogenicity and bioaccumulation (Muir and Eduljee, 1999; Yu et al., 2019), it is essential to obtain the pollution and distribution of chlorophenol compounds in aquatic environments of China and study the ecotoxicity to aquatic organisms for the prevention and control of their contamination. Information about pollution conditions of chlorophenol compounds could be obtained and analyzed from published articles related to chlorophenols found in different environmental media by literature retrieval, classification and analysis. Ecotoxicity of chlorophenol compounds to aquatic organisms could be conducted by toxicity experiments as shown in several previous studies (Zhang et al., 2008; Xing et al., 2012; Li et al., 2014; Chen et al., 2016; Ge et al., 2017; Yu et al., 2019), and then their effects and risk to aquatic organisms could be effectively evaluated based on the concentrations and distributions of chlorophenol compounds in aquatic environments and the acute toxicity or chronic toxicity data.

Suspended particles are widely distributed in aquatic ecosystems, and the suspended particles entering into waterbodies mainly come from the process of surface runoff from terrestrial ecosystems. Their concentrations in lakes or rivers are generally between 10 $\mu g \cdot L^{-1}$ and several hundred, or even up to several hundred $g \cdot L^{-1}$ (Boenigk et al., 2005). According to previous research, the time that suspended particles concentration exceeded 258 $mg \cdot L^{-1}$ in Taihu lake was more than 125 days (Zhu et al., 2005). Suspended particles are an important environmental factor that can play an important role in regulating pollutants toxicity to aquatic organisms (Ma et al.,2002). It is generally considered that suspended particles can change the bioavailability of chemicals through sorption processes, particularly, of the hydrophobic organic chemicals and heavy metals (Herbrandson et al., 2003a; Yang et al., 2006). In fact, suspended particles themselves also could influence aquatic organisms (Kirk and Gilber, 1990; Kirk, 1991). Kirk and Gilber (1990, 1991) demonstrated that suspended particles have acute toxicity to several species of planktonic rotifers and cladocerans. The survival of *Daphnia pulex* was significantly influenced while the concentration of suspended particles was 50 and 100 $mg \cdot L^{-1}$ respectively, and suspended particles concentration showed an inverse correlation with the reproduction, growth of *Daphnia magna*, and also with the survival of its larvae. Thus, suspended particles could increase stress on aquatic organisms in survival conditions or decrease the exposure conditions of chemicals by adsorption, and then the external stress could affect the toxicodynamic response of aquatic organisms to chemicals (Herbrandson et al., 2003a, b).

It should be noted that water quality standards or criteria were usually originated from toxicology experiment data. However, toxicity experiments on aquatic organisms to calculate acute toxicity or chronic toxicity data are normally carried out under laboratory conditions according to previous reports. The water used in the experiments generally is tap water or synthetic water which has a low ionic strength, simple inorganic chemistry, stable value of pH and temperature, and contain low amounts of suspended particles and dissolved organic matters (Yilmaz et al., 2004; Selviet al., 2005; Mihaich et al., 2009). The toxicity of chemicals to aquatic organisms can be modified by water hardness, pH, temperature, suspended particles, and dissolved organic matter. For example, previous research indicated that hardness and pH could change heavy metal toxic effects to aquatic species (Long et al., 2004), and pH could

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DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

affect chlorophenol's toxicity to green algae in the presence and absence of humic acid (Suzuki and Shoji, 2020). Bejarano et al. (2005) indicated that dissolved organic matters generally reduced chlorothalonil and chlorpyrifos toxicity to *Amphiascus tenuiremis* due to the decrease of bioavailability, while significantly enhancing the acute toxicity of fipronil because of the reduced light-dependent formation of fipronil-desulfinyl, a light or equivalently toxic metabolite. It is apparent that there are certain limitations that prevent us from protecting aquatic organisms caused by a lethal concentration of pollutants according to laboratory standard methods. Based on our review on the occurrence of chlorophenol compounds in aquatic environments of China and effects of suspended particles on toxicity of these chemicals to aquatic organisms, the results can be used as scientific basis for the toxic effect assessment of chlorophenol compounds to aquatic organisms and the development of water quality standards and criteria for better environmental protection.

Study methods

Literature retrieval methods are frequently used to collect sufficient and effective literatures from database according to the key words and setting time interval. In this study, these methods were used to summarize the literatures about the occurrence of chlorophenol compounds in aquatic environments of China including water, sediments and aquatic organisms in SCI journals and Chinese journals published from 1990 to 2019. Specific rivers or lakes, media, sampling date, levels and sources of chlorophenol compounds were mainly focused in this section. Literatures about the toxicity of these chemicals to aquatic organisms, including the effects of suspended particles on toxicity, were also concerned in the same time interval. Key words included China, chlorophenols, 2,4-dichlorophenol/2,4-DCP, 2,4,6-trichlorophenol/2,4,6-TCP, pentachlorophenol/PCP, toxicity and suspended particles/suspended solids, both retrieved separately and combined with the following terms including environments, rivers, lakes, waters, sediments, fish and aquatic organisms. Other available data and relevant information were from literature search. The obtained literatures were classified and analysed according to the target of each sub-chapter of the paper.

Results and analysis

Pollution and distributions of chlorophenol compounds in surface waters of China

Three literatures about the occurrence of chlorophenol compounds in surface water of rivers or lakes in China were found in SCI journals and nine literatures of that in Chinese journals. As shown in *Table 1,* PCP could be detected in all water environments of main rivers or lakes in China, which reflected that PCP are main chlorophenol pollutants. Concentrations of PCP showed significant difference in important rivers and lakes. The highest residue appeared in Dongting lake. However, it can be certain that the PCP pollution in surface water of the Dongting Lake tends to decrease gradually according to the data reported by Feng et al. (2014) in recent years. Gao et al. (2008) summarized the pollution levels of chlorophenols including 2, 4-DCP, 2, 4, 6-TCP and PCP in seven watershed of China. The results indicated that PCP was the most common chlorophenol in 85.4% of samples (median: 50.0 $ng \cdot L^{-1}$, range from 1.1 to 594 $ng \cdot L^{-1}$) and PCP contamination in the Yangtse River was the most serious

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DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

because of frequent industrial wastewater discharge and widely historical use of pesticide for killing snails in this schistosomiasis epidemic watershed. While the rivers with high 2,4-DCP and 2,4,6-TCP levels mainly occurred in the Yellow River, which may be for the reason that 2,4-DCP and 2,4,6-TCP originate from degradation processes of industrial activities or phenoxyacid herbicides that were extensively used in agricultural areas of these basin.

Table 1. *The occurrence of chlorophenol compounds in surface waters of China*

Location	Date	Sampling number	Compound	Levels ($ng \cdot L^{-1}$)		Main source	Reference
				Range	Mean		
Haihe River	—	10	PCP	ND~1800.00	189.00	Production and sewage discharge	Liu et al., 2006
Dongting Lake	1998	8	PCP	<5.00~103700.00	14949.79	Historical use	Zheng et al., 2000; Zhang et al., 2001
Qiantang River	2005	35	2,4-DCP and 2,4,6-TCP	—	876.86[a]	Historical use	Chen et al., 2005
Pearl River	2007	5	2-CP, 2,4-DCP, 2,4,6-TCP and PCP	41.20~54.40[a]	46.52[a]	Wastewater	Dong et al., 2009
Hengmen River				67.60~128.20[a]	86.72[a]		
Fish pond				6.90~19.10[a]	12.66[a]		
Songhuajiang River	2008	40	PCP	< 1.10~70.00	—	—	Gao et al., 2008
			2,4-DCP	< 1.10~250.00	—		
			2,4,6-TCP	< 1.40~250.00	—		
Liaohe River		58	PCP	< 1.10~60.00	—		
			2,4-DCP	< 1.10~170.00	—		
			2,4,6-TCP	< 1.40~30.00	—		
Haihe River		39	PCP	50.00~70.00	—		
			2,4-DCP	< 1.10~40.00	—		
			2,4,6-TCP	10.00~40.00	—		
Yellow River		50	PCP	< 1.10~70.00	—		
			2,4-DCP	< 1.10~19960.00	—		
			2,4,6-TCP	< 1.40~28650.00	—		
Yangtze River		150	PCP	< 1.10~594.00	—		
			2,4-DCP	< 1.10~380.00	—		
			2,4,6-TCP	< 1.40~30.00	—		
Huaihe River		39	PCP	< 1.10~351.00	—		
			2,4-DCP	< 1.10~246.00	—		
			2,4,6-TCP	< 1.40~70.00	—		
Pearl River		150	PCP	< 1.10~396.00	—		
			2,4-DCP	< 1.10~264.00	—		
			2,4,6-TCP	< 1.40~70.00	—		
Southeast drainage area rivers		74	PCP	< 1.10~32.40	—		
			2,4-DCP	< 1.10~26.60	—		
			2,4,6-TCP	< 1.40~22.00	—		
Northwest drainage area rivers		18	PCP	< 1.10~60.00	—		
			2,4-DCP	< 1.10~55.00	—		
			2,4,6-TCP	< 1.40~69.50	—		
Southwest drainage area rivers		5	PCP	< 1.10	—		
			2,4-DCP	< 1.10	—		
			2,4,6-TCP	< 1.40	—		
Yangtze River	2009	8	PCP	ND~220.00	—	Historical use	Han et al., 2009
			2,4-DCP	ND~130.00	—		

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DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

Songli spillway	2010	7	PCP, 2-CP, 2,4-DCP, 2,6-DCP,2,4,5-TCP,2,4,6-TCP, 2,4,5-TCP, 2,3,4,6-TECP and 2,3,5,6-TECP	12.19~2386.90[a]		Historical use	Wu et al., 2012a
Ouchi River		10		2.50~691.00[a]			
Tuojiang River		10		17.18~6148.28[a]			
Three Gorges Reservoir (Mainstream)	2010	9	2,4-DCP, 2,6-DCP, 2,4,5-TCP,2,4,6-TCP, 2,3,4,5-TeCP and 2,3,4,6-TeCP	1.82~16.10[a]	5.23[a]	Wastewater	Wu et al., 2012b
Three Gorges Reservoir (tributaries)		55		1.30~146.86[a]	11.93[a]		
Hangzhou Bay	2013	18	19 chlorophenol compounds	16.71~21810[a]	—	Wastewater	Qiu et al., 2016
Dongting Lake	2013	27	PCP, 2,4,6-TCP, 2,3,4-TCP,	19.75~111.49[a]	—	Historical use	Feng et al., 2014
Yangtze River in Chongqin City	2016	12	PCP	ND~2370	—	—	Yang et al., 2018
Poyang Lake wetland	2017	15	PCP	0.116~0.887	0.330	Historical use	Yang et al., 2019
Beitang Drainage River	2018	7	4-CP, 2,5-DCP, 2,4-DCP, 2,6-DCP	ND~4.63	—	Production and wastewater	Zhong et al., 2018
Dagu Drainage River		16		ND~1.57	—		
Yongdingxin River		14		ND	—		

"—" stands for not reported. "a" indicates the total concentrations of the target compounds." ND" stands for not detected

Pollution and distributions of chlorophenol compounds in sediment environments of China

As for the occurrence of chlorophenol compounds in sediment environment of rivers or lakes in China, five sources and eight articles were found in SCI journals and Chinese journals especially. As shown in *Table 2,* PCP was the main pollutant among chlorophenol compounds that were intensively reported in sediment environment of rivers or lakes in China, especially the pollutions of that in district of Yangtze River, Pearl River, Poyang Lake and Dongting Lake had generated great concern. Because of large amounts of PCP or NaPCP were used frequently and intensively historically in some area to control spread of snail borne schistosomiasis, or frequently used as a clean pond reagent in aquiculture areas, these phenomena were especially serious in Dongting Lake area. Due to the accumulation characteristics, sediment from Dongting Lake showed relatively higher PCP pollution levels than others, indicating an intensive history of pesticide usage in these areas. Pollution of chlorophenol compounds in sediment environment existed in many rivers or lakes in China, simultaneously the limited report on PCP levels around Jiaozhou Bay of Shandong province revealed their existence in coastal regions (Pan et al., 2008).

Pollution and distributions of chlorophenol compounds in organisms of China

Limited information could be searched about the occurrence of chlorophenol compounds in fish or other organisms survived in rivers or lakes in China compared to them that in water and sediment environment. Only four articles were found about pollutions of PCP both showed in SCI journals and in Chinese journals. Because of PCP was usually used in fish ponds cleaning for killing mussels and shellfish in South China, the residual amount of PCP in aquatic organisms could reflect the PCP pollution levels in waters indirectly, and indicate possible risk from human exposure due to diet. So the PCP accumulation levels in aquatic organism samples including

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DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

fish, shrimp, carp, crab and other aquatic organisms collected from fish ponds, farms or markets especially in Dongting Lake and Jiangsu province were detected. As shown in *Table 3,* Dongting Lake and Jiangsu province were the typical and key areas for long-term usage of NaPCP to clean ponds, which related to many literatures focused on these special districts, showed a much higher PCP presence in different aquatic organisms, such as fish, shrimp, frog, and so on. Similar to PCP pollution in water environment of the Dongting Lake, the pollution level of PCP in fish samples was also significantly reduced.

Table 2. *The occurrence of chlorophenol compounds in sediment environment of China*

Location	Date	Sampling number	Compound	Levels ($ng \cdot g^{-1}$)		Main source	Reference
				Range	Mean		
Yangtze River	—	4	PCP	0.49~4.57	—	Historic use	Xu et al., 2000
Qingdao coastal sea	1997, 1999	7	PCP	ND~3.7	0.50	Sewage sludge and historic use	Pan et al., 2008
Dongting Lake	1998	8	PCP	180.00~48300.00	20135.00	Historic use	Zheng et al., 2000; Zhang et al., 2001
Pearl River Delta	2000~2001	7	PCP	1.44~ 34.40	7.93	Historic use	Hong et al., 2005
Yangtze River	2005	11	PCP	< 0.64~1.68	0.38	Historic use	Tang et al., 2007
Hanjiang River		4		< 0.64~0.87	0.34		
Fuhe River		3		< 0.64~0.77	0.46		
Sheshui River		1		0.59	0.59		
Dongjing River		2		< 0.64~0.63	—		
Poyang Lake	2006	4	PCP	ND~23.80	20.75	Historic use	Liu et al., 2008a
		19	PCP	ND~35.28	26.61	Historic use	Liu et al., 2008b
Aojiang River	2006	6	PCP	ND~71.40	27.76	Historic use	Chen et al., 2008
Taihu lake	2008~2009	58	2,5-DCP	ND~116.80	26.90	Industry and agriculture	Zhong et al., 2010
			2,4,6-TCP	ND~840.40	35.90		
			2-CP	ND~166.10	6.00		
			2,4-DCP	ND~143.10	19.60		
			2,4.5-TCP	ND~65.30	14.60		
Huangpu River	2010	13	2-CP, 3-CP, 4-CP, 2,6-DCP, 2,4-DCP, 2,5-DCP, 2,3-DCP, 3,5-DCP, 3,4-DCP, 2,4,6-TCP, 2,3,5-TCP, 2,3,4-TCP, 2,4,5-TCP, 2,3,6-TCP, 3,4,5-TCP, 2,3,5,6-TeCP, 2,3,4,5-TeCP, 2,3,4,6-TeCP and PCP	3.79~47.10[a]	18.30[a]	Human activities	Wu et al., 2012c
Suzhou River		5		4.20~25.10[a]	14.80[a]		
Yunzao Brook		3		3.64~5.48[a]	4.44[a]		
Yangtze River in Chongqin City	2016	12	PCP	ND~6.36	—	—	Yang et al., 2018
Poyang Lake wetland	2017	15	PCP	0.062~0.0089	0.0234	Historical use	Yang et al., 2019
Beitang Drainage River	2018	7	4-CP, 2-CP, 2,5-DCP, 3,4,5-TCP, PCP	ND~9.71	—	Historical use	Zhong et al., 2018
Dagu Drainage River		16		ND~65.1o	—		
Yongdingxin River		14		ND	—		

"—" stands for not reported. "a" indicates the total concentrations of the target compounds." ND" stands for not detected

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

Table 3. *The occurrence of chlorophenol compounds in organisms of China*

Location	Date	Sampling number	Organism	Compound	Levels ($ng \cdot g^{-1}$)		Main source	Reference
					Range	Mean		
Jiangsu Province	1991~1993	2	Fish	PCP	0.02~2.13	0.15	PCP usage	Yang et al., 1996
Dongting Lake	1998	8	Silver carp (*Hypophthalmichthys molitrix*)	PCP	—	550.00	PCP usage	Zheng et al., 2000; Zhang et al., 2001
			Grass carp *(Ctenopharyngodon Idellus)*		—	630.00		
			White bream *(Parabramis pekinensis)*		40.00~340.00	50.00		
			Common carp *(Cyprinus carpio)*		60.00~110.00	85.00		
Qingdao coastal sea	1997, 1999	7	Common Mussel *(Mytilus edulis Linee)*	PCP	—	4.50	Sewage sludge and historic use	Pan et al., 2008
Jiangsu Province	2003~2004	4	Crucian carp *(Carassius auratus)*	PCP	—	53.00	PCP usage	Ge et al., 2007
		6	Grass carp *(Ctenopharyngodon idellus)*		—	3.60		
		4	Big head carp *(Aristichthys nobilis)*		—	2.40		
		13	Chinese mitten crab *(Eriocheir sinensis)*		—	0.90		
		1	Silver carp *(Hypophthalmichthys molitrix)*		—	2.40		
		1	Bull frog (*Rana catesbeiana)*		—	2.50		
		1	Turtle *(Amyda sinensis)*		—	2.50		
		6	Shrimp *(M. rosenbergii)*		—	1.00		
		13	Shrimp *(M. nipponense)*		—	0.90		
		6	Shrimp *(P. vanmamei)*		—	0.25		
Jiangsu Province	2008	6	Fish	PCP	0.10~0.35	0.22	PCP usage	Xu et al., 2010
	2009				0.09~0.32	0.21		
Dongting Lake	2013	27	Common carp *(Cyprinus carpio)*	PCP	—	92.33	Historic use	Feng et al., 2014
			Silver carp *(Hypophthalmichthys molitrix)*		—	87.76		
			White semiknife carp (*Ochetobius elongatus Kner*)		—	25.87		
			Crucian carp *(Carassius auratus)*		—	2.14		

"—" stands for not reported

Effect of suspended particles on chlorophenols toxicity to aquatic organisms

Aquatic organisms in natural waters are usually exposed to mixed pollutants and physical pressures, which could induce adverse effects such as behavioral, physiological or biochemical activity disturbance, and can even cause death. Suspended particles in natural waters are usually present and are the main stressors for aquatic organisms. Herbrandson et al. (2003b) and Zurek (1983) indicated that *Daphnia magna* could feed on suspended particles and the ingested particles may lead to decreased ability of daphnids to float in the water, causing a greater energy demand for extra effort and higher metabolism ability to maintain an appropriate position in the water area, and then

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

played a role in toxicodynamics by increasing the energy requirements of the organisms, thus the suspended particles indirectly enhanced the toxicity of pollutants. Herbrandson, Bradbury and Swackhamer (2003a, b) demonstrated that suspended particles could influence carbofuran toxicokinetics in *Daphnia magna* and contribute to greater energy expenditures. Zurek (1983) found that *Daphnia hyaline* increased 10.6%~32.4% of their metabolism when exposed to a solution containing 100~1000 $mg \cdot L^{-1}$ suspended particles and Kirk (1991) demonstrated that suspended clay sediment reduced the algal ingestion rate of *Daphnia ambigua* by 60%~70% and 27% respectively at low and high algal concentrations. The abnormal feeding activities induced by suspended particles can cause energy budget imbalance, which makes the organisms more fragile to additional pressures (Jeon et al., 2010). Furthermore, chemicals can enhance the toxicity to aquatic organisms through feeding on suspended particles with adsorbed chemicals (De Schamphelaere et al., 2007; De Schamphelaere and Janssen, 2004).

However, when the effect of suspended particles on chlorophenol compounds toxicity to aquatic organisms was focused, the above-mentioned theories were not applicable in some cases. Ra et al. (2008) investigated the acute toxicity of several pharmaceuticals, estrogens and phenols to *Daphnia magna* exposed to suspended particles containing humic acid, the results revealed that suspended particles did not adsorb pharmaceuticals and estrogens, as the toxicity of those chemicals exhibited no significant decreases to *Daphnia magna*, while the suspended particles adsorbed three phenolic compounds including octylphenol and pentachlorophenol, which promoted a significant toxicity decrease to *Daphnia magna*. As extremely important organisms in aquatic ecosystems, fish is widely distributed in the freshwater. Chemicals toxicity to fish could also be influenced by suspended particles. Yan et al. (2015) indicate that suspended particles (7500 and 15,000 mg/l) decreased dissolved chemicals by adsorption, thus decreasing atrazine toxicity to *Brachydanio rerio* (Zebrafish) through the reduction of bioavailability. The gill is an important organ that plays a vital role in respiration, and it is unquestionable that respiration (i.e. inhalation through gills) is the most significant way of pollutants exposure in water (Ge et al., 2017). A certain high concentration of suspended particles blocking the gills of fish may protect from the toxicity of chemicals.

In addition, the effect of suspended particles on aquatic organisms is dependent on several key factors including the concentrations, composition and particle size distribution of suspended particles, and also the duration of exposure to suspended particles (Bilotta and Brazier, 2008). Inorganic particles with sharp edges could stimulate the intestinal tract or externally-exposed tissues, leading to changes in energy resources allocation and food intake or respiratory rate reduction. Biochemical or behavioral reactions caused by the above stressors could imaginably aggravate the chemicals toxicity effects (Herbrandson et al., 2003b). Herbrandson et al. (2003b) demonstrated that suspended subsoil was more toxic than suspended decomposed peat to carbofuran exposed *Daphnia magna* and the main reason was that *Daphnia magna* required excessive amounts of energy to ingest suspended subsoil than suspended decomposed peat. The process of chemical poisoning to aquatic organisms is therefore more complex, particularly under natural conditions.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

Comparison analysis of water quality criteria and chlorophenol compounds toxicity

Water quality criteria is an important foundation to determine the limit values of water quality standards, which has extreme significance for environmental pollution and control. Based on existing toxicological data as well as numerical calculation and derivation, U.S. Environmental Protection Agency has established water quality criteria aiming at several typical chlorophenol compounds to protect human health and aquatic life (list in *Table 4*). Water quality criteria for human health represents the maximum allowable pollutants concentrations, which will not have deleterious health effects through eating aquatic animals or drinking water. The criteria for protecting aquatic life of specific pollutants represent the minimum concentrations that does not result in a significant risk to most aquatic organisms, including acute and chronic toxicity values.

Depending on the analysis results from the collected literatures, concentrations of several chlorophenol compounds in water environment of many important rivers or lakes such as Haihe River, Yellow River and Dongting Lake apparently exceeded the water quality criteria for human health and aquatic life, which posed potential risks and threats to local human health and ecological environments. However, for those that did not exceed the criteria in most rivers or lakes, suspended particles played an important part in the regulation of chemical toxicity on aquatic organisms as showed in the present analysis, which indicated that potential risk and threat would still exist while suspended particles were present. For a further protection of water quality and aquatic organisms, it is necessary to predict appropriate criteria for watershed with relatively high concentration of suspended particles based on our analysis.

Table 4. *Water quality criteria for protecting human health and aquatic life/μg·L^{-1}*

Compounds	Criteria for protecting human health consumption of water and organism		Criteria for protecting aquatic life (fresh water)	
	Water + organism	Organism only	Acute toxicity	Chronic toxicity
2,4-DCP	10	60	—	—
2,4,5- TCP	1.5	2.8	—	—
2,4,6-TCP	300	600	—	—
2-CP	30	800	—	—
PCP	0.03	0.04	19	15

Conclusions

The occurrence of chlorophenol compounds in aquatic environments including waters, sediments and in organisms were summarized through literature retrieval, classification and analysis. It was shown that various chlorophenol compounds were ubiquitously detected in aquatic environments including waters, sediments and organisms in China, particularly numerous applications of PCP or NaPCP present wide pollution and distributions. PCP was the most ubiquitous chlorophenol with the highest residue appearing especially in the water environment of Dongting lake and Yangtze River, and in the sediment environment and organisms of Dongting lake. Meanwhile, on the other hand, the latest research data also reflect that the pollution levels of PCP, especially in surface waters and organisms in some heavily polluted areas of China, have been greatly reduced compared with the past. Multiple comparison between occurrence, toxicity and water quality criteria of chlorophenol compounds showed more

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

appropriate criteria are needed for better protection of human health and aquatic organisms based on the analysis of chemicals toxicity to aquatic organisms in the case of suspended particles. In future studies, we need to focus on the influence of suspended particles in natural water setting on the toxicity of chlorophenol compounds. The joint acute toxicity and accumulation dynamics study of suspended particles and chlorophenol compounds in the future experiments can facilitate to develop an ecotoxicological model for the evaluation of the ecological harm of these chemicals and formulate relevant water quality criteria.

Acknowledgements. This work was supported by the CRSRI Open Research Program (Program SN: CKWV2018495/KY).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79017913

INFLUENCE OF URBAN CONSTRUCTION LANDSCAPE PATTERN ON $PM_{2.5}$ POLLUTION: THEORY AND DEMONSTRATION – A CASE OF THE PEARL RIVER DELTA REGION

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(Received 18th Mar 2020; accepted 20th Aug 2020)

Abstract. With the continuous improvement of China's urbanization level, the huge expansion of construction land has a negative impact on urban air quality, especially $PM_{2.5}$ pollution. In this paper, the relationship between the landscape pattern of urban construction land and $PM_{2.5}$ concentration is studied by using spatial exploratory analysis (Moran I) and spatial econometric analysis (SLM/SEM). The results show that: ① From 2000 to 2016, $PM_{2.5}$ pollution in the region of Pearl River Delta rose first and then dropped; In terms of spatial distribution, it showed a trend of high in the middle and low in the peripheral areas. ② The construction land in Pearl River Delta expanded obviously from 2000 to 2016, with the overall increase as high as 75.37%. At the same time, the advantages, integrity and accumulation of construction land patches in Pearl River Delta were strengthened continuously, while the degree of size breakage dropped to some extent. ③ The landscape pattern of the construction land in Pearl River Delta has obvious influences on $PM_{2.5}$ pollution: the construction area (CA) and the proportion of construction land (PLAND) have positive correlations with $PM_{2.5}$ pollution; patch density (PD) and mean nearest distance (MNN) show negative correlations with $PM_{2.5}$ pollution; ④ The increase of economic factors, including road network density (RD), average gross domestic product (AGDP) and the proportion of secondary industry output (IND), all could cause $PM_{2.5}$ pollution to rise. Among the natural factors, increase of precipitation could increase $PM_{2.5}$ pollution, while increase of wind speed could reduce $PM_{2.5}$ pollution.
Keywords: *urbanization, construction land expansion, hazy weather, $PM_{2.5}$ concentration, spatial panel model*

Introduction

As Chinese economy and urbanization keep developing continuously, urban air pollution has become one of the serious environmental problems of China (Yang et al., 2012a). As the major air pollutant and the chief culprit of hazy weather, $PM_{2.5}$ could seriously affected on atmospheric visibility (Hyslop, 2009) and human health (Yang et al., 2012b). According to the data issued by the Ministry of Environmental Protection, the average concentration of $PM_{2.5}$ in cities at prefecture level or above in China was 43 ug/m^3 in 2017, dropping 40.3% compared with 72 μg/m^3 in 2012. However, 338 cities of China at prefecture level or above had 2,311 day-time severe pollution and 802 day-time gross pollution, among which the days when $PM_{2.5}$ was the primary pollutant for severe or more serious pollutions accounted for 74.2%. Therefore, $PM_{2.5}$ pollution has become a serious problem in China. In fast development of urbanization, the land use

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DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

types and landscape patterns of urban land also change greatly (Liu et al., 2003), and the emission source and the emission area of urban landscape play decisive roles in $PM_{2.5}$ pollution; so, changes of land use types and the landscape patterns could reflect directly the spatial differences of the emission source of pollutants (Querol et al., 2004; Li et al., 2011; Zhou et al., 2011). The most remarkable feature of urbanization is continuous increase of impervious landscapes, which could result in increase of urban pollution emissions and decrease of absorptivity, thereby leading to degradation of the ecosystem function. Therefore, the construction land with impervious landscape as the major landscape type as well as the changes of its landscape pattern are related closely with $PM_{2.5}$ pollution. So, to study the influences of landscape patterns of urban construction land on $PM_{2.5}$ pollution (Wu, 2000a; Zhang et al., 2006; Strohbach and Haase, 2012; Kadish and Netusil, 2012; Xie and Wu, 2017) is of great significance to exert the controlling effects of construction land type and its landscape pattern on $PM_{2.5}$ pollution and to promote healthy development of urban land use as well as regional ecological and security construction by optimizing spatial configuration.

Nowadays, scholars have begun to study $PM_{2.5}$ pollution together with land use type (Hankey and Marshall, 2015; Fan et al., 2018; Tian et al., 2018). Research finds that the influences of different land use types on $PM_{2.5}$ pollution differ greatly. For example, Tang et al. (2015) conducted analysis on correlations between $PM_{2.5}$ pollution distribution and land use types of Wuhan in 2013 and concluded that the construction land had significant positive influence on $PM_{2.5}$ pollution and that $PM_{2.5}$ pollution could be reduced by increasing the green area (Tang and Liu, 2015). By studying the influences of changes of urban land use on atmospheric particulate pollution, Cui (2013) concluded that the correlation coefficients between residential land and road traffic land and atmospheric particulate pollution are 0.789 and 0.743, respectively. Some other studies analyze the relations between single land use type and $PM_{2.5}$ pollution, such as urban forest (Wu et al., 2008) and types of vegetation coverage of urban green spaces (Wu, 2007; Li et al., 2014; Chen et al., 2014; Weber et al., 2014; Tang et al., 2015; McCarty et al., 2015). In addition, Vegetation cover types on urban green land and urban morphology and landscape pattern could also affect atmospheric particulate pollution, and the landscape indexes have good indicating effect on atmospheric particulates (Tang and Wang, 2007). For example, research has found that patch density in landscape pattern and landscape shape index show negative correlations with $PM_{2.5}$ pollution, while marginal density and atmospheric particulate pollution have positive correlation (Shao et al., 2004). Some other studies investigate the quantitative relations of the proportions of construction land to road area, walking and parallel index, landscape diversity and other indicators of landscape patterns with $PM_{2.5}$ pollution (Jiao et al., 2015). Generally, most of the existing researches discuss the relations between various land use types and $PM_{2.5}$ pollution from the perspective of land use changes. Though some scholars have noticed that landscape indicators have significant influences on atmospheric particulates, there are still less studies on relations between landscape pattern indexes and $PM_{2.5}$ pollution in terms of construction land systematically. As the major place for human activity and energy consumption, construction land is the most important emission source of particulate matters (Xu et al., 2016). Therefore, it is of great significance to study the influences of landscape pattern of construction land on $PM_{2.5}$ pollution. Moreover, in terms of the methods of empirical study, most of the studies are conducted on the data of time sequence; however, development of different regions is unequal and the environmental condition and land use conditions of one area could affect the neighboring regions. Time

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DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

sequence-based analysis method neglects such spatial effect and has certain disadvantages theoretically. So, based on related theories of spatial metrology, this essay studies the spatial effects of landscape pattern of urban construction land on $PM_{2.5}$ pollution from the perspective of landscape pattern of urban construction land, and puts forward $PM_{2.5}$ governance policies, with the aim to provide theoretical basis for region control of $PM_{2.5}$ pollution.

Materials and Methods

Study area

The region of Pearl River Delta has developed economy, high level of urbanization, and strong regional overall strength. In 2016, its GDP accounted for 9.2% of the total amount of the national economy, with the level of urbanization reaching 69.2%, The details are shown in *Table 1*. However, $PM_{2.5}$ pollution in Pearl River Delta is not promising in the process of fast development of economy and urbanization. In 2016, the days when the air quality exceeds the standard of 9 cities in Pearl River Delta accounted for 10.6%, among which mild pollution covered 9.0%, moderate pollution 1.4% and severe pollution 0.2%, and the average concentration of the cities ranges within 26-38 $\mu g/m^3$. On February 18, 2019, the Central Committee of the Communist Party of China and the State Council issued officially Outline of the Development Plan for Guangdong, Hong Kong, Macao Great Bay Area, in which the guiding thought and strategic location of Guangdong, Hong Kong, Macao Great Bay Area are made clear, pointing out that it is necessary to promote coordinated development of Pearl River Delta with Hong Kong and Macao so as to improve the economic development level and international competitiveness of the region of Pearl River Delta; meanwhile, the development principles of green development and environmental conservation are also put forward. Therefore, it is of great significance to analyze the correlations between $PM_{2.5}$ pollution and the landscape pattern of urban construction land in the region of Pearl River Delta and to put forward policy recommendations for high-quality development of Pearl River Delta and control of haze pollution.

Table 1. *Basic situation of cities in the Pearl River Delta region in 2016*

City	Construction land area/hm^2	GDP/100 million yuan	The level of urbanization/%	Permanent Population at the Year-end/Ten thousand
Zhaoqing	40148.1	2084.02	46.08	454.4
Guangzhou	141027.3	19547.44	86.06	1404.35
Huizhou	72609.39	3412.17	69.05	477.5
Jiangmen	76120.02	2418.78	65.06	454.4
Shenzhen	87642.45	19492.6	100	1190.84
Dongguan	122226.39	6827.69	89.14	826.14
Zhongshan	49771.62	3202.78	88.2	323
Zhuhai	26507.97	2226.37	88.8	167.53
Foshan	116813.52	11423.23	96.77	1000.73

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Connotation of landscape pattern index of construction land and the mechanism of its action on $PM_{2.5}$ pollution

Landscape pattern index refers to the quantitative index that could reveal different landscape types, quantity and characteristics of spatial arrangement. As the specific manifestation of landscape heterogeneity, specifically, it refers to differences in size, shape and spatial combination of landscape patches that are formed naturally or artificially in spatial arrangement; and it is also comprehensive performance of interactions of various ecological actions in different dimensions (Wu, 2000b). Landscape pattern of urban construction land is usually divided into landscape scale, landscape structure and landscape layout. Landscape scale refers to the quantity of landscapes, landscape structure refers to the proportions of the compositions of each landscape type in the landscape pattern of some region, and landscape layout emphasizes the combination effect of patch types as well as the layout effect of the overall landscape (Beckett et al., 1998). Landscape pattern index has been applied extensively not only in the study of urban morphology and urban landscape, but also in study of the effects of the composition and structure of different land use types on biodiversity and habitats. Because landscape index is highly concentrated, easy to express, and easy to acquire (Zhou et al., 2011; Wu et al., 2015; Sun et al., 2015), it is appropriate and feasible to use the landscape index to study the influences of urban landscape patterns on $PM_{2.5}$. On one hand, differences in the attributes of different landscape structures and landscape patterns could cause local $PM_{2.5}$ to increase or decrease directly; on the other hand, changes of the surface landscape structure could cause local climate to change, which could cause the energy and material to be distributed and transmitted unevenly, thereby affecting spatial distribution of urban atmospheric pollutants (Liu et al., 2015; Tong et al., 2016).

Type, shape, size, quantity and spatial combination of landscape patches are results of interactions of various interfering factors; meanwhile, they could also affect the ecological process and peripheral effects of the region (Wu, 2007). The existing studies mainly quantize landscape patterns from the perspectives of patch level, type level and landscape pattern (Loehle and Wein, 1994). On account of this, in this study, analysis framework of the influence of landscape pattern on $PM_{2.5}$ pollution is constructed from three aspects, including landscape area (scale), landscape composition (structure) and landscape layout (see *Fig. 1*). With the continuous progress of urbanization, the landscape pattern will evolve accordingly and affect the concentration of air pollutants. First of all, progress of urbanization will cause the expansion of urban construction land area (CA), increase the landscape of emission sources of air pollutants, and affect the concentration of air pollutants (Tan, 2009). At the same time, this process will lead to increase of the proportion of construction land (PLAND) and landscape dominance of air pollutant emission sources, as well as decrease of landscape dominance of sinks, which will also have an impact on air pollutant emissions (Wang, 2019). In addition, with gradual improvement of the urbanization level, the compactness and integrity of the landscape patch layout of construction land will continue to strengthen. For example, the patch density (PD) and the mean nearest distance (MNN) will decrease, and the largest patch index (LPI) will increase. The change of the landscape layout of construction land means that the distribution of air pollutant emission sources will be more concentrated and the scale effect will be enhanced, which would in turn affect the concentration of air pollutants (Zhang et al., 2019).

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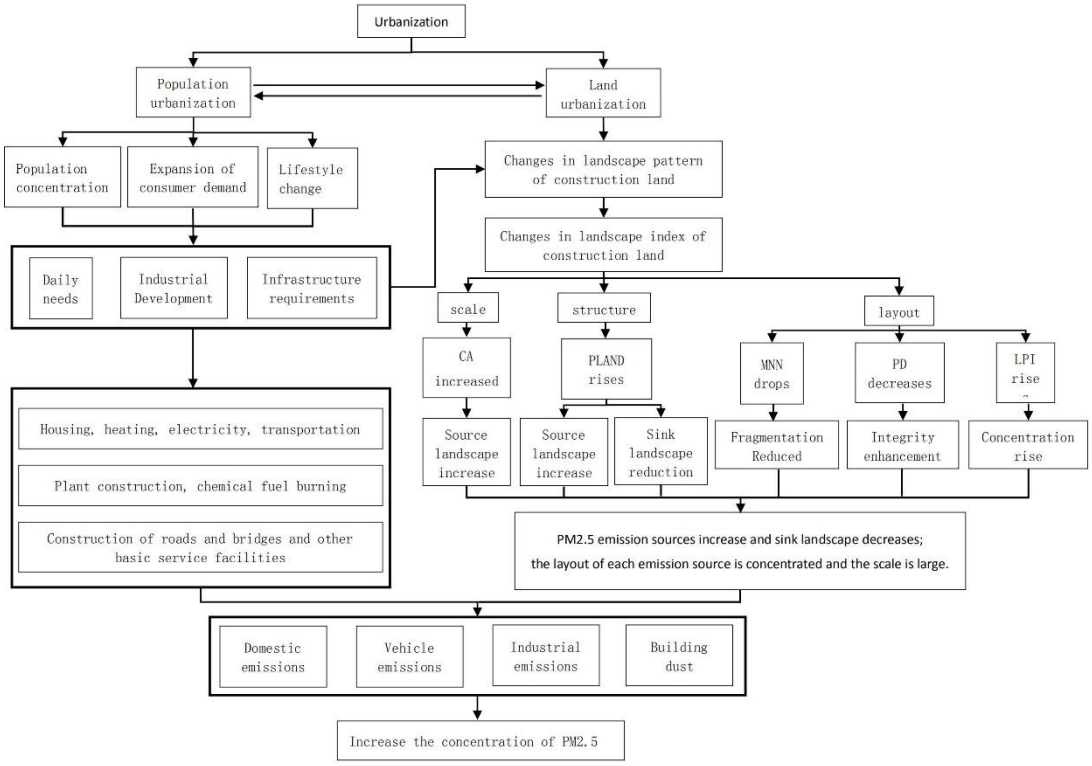

Figure 1. *Analysis framework of the influencing mechanism of landscape pattern index of construction land on $PM_{2.5}$ pollution*

Urbanization is a comprehensive and complex process, which includes mainly land urbanization and population urbanization (Liang et al., 2019). The most direct result of land urbanization is expansion of construction land, which could cause changes of the landscape pattern of urban construction land (Chen et al., 2013). In the process of land urbanization, as the carrier of human survival and development, land could affect population urbanization by attracting the population to gather continuously. Continuous improvement of population urbanization not only could promote population gathering, but also could change the residents' life styles and increase their consumption demands. All these changes could increase demands for capital construction, living needs and requirement for industrial development, resulting in sharply increase of demands for houses, commercial service and industrial lands, which could change the landscape patterns of urban construction land to a large extent. Therefore, it is required synchronous development of land urbanization to meet the survival and development of the population (Fan et al., 2016), that is to say, population urbanization and land urbanization are interactive. Since emission of $PM_{2.5}$ pollution is determined mainly by the emission source and area of urban landscape (Sun, 2017), changes of landscape pattern of urban construction land caused by urbanization could reflect indirectly the changes of $PM_{2.5}$ pollution as well as the spatial differences. Relevant studies show that the changes of the landscape pattern of urban construction land are mainly manifested in three aspects: changes in landscape scale, changes in landscape structure, and changes in landscape layout (Chen et al., 2002). Based on this, this study selects the construction area (CA) to represent the scale of the landscape pattern of urban construction land, the proportion of

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construction land (PLAND) to represent the structure of the landscape pattern of urban construction land, the largest patch index (LPI), patch Density (PD) and mean nearest distance (MNN) to represent the layout of urban construction land. With these five specific quantitative indexes, the law of functions between landscape pattern of urban construction land and $PM_{2.5}$ pollution are studies from different dimensions.

Specific effects of landscape pattern of urban construction land on $PM_{2.5}$ pollution are as follows: ① At the level of scale, with continuous improvement of urbanization, the scale of urban construction land keeps expanding, with increase of impervious surface as well as flying dust and construction dust, which could promote $PM_{2.5}$ pollution. In addition, as the major place for human activities, expansion of construction land has significant gathering effect of population. With the population keeps flowing in and gathering, plenty of demands for electricity, heating, traffic, industrial development and garbage disposal come up one after another, which could cause burning of abundant fossil fuel, resulting in increase of $PM_{2.5}$ pollution, therefore, the increase of urban construction land could cause increase of $PM_{2.5}$ pollution. ② At structural level, increase of the proportion of construction land shows that the land use types of $PM_{2.5}$ sourced landscape which is the carrier of various $PM_{2.5}$ emission sources are increasing, while the land use types of converged landscape represented by greenbelt and forest are decreasing. Significant changes in physical characteristics of the earth surface (roughness, reflectivity, soil water content, etc.), drive changes in climate, soil, hydrology, and landform eco-environmental factors, which in turn could affect regional material energy cycles and eco-chemical process, and affect $PM_{2.5}$. Because of finiteness of land resource, increase of "$PM_{2.5}$-sourced landscape" land use types and decrease of "converged landscape" land use types in the region will inevitably result in rise of $PM_{2.5}$ pollution. So, increase of the proportion of construction land could bring in rise of $PM_{2.5}$ pollution. ③ At the level of layout, larger largest patch index represents stronger integrity of urban construction land patches in the region, and the centralized continuity between the construction land patches is increased, which means that the scale of the city is keeping expanding and enlarging. During this process, the attracting and gathering effects of cities to population and industries are enhanced, which could cause a large increase of domestic emission and industrial waste gas, forming scale effect on $PM_{2.5}$ emission, which could promote $PM_{2.5}$ pollution. The greater the mean nearest distance and the larger the patch density, the worse the agglomeration between patches of the construction land in the area would be, and the stronger the fragmentation would be. Since the distance between the patches of the construction land is large and the construction land of unit area is segmented into small patches artificially or naturally, the scale of such city is relatively smaller and it is hard to form large-scale living quarters, commercial service areas and industrial parks. $PM_{2.5}$ emission sources are distributed in discrete type and it is hard to form large-scale emission of domestic gas and industrial waste gas, resulting in weak urban heat island effect. Therefore, increase of mean nearest distance and patch density could inhibit $PM_{2.5}$ pollution. Based on the above analysis, it could be seen that the stronger integrity of urban construction land and higher aggregation extent could improve $PM_{2.5}$ pollution; otherwise, large fragmentation could inhibit $PM_{2.5}$ pollution.

Data sources

In this paper, the annual average $PM_{2.5}$ concentration of prefecture-level cities in Pearl River Delta from 1998 to 2016 is taken as the explanatory variable, the landscape pattern index of construction land in prefecture-level cities in Pearl River Delta is taken as the

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DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

core explanatory variable, and social and economic factors (road density, population density, per capita GDP, proportion of secondary industry) and natural factors (wind speed, rainfall) are added as the relevant control variables, to explore the impact mechanism of construction land landscape pattern on $PM_{2.5}$ in the Pearl River Delta with spatial analysis method. The specific attributes of each indicator are shown in *Table 2*.

Table 2. *Variable description*

Variables	Level I index	Level II index	Definition of index
Landscape pattern	Landscape area	Construction area (CA)	Total area of construction land, which could reflect the landscape scale of construction land.
	Landscape composition	Proportion of construction land (PLAND)	Proportion of construction area to total land area, which could reflect the landscape dominance of construction
	Landscape layout	Patch density (PD)	Number of patches of construction land in unit area, which could reflect the fragmentation of the construction land.
		Largest patch index (LPI)	Proportion of the area of the largest patch of the construction land to the area of the total construction land, which is used to determine the overall degree of construction land patches.
		Mean nearest distance (MNN)	Sum of the nearest distances between the patches of the construction land divided by total number of the patches, which is used to reflect the aggregation degree of the construction land
Control variables	Road factors	Road density (RD)	Road mileage/total land area, which could reflect the intensity of automobile exhaust emissions
	Population factors	Population density (POPD)	Population/total land area, which could reflect the life emission intensity
	Economical factors	GDP per capita (AGDP)	GDP/total population, which could reflect the intensity of economic energy consumption
	Industrial structure	Proportion of secondary industry (IND)	Yield of secondary industry/GDP, which could reflect the industrial emission intensity.
	Natural factors	Rainfall (RAIN)	Mean annual precipitation, which could reflect the intensity of humidity.
		Wind speed (WIND)	Annual average wind speed, which could reflect the intensity of air flow
Explained variables	$PM_{2.5}$	Average $PM_{2.5}$ concentration ($PM_{2.5}$)	Annual average $PM_{2.5}$ concentration, which could reflect the $PM_{2.5}$ pollution degree

The data sources involved in this paper are as follows:

(1) Land use data: The land use data come from Landsat series remote sensing image data with 30 m × 30 m resolution published by Data Center for Resources and Environmental Sciences of Chinese Academy of Sciences. Based on the availability, time-efficiency and expressivity of data, the image data of 2000, 2005, 2010 and 2016 are adopted as the land use data interpreting map. At the same time, to guarantee the abundance of the expressions of the remote-sensing images as well as the accuracy of the interpreting results, the shooting time of the images is selected to be within the last ten days of May and the middle ten days of June, when the cloudage is less than 10%. Based

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on the above image data, the urban construction land use data of all stages in the research area are sorted, extracted and calculated with Arcgis10.3 and Fragstats4.2 software.

(2) $PM_{2.5}$ data: The data of $PM_{2.5}$ pollution adopted in this essay are acquired based on the global historical $PM_{2.5}$ annual mean raster dataset (http://sedac.ciesin.columbia.edu) from 1998 to 2016 issued by International Earth Science Information Network Center of Columbia University, the aerosol optical depth (AOD) of which is inversed by geographical weighted regression method with various satellite instruments, including NASA MODIS, MISR and Sea-WIFS, to obtain the mean concentration of $PM_{2.5}$ of all cities in the Pearl River Delta. The raster data have the characteristics of wide coverage area (55°S~70°N), long time sequence (19a) and high precision (with the resolution of 1 km × 1 km). Compared with the data of air pollutant obtained based on the ground monitoring point, the global air pollution data retrieved by remote sensing have better spatial continuity, with which the spatial distribution and changes of the pollutants can be detected conveniently for numerical computation. It has been proved that the data can be used to study the relations between landscape pattern and air pollutant (Bechle et al., 2017). In this essay, $PM_{2.5}$ pollution is analyzed from the perspective of landscape pattern index of the construction land, so, emphasis should be laid on the integration with the land use data in model construction. That is why the data of 2000, 2005, 2010 and 2016 are taken as the explained variables in this study.

(3) Socioeconomic data: Based on previous studies (Guo et al., 2018; Han, 2018), this essay adopts road, population, economy and industrial structure as the control variables of socioeconomic attributes, among which GDP per capita, industrial structure and population data come from Statistical Yearbook of Guangdong Province of the corresponding year, and the road mileage comes from China City Statistical Yearbook of the corresponding year. Specific processing methods and attributes of the indexes are shown in *Table 2*.

(4) Natural condition data: Studies have shown that the humidity and flow speed of air have a significant effect on $PM_{2.5}$ (Sun et al., 2020). However, due to the high collinearity of the humidity data in the Pearl River Delta, this essay adopts amount of precipitation and wind speed as the control variables to study the influences of natural weather conditions. The data comes from the annual value of the prefecture-level cities in Pearl River Delta in 2000, 2005, 2010 and 2016 China Meteorological Data Network (http://data.cma.cn/).

Methodology

Landscape pattern index

The landscape pattern indexes are selected from three aspects, landscape area, landscape composition and landscape layout. Fragstats4.2 software is used to calculate the interpreted remote-sensing image of the land utilization to obtain the landscape indexes. Calculation formula of landscape index is as the *Table 3*.

The larger the value of CA, the larger the scale of construction land; the larger the value of PLAND, the higher the proportion of the urban construction land to the total area is; PD could reflect the fragmentation of urban construction land, the bigger the value is, the higher the degree of fragmentation is; LPI could reflect the integrity of urban land, the bigger the value is, the higher the integrity of the urban land is; MNN could reflect the aggregation degree of regional urban land, the smaller the value is, the stronger the cohesiveness of the urban land is.

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Table 3. *Calculation formula of landscape index*

Categories	Metrics	Formulas
Landscape scale	Construction land area (CA)	$\mathrm{CA} = \sum_{i=1}^{n} a_i$
Landscape composition	Proportion of construction land (PLAND)	$\mathrm{PLAND} = \frac{\sum_{i=1}^{n} a_i}{A} \times 100$
Landscape layout	Construction land Patch density (PD)	$\mathrm{PD} = \frac{n}{A}$
	Construction land Largest patch index (LPI)	$\mathrm{LPI} = \frac{\max\limits_{0<i\le n} a_i}{A}$
	Construction land Mean nearest distance (MNN)	$\mathrm{MNN} = \frac{1}{n} \min\limits_{0<i\le n} h_i$

i refers to some patch of urban construction land within the research unit, *n* is the number of the patches of urban construction land within the research unit, and a_i is the area of the *i*-th patch of urban construction land within the research unit (hm^2), *A* is the total area of the research unit (hm^2), h_i is the Euclidean distance of the patch that is the nearest to the *i*-th patch (m)

Spatial econometric model

Spatial autocorrelation refers to potential interdependency between the observed data of some variables within the same distribution area. Tobler (1970) once pointed out "The first law of geography: Everything is related to everything else, but things closer are more relevant than things far away." So, this essay adopts spatial autocorrelation test to measure whether $PM_{2.5}$ pollutions in different regions are interdependent. If the spatial autocorrelation test is passed, a spatial econometric model is adopted for modeling; otherwise, a linear regression model is used for regression analysis. At present, two common indexes for spatial autocorrelation test are Moran's index I and Geary's index C. In this essay, Moran's I index is adopted to conduct spatial test, as shown by formula *Eq.1*:

$$Moran's\ I = \frac{\sum_{i=1}^{n}\sum_{j\neq i}^{n} w_{ij}(x_i - \bar{x})(x_j - \bar{x})}{\sigma^2 \sum_{i=1}^{n}\sum_{j\neq i}^{n} w_{ij}} = \frac{\sum_{i=1}^{n}\sum_{j\neq i}^{n} w_{ij}(x_i - \frac{1}{n}\sum_{i=1}^{n} x_i)(x_j - \frac{1}{n}\sum_{i=1}^{n} x_i)}{\frac{1}{n}\sum_{i=1}^{n}(x_i - \frac{1}{n}\sum_{i=1}^{n} x_i)^2 \sum_{i=1}^{n}\sum_{j\neq i}^{n} w_{ij}} \quad \text{(Eq.1)}$$

where, x_i and y_i are the attribute values of regions i and j; $\bar{x}$ is the mean value of the attribute value of the regions ; w_{ij} is the weight matrix of spatial position. when i is adjoined to j, w_{ij}=1; if i is not adjoined to j, w_{ij}=0; σ^2 is the variance of the attribute value; n is the sum of the spatial units.

Environmental pollution has high spatial autocorrelation (Maddision, 2005; Zhu et al., 2010), while high geographical aggregation of construction landscape has strengthened the spatial dependency of environmental pollution, so, spatial correlation should be included in studies on correlations between landscape pattern index of construction land and particle pollution. The premise in the classical econometric model is the premise of strict assumptions of spatial homogeneity and independence of the sample to be studied, and fixed explanatory variables. At the same time, common econometric model ignores

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the spatial correlation of the residual terms when applying ordinary least square for parameter simulation, resulting in large deviation between the estimation result of the model and the actual meaning. Therefore, the spatial econometric model is required to effectively solve the problems of the variables, such as spatial dependence and spatial correlation. While, conducting empirical studies by using spatial measurement method, the model could be divided into two types based on different impact ways of spatial terms, that is, Spatial Lag Model (SLM) and Spatial Error Model (SEM). The SLM and SEM established in this essay are shown by Formula *Eq.2* and Formula *Eq.3*.

$$P_{it} = \rho W_{P_{it}} + \alpha_0 + \alpha_1 CA + \alpha_2 PLAND + \alpha_3 PD + \alpha_4 LPI + \alpha_5 MNN + \alpha_6 X_{it} + \varepsilon_{it} \quad \text{(Eq.2)}$$

where, $\varepsilon_{it} \sim N(0, \sigma_{it}^2)$,
and where, i and t are the data of the i-th prefecture-level city in the region of Pearl River Delta in the t-th year. P refers to $PM_{2.5}$ pollution, ρ is the spatial regression coefficient, which reflects the spatial dependency between the observed values of the samples, i.e., the direction and strength of the functions of $PM_{2.5}$ pollution value W_P of the neighboring prefecture-level city in Pearl River Delta on the W_P pollution value P of the local region. W is $n \times n$ spatial weight matrix. In this essay, spatial adjacency weight matrix is adopted, that is, when local prefecture-level city i is adjourned to prefecture-level city j, W=1; if city i is not adjourned to city j, W=0. W_P is the spatial lag dependent variable, which reflects the extent of the influence of spatial distance on $PM_{2.5}$ in prefecture-level cities in the Pearl River Delta. CA is the area of the construction land, PLAND is the proportion of construction land types, PD is the patch density of the construction land, LPI is the largest patch index and MNN is the mean nearest distance between the patches. X is the other control variable that could affect environmental pollution, including road density, population density, GDP per capita, proportion of secondary industry, precipitation, and wind speed. ε_{it} is random error term vector.

$$P_{it} = \alpha_0 + \alpha_1 CA + \alpha_2 PLAND + \alpha_3 PD + \alpha_4 LPI + \alpha_5 MNN + \alpha_6 X_{it} + \varepsilon_{it} \quad \text{(Eq.3)}$$

where, $\varepsilon_{it} = \lambda W_{it} + \mu_{it}$, $\mu_{it} \sim N(0, \sigma_{it}^2)$.

In the formula *Eq.3*, parameter λ is spatial error coefficient, which evaluates the spatial dependency of the observed value of the sample, that is, the degree and direction of the influences of $PM_{2.5}$ of the neighboring prefecture-level city in Pearl River Delta on $PM_{2.5}$ pollution of the local region. Different from SLM model, there is error term in the spatial dependency in SEM model, which could measure the influence degree of error impact of dependent variable of the neighboring region on the observed value of the local. μ_{it} is the random error vector in normal distribution.

Results

Spatial-temporal change characteristics of $PM_{2.5}$ pollution in Pearl River Delta

Time change characteristics

It can be seen from *Fig. 2* that $PM_{2.5}$ pollution in the region of Pearl River Delta generally increases first and then decreases, which is mainly divided into three phases:

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① Phase of fast growing: During 2000 to 2005, prefecture-level cities in Pearl River Delta experienced a phase of fast growing, when the annual growth of Guangzhou was 3.84 $\mu g/m^3$, the highest growth of all the cities. Zhuhai had the smallest growth, which was 1.86 $\mu g/m^3$. ② Phase of slow growing: During 2005 to 2010, the $PM_{2.5}$ pollution values of most cities in Pearl River Delta were rising slowly, among which Zhaoqing had the largest annual growth, which was 0.38 $\mu g/m^3$, and Zhongshan had the smallest growth, which was 0.02 $\mu g/m^3$. The maximum annual growth was 1/5 of the minimum annual growth of the phase of fast growing. During this period, $PM_{2.5}$ pollution in Guangzhou, Shenzhen and Dongguan showed descending tendency, with the annual descending degree of 0.44 $\mu g/m^3$, 0.16 $\mu g/m^3$ and 0.18 $\mu g/m^3$, respectively. The possible reason could be that the year of 2010 was the year when the Asian Games was held in Guangzhou, when air quality assurance measures were taken on pollution sources of industry, automobile and flying dust in the city of Guangzhou and the surrounding areas, which has inhibited to some degree urban particle pollution; so, $PM_{2.5}$ pollution in Guangzhou as well as Shenzhen and Dongguan (which are display windows to the outside world) were reduced to some degree (Hu et al., 2013). ③ Phase of slow declining: From 2010 to 2016, $PM_{2.5}$ pollution in all the prefecture-level cities in Pearl River Delta declined slowly, among which Zhaoqing had the largest descent degree, with the annual descent value of 1.78 $\mu g/m^3$, and Huizhou had the smallest descent degree, with the annual descent value of 0.98 $\mu g/m^3$. Compared with the annual growth during the phase of fast growing, the annual descent degree of this phase was low, so, it was the phase of slow declining. However, by 2016, $PM_{2.5}$ pollution of all the prefecture-level cities was higher than that in 2000. The main reason for this change is that industrialization and urbanization in the region of Pearl River Delta was developing rapidly during 2000-2005, which was manifested mainly in rapid development of the secondary and tertiary industries, expansion of construction land area, significant accumulation of population and industries, as well as a series of domestic emissions, industrial waste gas, and automobile exhaust that were generated in large quantities, causing rapid increase of $PM_{2.5}$ pollution (Zhou et al., 2019). During 2005 to 2010, the region of Pearl River Delta began to upgrade and transform it industries for the first time in China, so the proportion of the secondary industry decreased, while the proportion of the tertiary industry represented by high-tech technology and services increased, resulting in significant changes of the industrial structure (Zhao, 2011). Different industrial structures have different influences on environment. Generally speaking, in the three industrial structures, the pollution intensity of the secondary industry is significantly higher than that of the primary and tertiary industries. This is because industrial production of the secondary industry is mostly extensive production with high consumption intensity and high consumption amount, while the tertiary industries are mainly intelligent-intensive enterprises and service industries, whose energy-consuming intensity and emission intensity are far lower than those of the second industry (Zhang, 2009). Therefore, with the optimization and upgrading of the industrial structure in the region of Pearl River Delta, the $PM_{2.5}$ pollution in this region was slowed down also. During the period of 2010-2016, with continuous deepening of government regulations and ecological development concept, the industrial structure of the region of Pearl River Delta had been continuously optimized and upgraded, the popularity rate of clean energy technology was increased continuously, and tasks of energy conservation and emission reduction were introduced, so, $PM_{2.5}$ pollution started to decline (Luo et al., 2018).

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Figure 2. *Bar graph of time sequential changes of $PM_{2.5}$ concentration in Pearl River Delta*

Evolution of spatial features

Natural breakpoint method is adopted to classify the $PM_{2.5}$ pollution data of four phases of the region of Pearl River Delta into four types, the spatial distribution characteristics of which are shown in *Fig. 3*. It could be seen from *Fig. 3* that $PM_{2.5}$ pollution values in the region of Pearl River Delta generally shows the feature of "high in the middle and low in the surrounding areas". In 2000, the $PM_{2.5}$ pollution value in the region of Pearl River Delta was at a relatively low level. Even Foshan and Zhongshan, which had high $PM_{2.5}$ pollution values in the central region, reached only moderate pollution level, Guangdong, Dongguan, Shenzhen, Zhuhai and Jiangmen were at low pollution levels, and Zhaoqing and Huizhou on both wings of the region were in areas with low $PM_{2.5}$ pollution level. Till 2005, $PM_{2.5}$ pollution value of the region of Pearl River Delta began to rise and the area with high pollution showed expanding tendency in space. Guangzhou, Shenzhen, Foshan and Zhongshan in the middle area had developed into highly $PM_{2.5}$-polluted area, Zhaoqing, Jiangmen, Zhuhai and Shenzhen became highly polluted area from the original low-polluted area or lower polluter area, and Huizhou became moderate polluted area from the original low polluted area. In 2010, the spatial pattern of $PM_{2.5}$ pollution in the region of Pearl River Delta did not change much, with only Shenzhen descending from the original higher polluted area to moderately polluted area; In 2016, compared with the previous period, the $PM_{2.5}$ pollution value in the region of Pearl River Delta had an overall descending trend. Guangzhou, Dongguan, Foshan, and Zhongshan in the middle had lowered from the original highly polluted areas to areas with higher pollution, Zhaoqing and Jiangmen lowered from higher polluted area to moderately polluted area, and Huizhou and Shenzhen descended from moderately polluted area to less polluted area. It could be seen from the four-phase spatial pattern of $PM_{2.5}$ pollution in the region of Pearl River Delta that though it had the rule of rising first and then descending in the sequence of time, it still maintained the spatial pattern of "high in the middle and low in the surrounding area". The major reason for such spatial distribution characteristic is that in middle area of Pearl River Delta, the level of economic development and urbanization are higher, the population size is larger, the secondary and tertiary industries occupy a higher proportion, the industrial waste gas, the domestic discharge and automobile exhaust are more severe, and $PM_{2.5}$-sourced landscape dominance is higher and distributed in concentration. All of these factors are important emission source of particle matters; thereby high-valued aggregation areas of $PM_{2.5}$

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pollution are formed. With relatively low level of economic development and urbanization, the scale effect of the population and the proportion of secondary and tertiary industries in the surrounding areas of Pearl River Delta are relatively low, so, are also relatively less industrial waste gas, domestic discharge and automobile exhaust. In addition, the $PM_{2.5}$ sourced landscape in these areas are scattered and their dominance is low, so the $PM_{2.5}$ pollution value is low (Wang, 2017).

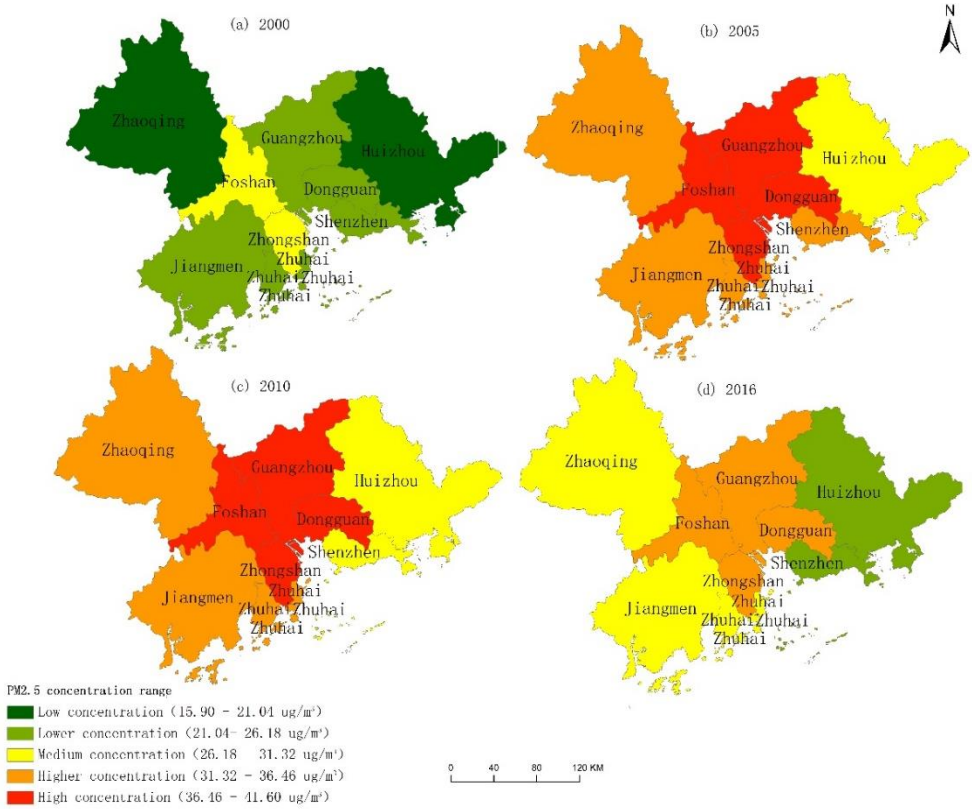

Figure 3. *Spatial changes of $PM_{2.5}$ pollution in the region of Pearl River Delta*

Change characteristics of landscape pattern of urban construction land in the region of Pearl River Delta

Landscape area

As shown in *Fig. 4*, the construction area (CA) shows increasing tendency with years in the cities of Pearl River Delta. Through analysis, it is found that changes of the construction area in the region of Pearl River Delta experience mainly two stages: ① Stage of rapid growth (2000-2005): During this stage, the process of urbanization was accelerated and urban expansion was serious. ② Stage of slow growth (2005-2016): Affected by economic and other factors, the process of urbanization had slowed down, and urban expansion had declined. In addition, with continuous progress of the society, the public began to pay more attention to resource conservation and ecological development, urban intensive and efficient development had gradually become a consensus, and urban expansion contracted further. From 2000 to 2016, Guangzhou was the city where the area of construction land increased the most, rising from 79,904.43 hm^2 to 141,027.30 hm^2, with the percentage gain of 76.49%; while Zhuhai was the region

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where the area of the construction land increased the least, from 18,502.92 hm^2 in 2000 to 26,507.97 hm^2 in 2016, increasing by 8,005.05 hm^2, with the percentage gain of 43.26%. Generally, the total area of construction land in the region of Pearl River Delta was increased obviously. In 2000, the total area was 417,908.61 hm^2, and till 2016, it expanded to 732,866.76 hm^2, increasing 314,958.15 hm^2, with the percentage gain of 75.37%. Thus, it could be seen that the region of Pearl River Delta had experienced a rapid process of urbanization from 2000 to 2016, during which the area of construction land increased significantly.

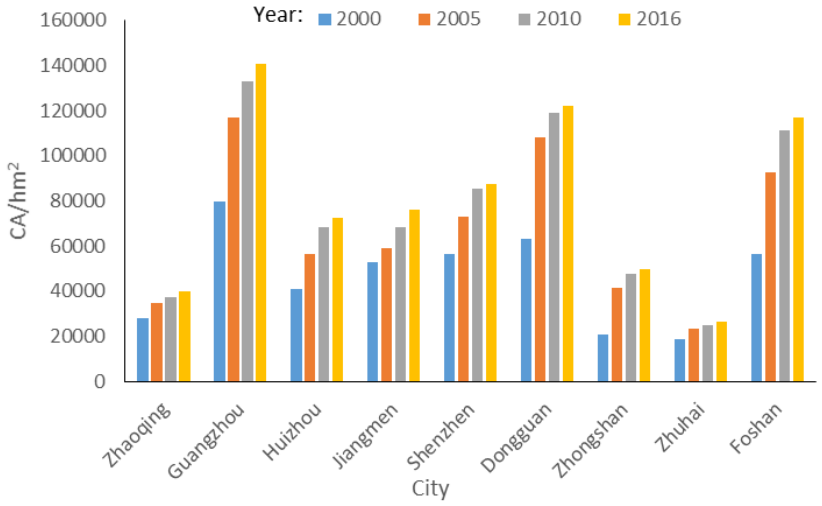

Figure 4. *Pearl River Delta Region Urban Construction Land Area (CA) Change Chart*

Landscape composition

It could be seen from *Fig. 5* that the proportion of construction land (PLAND) of the cities in the region of Pearl River Delta from 2000 to 2016 is basically similar in the changing law of the construction land area (CA), showing increasing tendency with years. Through comparison, it is found that the proportion of construction land (PLAND) in the Pearl River Delta experienced a period of rapid growth from 2000 to 2005; during 2005-2010, the growth slowed down; and till 2010-2016, the growing speed declined further, tending to steady slow growth. The possible reason might be that from 2000 to 2005, Chinese economy grew rapidly, and the process of urbanization was accelerated. As one of the economic development centers of China, it is inevitable that the region of Pearl River Delta would have more demands for construction land, resulting in rapid increase of the proportion of construction land (PLAND). Till 2005-2010, affected by market environment, economic crisis and other factors, the process of urbanization began to slow down and the demands for construction land were reduced also. During 2010-2016, Pearl River Delta had stricter requirements for resource conservation and green development, the concept of urban development transformed from extensive type to descending, digging and other vertical development, and the expansion rate of construction land began to slow down further. By 2016, the proportion of construction land (PLAND) of Dongguan increased the most, increasing 24.57%, while that of Zhaoqing increased the least, increasing 0.80%. Generally, PLAND in the region of Pearl River Delta was increased obviously, averaging 10.58%.

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Figure 5. *Pearl River Delta Region Proportion of Construction Land (PLAND) Change Chart*

Landscape layout

Landscape layout emphasizes the combined effect of patch types in space as well as the layout effect of the entire landscape (Peng et al., 2006). The characterization factors are mainly divided into three aspects: fragmentation, integrity and cohesiveness. So, in this essay, patch density, largest patch index and mean nearest distance are adopted to assess the characteristics of landscape layout of the construction land in the region of Pearl River Delta.

Fragmentation is mainly expressed by patch density (PD) of the landscape. The changing laws (*Fig. 6*) of patch density in the cities are mainly classified into two types: one is U-shaped trend, that is, the patch density decreases first and then increase; and the other is increase by year. Regions that show U-shaped trend include Dongguan, Foshan, Guangzhou, Shenzhen, Zhongshan, and Zhuhai; regions where PD increases by year include Huizhou, Jiangmen and Zhaoqing. Through further analysis, it could be found that regions showing U-shaped trend are mainly cities with higher economic development level, while Huizhou, Jiangmen and Zhaoqing showing the trend of increase by year have relatively lower economic development level. The possible reason could be that with continuous development of economy and urbanization, the city size is keep increasing, the concentration of urban construction land is strengthened and the patch density is lowered. When the level of urbanization is increased further, population, resources, and ecological capacity of the central city reach their limits. To alleviate the pressure on the downtown, development of new urban areas would become an inevitable choice, and so the patch density begins to increase gradually again. Due to relatively lower economic and urbanization levels as well as weak agglomeration effects, Huizhou, Jiangmen, and Zhaoqing have not formed large-scale central cities. The urban layout is relatively scattered and the patch density is high. Compare with 2000, the patch density of Dongguan in 2016 descended the most, dropping 0.14, and PD of Zhuhai descended the least, dropping only 0.01. Zhaoqing, Huizhou and Jiangmen showed increasing trend, rising 0.01. Generally, the patch density (PD) of urban construction land in Pearl River Delta decreased 0.24, indicating that the degree of breakage of the urban construction land in Pearl River Delta decreased.

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Figure 6. *Pearl River Delta Region Urban Construction Land Patch Density (PD) Change Chart*

Integrity is represented by the area of the largest patch. During 2000-2016, the largest patch index (LPI) in the region of Pearl River Delta showed the trend of increase by year (*Fig. 7*), among which, during 2000-2005, the increasing tendency was the most obvious while during 2005 to 2016, the integrity grew slowly. The main reason is that during the period of rapid development of economy, the level of urbanization in various cities increases significantly, large-scale urban districts have begun to form, the collaboration and integrity of cities are improved significantly, and the largest patch index also increases rapidly; with the slowdown of economic development and urbanization, the expansion of large-scale cities begins to decrease, and the growth rate of the largest patch index is slowed. By 2016, the largest patch index of Dongguan increased the most, increasing 26.3698, while that of Zhaoqing increased the least, increasing 0.05. On the whole, during 2000 to 2016, the largest patch index increased by an average of 7.17 in the region of Pearl River Delta, indicating that the integrity of urban construction land in the region of Pearl River Delta is strengthened.

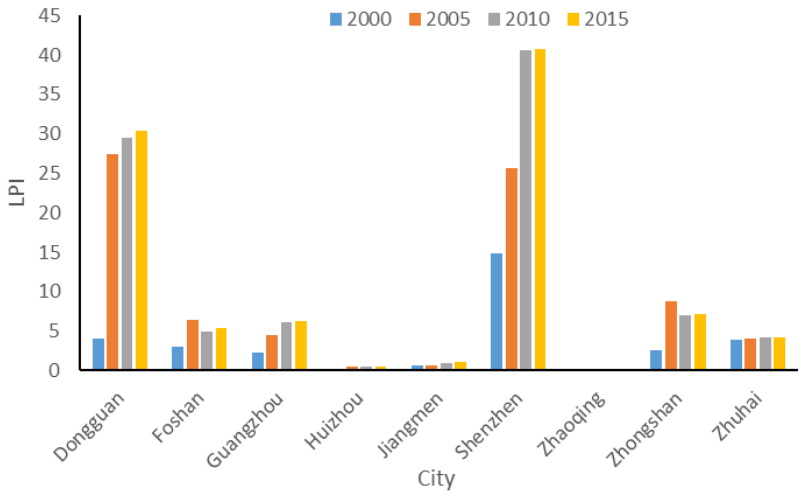

Figure 7. *Pearl River Delta Region Urban Construction Land Largest Patch Index (LPI) Change Chart*

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Cohesiveness is represented by the distance between the patches of the landscape. The mean nearest distance (MNN) of the construction land in the region of Pearl River Delta generally show U-shaped variation (*Fig. 8*), i.e., decreasing first and then rising. The reason is that the aggregation effect and driving effect caused by continuous increase of urbanization, expansion of urban scale and large-scale cities are enhanced continuously, the construction lands develop gradually into concentrated areas, and the average nearest distance keeps decreasing. After the scale of the city reaches certain level, the potential of urban development begins to decline, the government begins to plan construction of sub-central cities and satellite cities, therefore, concentration of the construction land begins to decline, and the average nearest distance increased. During 2000 to 2016, the city where the mean nearest distance of construction land in Pearl River Delta declined the most was Shenzhen, dropping by 95.8791 m, while the city with the smallest decline was Guangzhou, dropping by 1.40 m. Generally, the mean nearest distance of the construction land in the region of Pearl River Delta decreased 42.66 m, indicating that the cohesiveness of the urban construction land of the region of Pearl River Delta from 2000 to 2016 was increased.

Figure 8. *Pearl River Delta Region Urban Construction land Mean Nearest Distance (MNN) Change Chart*

Spatial panel econometric analysis of the influences of landscape pattern of urban construction land on $PM_{2.5}$ pollution

Spatial autocorrelation test

Moran's I statistic is adopted in this essay to conduct spatial autocorrelation test on $PM_{2.5}$ pollution of four phases in 9 cities in the region of Pearl River Delta. If the spatial autocorrelation test is passed, spatial econometric model would be used to conduct regression analysis on $PM_{2.5}$ pollution in the region of Pearl River Delta, the landscape pattern of urban construction land and relevant control variables; otherwise, OLS method would be adopted to conduct modeling analysis. According to the results of the spatial autocorrelation test shown in *Table 4*, there is a significant spatial correlation between $PM_{2.5}$ pollution in 9 cities in the region of Pearl River Delta. Moran's I values of these four phases are 0.496, 0.411, 0.333 and 0.483, respectively, all passing the significance test at 5% significance level, indicating that $PM_{2.5}$ pollution in the region of Pearl River

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DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

Delta has significant positive spatial correlation. Therefore, when studying the correlation between $PM_{2.5}$ pollution in the region of Pearl River Delta and landscape pattern of urban construction land, it is necessary to take spatial correlation into consideration.

Table 4. *Spatial autocorrelation test of $PM_{2.5}$ pollution in the region of Pearl River Delta*

Years	Moran's I		
2000	Statistics	Z	P-value
	0.496	2.701	0.0070
2005	Statistics	Z	P-value
	0.411	2.22	0.026
2010	Statistics	Z	P-value
	0.3330	1.9890	0.047
2016	Statistics	Z	P-value
	0.483	2.511	0.0120

Analysis of spatial panel econometric results

Based on the results of the spatial autocorrelation test mentioned above, two classical models for spatial econometrics are adopted in this essay, spatial lagging model (SLM) and spatial error model (SEM), to analyze the correlations between $PM_{2.5}$ pollution and the landscape pattern of urban construction land as well as the related control variables in the region of Pearl River Delta. According to the results of LM test and Hausman test, it is determined which model is better among SLM / SEM model, fixed effect model/random effect model. According to the LM test results reported in *Table 4*, it could be seen that LM (lag) statistics is larger than LM (errow) and more significant, and based on the principle of LM test (Burridge, 1980), it could be determined that SLM model adopted in this essay is better. The results of Hausman test rejected the null hypothesis of random effects at the significance level of 1%, that is, fixed effect is more suitable in this essay. So, the regression results of the fixed effect model of SLM model as the optimal fitting results are adopted in this essay for analysis and discussion. Specific fitting results see *Table 5*.

According to the optimal fitting results in *Table 5*, it can be seen that the landscape pattern index of each construction land has different influence mechanisms on $PM_{2.5}$ pollution. ① Landscape scale: The construction area (CA) has positive effects on $PM_{2.5}$ pollution, with the regression coefficient of 0.298, and it is significant at 10% level, which accords with the theoretical expectation, indicating that increase of the area of urban construction land could improve $PM_{2.5}$ pollution of the region. ② Landscape composition: Proportion of construction land (PLAND) also has positive effects on $PM_{2.5}$ pollution, with the regression coefficient of 3.299, and passes the significance test at 5% level. This result coincides with the theoretical analysis mentioned above, indicating that expansion of landscape pattern of urban construction land could cause rise of $PM_{2.5}$ pollution. ③ Landscape layout: The regression coefficient of the largest patch index (LPI) is 0.037, however, it fails the significance test, indicating that though increase of LPI of construction land could promote $PM_{2.5}$ pollution, the promoting effect is still weak and has not been displayed fully. The possible reason is that increase of the largest patch index of construction land is the result of the enhancement of urban integrity, which could bring about scale effect of $PM_{2.5}$ emissions; however, since the region of Pearl River Delta has

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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higher economic development level, especially in large-scale cities with higher integrity, with continuous optimization and upgrading of the industrial structure as well as the development of scientific technology, the proportion of the tertiary industry, the clean energy technology and waste gas treatment technology are all at higher level; therefore, the scale effect of $PM_{2.5}$ emission in large-scale cities weakens, and the promoting effect of largest patch index of construction land on $PM_{2.5}$ pollution also decreases, resulting in non-significant state finally. Patch density (PD) and mean nearest distance (MNN) have negative inhibiting effect on $PM_{2.5}$ pollution, which accords with the theoretical expectation. The inhibiting effect of patch density (PD) on $PM_{2.5}$ pollution is most obvious, with the regression coefficient of -40.672, and it is significant at 5% level, indicating that with the increase of the fragmentation degree of construction land in the region of Pearl River Delta, $PM_{2.5}$ pollution decreases obviously. The regression coefficient of the mean nearest distance (MNN) is -2.278 and is significant at 5% level, indicating that $PM_{2.5}$ pollution decreases with the increase of the mean nearest distance. Increase of MNN means that the aggregation of the patches of the construction land decreases. $PM_{2.5}$ emission sources are distributed in scatter and the scale effect decreases, which could result in decrease of $PM_{2.5}$ pollution.

Table 5. *Spatial analysis results of landscape pattern of construction land on $PM_{2.5}$ pollution*

Index type	Index name	Model			
		SLM		SEM	
		fe	re	fe	re
Landscape area	CA	0.298* （-1.86）	0.088** （-2.46）	0.383** （-2.51）	0.090** （-2.45）
Landscape composition	PLAND	3.229** (-2.25)	0.053 （-0.19）	-5.116*** (-3.25)	0.028 （-0.11）
Landscape layout	PD	-40.672** (-2.03)	-5.169* (-1.85)	-67.216*** (-2.92)	-5.327** (-2.08)
	LPI	0.037 （-1.2）	-0.006 (-0.17)	0.043 -1.44	-0.008 (-0.25)
	MNN	-2.278** (-2.37)	0.208 （-0.84）	-3.645*** (-3.38)	0.181 (-0.73)
Social-economic factors	RD	0.358** （-2.56）	0.302** （-2.05）	0.245 （-1.59）	0.332** (-2.26)
	POPD	-0.555 (-1.59)	-0.255** (-2.08)	-0.33 (-0.84)	-0.241** (-2.04)
	AGDP	0.183** （-1.97）	-0.002 (-0.07)	0.144 （-1.5）	0.006 (-0.17)
	IND	4.725*** （-2.8）	3.633*** （-2.62）	2.365 （-1.28）	4.077*** (-2.93)
natural factors	RAIN	0.619** （-2.05）	0.034 （-0.12）	1.001*** （-3.27）	-0.029 (-0.10)
	WIND	-1.080*** (-3.95)	-0.788*** (-3.36)	-0.884*** (-3.32)	-0.820*** (-3.55)
LM test	LM(lag)	3.168*			
	R-LM(lag)	2.995*			
	LM(errow)	2.293			
	R-LM(errow)	2.121			
Hausman test	chi2(6)	114.39		83	
	Prob>chi2	0		0	

The t values ***, ** and * represent significant at the 1%, 5%, and 10% levels

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DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

Social-economic factors that have positive influences on $PM_{2.5}$ pollution include road density (RD), average GDP (AGDP), proportion of the output of the secondary industry (IND), the regression coefficients of which are respectively 0.358, 0.183 and 4.725. RD and AGDP) are significant at 5% level, and IND is significant at 1% level. This result shows that the positive influence of IND on $PM_{2.5}$ pollution is the most significant of all the social-economic factors. The major reason is that the secondary industries are mostly chemical industry, manufacturing and other industries which could generate more waste gases, so increase of IND will inevitably bring in increase of $PM_{2.5}$ pollution, with significant influences. The increase of per capita income is based on the increase of the yields of primary, secondary and tertiary industries; therefore, the increase of AGDP can also promote $PM_{2.5}$ pollution. However, with continuous optimization and adjustment of the industrial structure as well as continuous increase of the proportion of the tertiary industry, AGDP has the smallest positive influencing coefficient on $PM_{2.5}$ pollution. Increase of RD means increase of vehicles. At present, most vehicles in China are fuel vehicles and the exhaust emission of the fuel vehicles will inevitably cause increase of $PM_{2.5}$ pollution. The regression coefficient of population density (POPD) is -0.555, indicating increase of population density could lower $PM_{2.5}$ pollution; however, this index fails the significance test. The reason for such result may be that with continuous increase of population density, regional land use efficiency keeps increasing and the public transport is developed; meanwhile, the municipal departments are more active in environmental governance with higher efficiency (Hu, 2019; Chang and Zhao, 2019). A series of such measures could reduce emission of automobile exhaust and industrial gas as well as unlimited sprawl of construction land, which could in turn reduce $PM_{2.5}$ pollution to some degree; however, the significance of the effects is still poor, which cannot improve regional $PM_{2.5}$ pollution obviously.

Natural factors: Rainfall (RAIN) has positive effect on $PM_{2.5}$ pollution, with the regression coefficient of 0.619, passing the significance test at 5% level. The main reason is that the increase of rainfall will increase relative humidity in the air, and the diameter of $PM_{2.5}$ particles is small, which mainly grows by moisture absorption and aggregation to cause rise in pollution; meanwhile, to improve the electric property of particulate matters is more beneficial to aggregation of $PM_{2.5}$ particles, which could increase $PM_{2.5}$ pollution (Tang et al., 2013). Wind speed (WIND) has significant negative effect on $PM_{2.5}$ pollution, with the regression coefficient of -1.080, and is significant at 1% level, indicating that increase of wind speed could reduce $PM_{2.5}$ pollution. This is mainly because wind has good diffusing and purifying effects. The region of Pearl River Delta is in the coastal strip. Especially in summer, the wind blows from the southeast ocean and the clean air brought by it could dilute and purify the air inland, thereby reducing $PM_{2.5}$ pollution (Zhou and Liang, 2013).

Discussion

According to the analysis results of spatial-temporal evolution of $PM_{2.5}$ concentration and landscape pattern index of the construction land in the Pearl River Delta, and the spatial econometric analysis results of the impact of the landscape pattern of construction land on $PM_{2.5}$, some suggestions are put forward on rational utilization of construction land in the Pearl River Delta, with the hope to exert the controlling effect of construction land in $PM_{2.5}$ pollution control and improve the air quality level of the Pearl River Delta. The details are as follows:

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

(1) From the influencing results of landscape scale of construction land on $PM_{2.5}$ pollution, it could be seen that expansion of construction land could significantly improve $PM_{2.5}$ pollution in the region of Pearl River Delta. So, in the process of urban development: **a.** The area of construction land should be increased rationally and orderly to avoid urban sprawl in urban development; **b.** Emphasis should be laid on improvement of land utilization efficiency and shift towards three-dimensional urban development model; **c.** Reform of the industrial structure should be promoted further, backward industries with high pollution, high consumption and more land capital input should be weeded out gradually, and the proportion of intelligence-intensive high-tech industries should be increased, so as to improve the level of intensive land use in the region of Pearl River Delta.

(2) From the influencing results of landscape composition of construction land on $PM_{2.5}$ pollution, it could be seen that increase of the proportion of construction land has significant positive effect on $PM_{2.5}$ pollution in the region of Pearl River Delta. So, in the process of urban planning, not only the expansion of construction land should be controlled, it is also necessary to increase the proportions of road greening, park green space and urban forests in the design of urban landscapes. In the premise of guaranteeing healthy development of the city, the proportion of $PM_{2.5}$ converged landscape should be improved rationally to exert the dust reducing effect of land use and control efficiently $PM_{2.5}$ pollution.

(3) From the influencing results of landscape layout of construction land on $PM_{2.5}$ pollution, it could be seen that increase of the integrity and aggregation of construction land could improve significantly $PM_{2.5}$ pollution in the region of Pearl River Delta. Since mononuclear city has low efficiency in spatial allocation of the resource factors and that the social resources cannot be integrated effectively, excessive expansion of urban scale could result in increase of traffic cost and reduce of energy utilization efficiency; therefore, when selecting the pattern of urban development, it is necessary to adopt multi-core urban development policy of satellite town, that is, to develop sub-center city and multi-center city so as to avoid severe $PM_{2.5}$ pollution caused by high aggregation of the city.

Conclusion

In this essay, remote sensing is used to revert $PM_{2.5}$ pollution data as the explained variable, the CA, PLAND, PD, LPI, and MNN landscape indexes of the construction land are calculated based on the data of land use as the core explanatory variable, and the social-economic factors and natural factors are taken as the control variables, based on which the spatial-temporal variation rules of $PM_{2.5}$ pollution in the region of Pearl River Delta from 2000 to 2016 as well as the variation law of landscape pattern of the construction land are analyzed, and the correlations between $PM_{2.5}$ pollution and the landscape index of construction land are also analyzed with spatial econometric model. The major conclusions are as follows:

(1) During 2000 to 2016, $PM_{2.5}$ pollution in the region of Pearl River Delta rose first and then decreased, however, by 2016, $PM_{2.5}$ pollution in all regions of Pearl River Delta was higher than that in 2000. Spatially, $PM_{2.5}$ pollution in the region of Pearl River Delta shows the spatial distribution law of high in the middle and low in the surrounding area. The regions with higher value are focused mainly cities neighboring Guangzhou, while regions with low value are mainly Zhaoqing and Huizhou in the east and west wings of Pearl River Delta.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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(2) Construction land area (CA) in the region of Pearl River Delta expanded obviously during 2000 to 2016, with an overall increase of 75.37%. Patch density (PD) and mean nearest distance (MNN) of the construction land decreased to varying degree, indicating that fragmentation of landscape of the construction land in Pearl River Delta decreased while the aggregation increased. Proportion of construction land (PLAND) and largest patch index (LPI) showed the tendency of increasing, indicating that the dominance and integrity of landscapes of the construction land in the region of Pearl River Delta kept increasing.

(3) The landscape pattern index of construction land mainly affects $PM_{2.5}$ pollution from three aspects, landscape scale, landscape structure, and landscape layout. **a.** Landscape scale: Construction area (CA) has positive correlation with $PM_{2.5}$ pollution. **b.** Landscape composition: Proportion of construction land (PLAND) also has positive correlation with $PM_{2.5}$ pollution. **c.** Landscape layout: Patch density (PD) and mean nearest distance (MNN) has negative correlation with $PM_{2.5}$ pollution, i.e., increase of PD and MNN could reduce $PM_{2.5}$ pollution. Though the largest patch index (LPI) shows positive correlation with $PM_{2.5}$ pollution, but it is not significant.

(4) Economic factors and natural factors also have great influences on $PM_{2.5}$ pollution. **a.** Increase of road density (RD), average GDP (AGDP) and the proportion of the output of the secondary industry (IND) could cause rise of $PM_{2.5}$ pollution. Population density (POPD) has negative correlation with $PM_{2.5}$ pollution, but not significant. **b.** In the natural factors, rainfall could increase $PM_{2.5}$ pollution, while wind speed could lower $PM_{2.5}$ pollution.

Acknowledgments. This paper supported by the National Natural Science Foundation of China (41771565), Supporting Plan for Scientific and Technological Innovative Talents (Humanities and Social Sciences) in Colleges and Universities of Henan Province (2019-cx-014), Jiangsu Province Natural Resources Think Tank Open Cooperation Project in 2019 (2019TDZY10).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79157939

THE INFLUENCE OF COMBINATIONS OF OPERATION PARAMETERS ON SEWAGE TREATMENT IN VERTICAL-FLOW CONSTRUCTED WETLANDS

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(Received 18th Mar 2020; accepted 20th Aug 2020)

Abstract. This paper aims to research the relationship between design parameters and purification effect. An Orthogonal experiment was conducted on the aeration mode, hydraulic load rate and organic load, to study the influence of operation parameters on the pollutant removal rate and substrate enzyme activities of vertical-flow constructed wetlands. These results revealed that the best treatment under the conditions of 20 cm/d hydraulic, 60 g/(m^2·d) organic load and intermittent aeration has outstanding decontamination performance and the removal efficiency of total nitrogen, total phosphorous and chemical oxygen demand reached 61%, 31% and 92%, respectively. It was higher than those of other treatments significantly ($p < 0.05$), indicating that proper improvement of organic load, reduction of hydraulic load and auxiliary intermittent aeration were beneficial to the improvement of sewage removal capacity of vertical flow constructed wetland. By analyzing the correlation of pollutant removal efficiency and enzyme activity, urease and catalase appear to have significant correlation with total nitrogen removal efficiency ($p < 0.01$), while catalase appear to have significant correlation to chemical oxygen demand removal efficiency ($p < 0.01$). The results for the use of enzymes as evaluation indices for the purification effect and the improvement of the decontamination capability in vertical-flow constructed wetland provides a theoretical basis.

Keywords: *vertical-flow constructed wetland, orthogonal experiment, operation parameters, enzyme, removal efficiency*

Introduction

Constructed Wetlands (CW) are used extensively for the removal of contaminants from sewage, improving the environment in the world. Compared with traditional sewage treatment technologies, CW has the advantage of being simple, low cost and high efficiency, which makes it very suitable for application in developing countries. CW can remove nitrogen, phosphorus, organic and inorganic pollutants therefore, it could prevent the spread of germs in water (Kivaisi, 2001). It can generally be divided into three types: surface-flow constructed wetland (SFCW), horizontal subsurface-flow constructed wetland (HSFCW) and vertical-flow constructed wetland (VFCW) according to the difference of bed fabric water method and flow mode (Cui et al., 2009). Vertical-Flow constructed wetland (VFCW) has been widely used because of its small area and high processing efficiency. Moreover, recent studies have shown that VFCW not only performs well in the treatment of BOD (biological oxygen demand) and TSS (total suspended solids), but also exhibits strong nitrification at low temperature and high load conditions (Cooper, 2005; Prochaska et al., 2007). Hydraulic load and organic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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load could affect the treatment efficiency of COD (chemical oxygen demand) and orthophosphate in VFCW (Sani et al., 2013). There are many studies on the purification effect of wetland plants, microorganisms and enzymes on wetland pollutants (Martens et al., 1992; Freeman et al., 1997; Kang et al., 1998; Shackle et al., 2000; Cheng et al., 2002). Among them, the enzyme activity of wetland soil directly affects the rate of material transformation and circulation in the environment, which plays a crucial role in maintaining the balance of the wetland ecosystem (Hill et al., 2006; Sun et al., 2018). Shackle (2006) demonstrated that extracellular enzymes can improve the biodegradation process. Soil enzymes together with microorganisms promote the conversion of substances. There are many factors influencing soil enzyme activity, including biological factors, soil factors and environmental factors (Duarte et al., 2008; Reboreda et al., 2008). In the study of soil enzymes, it was found that phosphatase can promote the hydrolysis of organophosphates. Urease is the hydrolase of C-N and catalase can convert hydrogen peroxide in the organism and matrix into water and oxygen. Sun (2018) studied the soil of reed community in Huishan karst wetland in Guilin, and found that the content of TOC (total organic carbon), TN (total nitrogen) and TP (total phosphorous) were significantly positively correlated with the activities of acid phosphatase, catalase, cellulase and other enzymes, which to some extent represented the changes in soil quality of reed community. Li (2015) proved that there was a significantly positive correlation between catalase activity and organic matter, total nitrogen, and alkali-hydrolyzed nitrogen in decanting wetlands in Baiguishan reservoir area, indicating that catalase activity was consistent with soil fertility changes. The above studies have proved that enzymes are necessarily related to nutrient cycling in soil. Therefore, it is necessary to study the relations among the substrate enzymes, pollutant removal and operating parameters to improve the research on wetland decontamination.

At present, most researchers improve the decontamination efficiency through aeration, parameter optimization, wetland plant selection and other methods (Green et al., 1998; Ouellet-Plamondon et al., 2006; Nivala et al., 2012; Abou-Elela et al., 2012; Guo et al.,2014; Ma et al., 2019; Pu et al., 2019; Kang et al., 2019). The research studies mainly concentrated on the aeration rate, aeration location, load size, the plant collocation on pollutants removal, but regarding the hydraulic load, organic load and combinatorial optimization in VFCW, and relationship between the pollutants removal and substrate enzyme activity of VFCW there are fewer investigations. Therefore, by carrying the different operation parameters of orthogonal test and monitoring the change of phosphatase, urease and catalase among the removal efficiency of TN, TP and COD (chemical oxygen demand), the effect of operation parameters combination optimization of VFCW on N, P, COD removal and the correlation between enzyme activity and pollutant removal was studied. This could provide theoretical basis for improving the decontamination of VFCW and using enzyme activity as the index to evaluate the purification effect of wetland.

Materials and methods

Construction of the vertical-flow constructed wetlands

In this experiment, PVC column was used to simulate the vertical-flow constructed wetland, with the following specifications: diameter of 30 cm × height of 45 cm. Two Hybrid Giant Napier were planted in each column. From bottom to top, the packing was

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5 cm gravel layer and 35 cm mixed substrate (river sand + yellow soil). The experimental equipment was built in southwest China and shown in *Figure 1*.

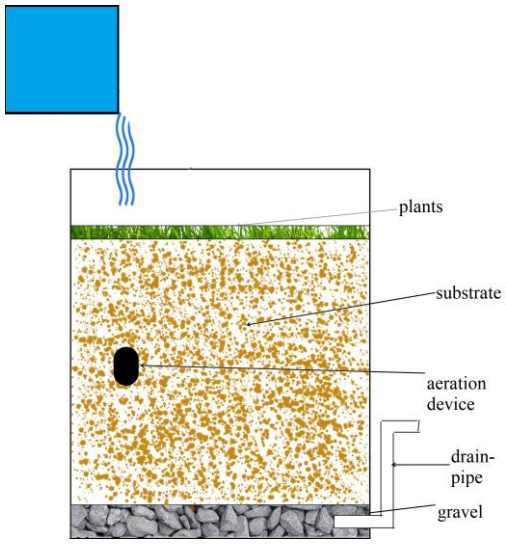

Figure 1. *Simulation diagram of experimental equipment*

Experimental process

The test settings were built outdoors in May 20th, and began to run in early June, and ended in September 20th. Quantitative peristaltic pump was used for continuous irrigation for 12 h. Three factors (1: hydraulic load, 2: organic load, 3: aeration mode) were set for the test. Hydraulic load was set at 3 levels and the corresponding hydraulic load levels were 20, 40 and 60 cm/d respectively. In addition, the level of organic load was set at 3 levels as follows: 20 g/(m^2·d), 40 g/(m^2·d) and 60 g/(m^2·d) (see *Table 1* for details). At the same time, aeration mode was also set at 3 levels: continuous aeration, no aeration and intermittent aeration. The aeration device was installed with a timer to control the aeration time and the aeration mode, the specific operations were as follows: the continuous aeration in this experiment refers to the continuous aeration for 12 h during the irrigation period. The aeration volume was set to 1 L/min, and the total amount of aeration was 720 L/d. Intermittent aeration refers to aeration of 1 h every 3 h during irrigation, and the total aeration time was 3 h. The aeration volume was set to 4 L/min, and the total amount of aeration was 720 L/d. Orthogonal design of three factors three levels, a total of 9 treatments, 3 repetitions, were presented in *Table 1*.

During the experiment period, the effluent water samples were collected every 10 day. After the experiment, soil samples were collected in each VFCW system and mixed to store.

Influent quality

The test sewage was synthetic simulated domestic sewage, the concentrations of TN and TP were 30-50 mg/L and 3-5 mg/L, respectively. COD concentrations of the 9 treatments were 100 mg/L, 150 mg/L, 66 mg/L, 200 mg/L, 50 mg/L, 100 mg/L, 300 mg/L, 100 mg/L, 33 mg/L, and correspondingly the organic load were 20 g/(m^2·d),

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60g/(m^2·d), 40 g/(m^2·d), 40 g/(m^2·d), 20 g/(m^2·d), 60 g/(m^2·d), 60 g/(m^2·d), 40 g/(m^2·d), 20 g/(m^2·d).

Table 1. *Orthogonal design of three factors at three levels*

Test number	Hydraulic load (cm/d)	Organic load (g/(m^2·d))	Aeration mode
A ($H_1O_1A_1$)	20	20	Continuous aeration
B ($H_2O_3A_1$)	40	60	Continuous aeration
C ($H_3O_2A_1$)	60	40	Continuous aeration
D ($H_1O_2A_2$)	20	40	No aeration
E ($H_2O_1A_2$)	40	20	No aeration
F ($H_3O_3A_2$)	60	60	No aeration
G ($H_1O_3A_3$)	20	60	Intermittent aeration
H ($H_2O_2A_3$)	40	40	Intermittent aeration
I ($H_3O_1A_3$)	60	20	Intermittent aeration

Orthogonal design of three factors three at levels: the three levels of hydraulic load were H1, H2 and H3 successively, namely: 20 cm/d, 40 cm/d and 60 cm/d; The three levels of organic load were O1, O2 and O3, namely: 20 g/(m^2·d), 40 g/(m^2·d), 60 g/(m^2·d); The three levels of aeration level were A1, A2 and A3 successively, that was, continuous aeration, non-aeration and intermittent aeration

Analytical methods

For COD, TN and TP methods prescribed by national standards (State Environmental Protection Bureau, 1989) were adopted. Phosphatase, urease and catalase were tested by phenyl disodium phosphate, naismith colorimetry, and potassium permanganate titration methods, respectively (Guan, 1986).

Statistical method

The software of SPSS 17.0 and Excel 2007 were used to analyse the variance and calculate the mean and standard deviation of the correlation analysis. The main factors were analyzed by visual analysis (Li and Hu, 2009).

Results and discussion

Removal efficiency of TN

The removal efficiency of TN in nine VFCW systems have rapidly declined in different degree in the early days (*Fig. 2*). The main reason for this result is that the microorganisms are in the adaptive period and the overall activity is unstable. After the 12th July, the removal effect of TN in 9 systems became stable, and the effect continued until the end of the experiment. It was also proved that the microorganisms meet the stable period in these systems. It is generally believed that the main mechanism of nitrogen removal is nitrification and denitrification by microorganisms (Gao, 2017). The removal rate of TN were G > A > B > C > D > H > E > I > F. At the end of the experiment, there was a significant difference between G ($H_1O_3A_3$) and other treatments ($p < 0.05$). It could be seen that increasing the organic load of inlet water and assisting intermittent artificial aeration are helpful for TN removal. The removal rate of TN in the

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system G was above 60%. It was higher than in previous studies on the removal rate of TN (Vymazal, 2002; Arias et al., 2005; Liu et al., 2005).

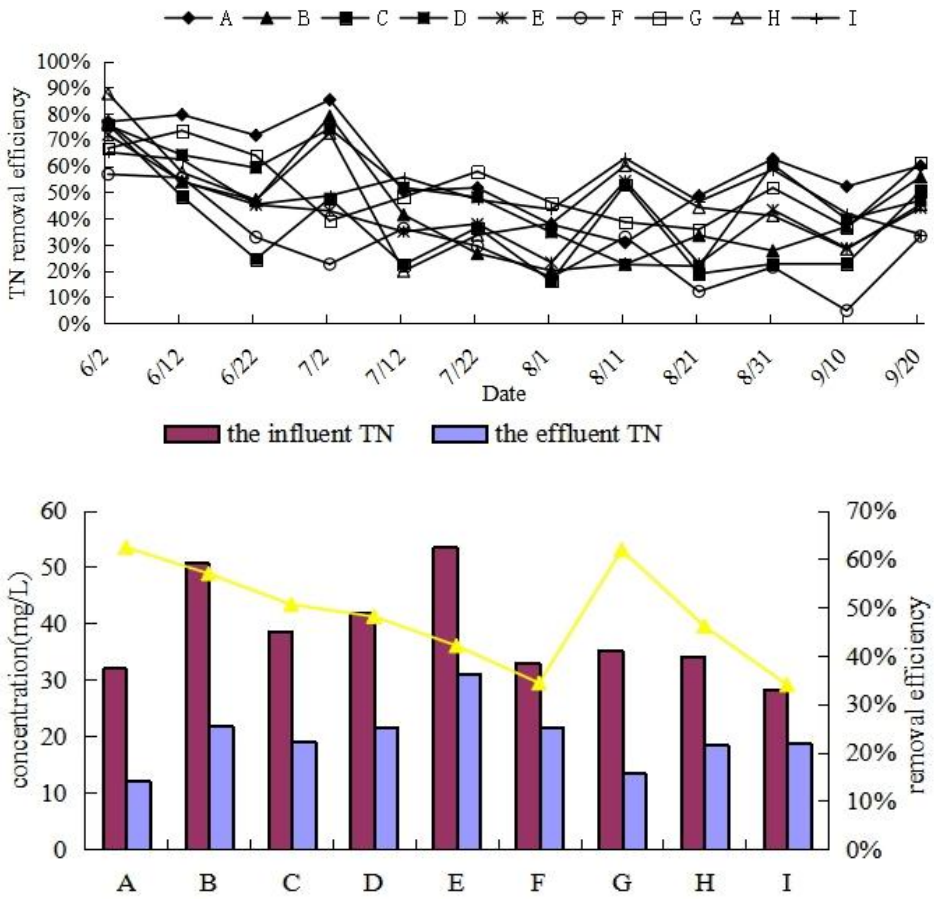

Figure 2. *Removal efficiency of TN in systems*

Removal efficiency of TP

As seen from *Figure3,* in the first week of the experiment, the removal rate of TP was high, ranging from 55% to 91%. After one week, the TP removal rate dropped sharply, and after the 2nd July, the TP removal rate was below 30% except for G. This influence continued to the end of the test, the removal rate of TP in the 9 treatments was G > A > D > H > F > E > I > B > C, and the first two systems (G and A) were significantly different from other systems ($p < 0.05$). Brix and Arias (2005) found that the average removal rate of TP in constructed wetlands was about 20%-30%. The removal efficiency of TP was relatively low in this test, it could be due to more rain during the experiment or a slightly thinner substrate layer.

Removal efficiency of COD

The COD removal efficiency of these 9 treatments were excellent (*Fig. 4*). the removal rate of COD in these systems reached up to 60%~90%, which were significantly higher than in other studies (Chazarenc et al., 2009; Tao et al., 2010; Li et al., 2011; Liu et al., 2011). The reason for this may be that the experiment was in summer, and the temperature was suitable for the growth of wetland plants and

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microorganisms, creating a favorable environment for COD removal. At the end of the experiment, D and G were as high as 90%, this is due to the higher concentrations of influent in D and G. Liao (2002) studied the effect of constructed wetlands on organic matter treatment of pig farm wastewater, and found that the removal effect of COD and BOD in wetlands was significantly improved under the operation condition that the concentration of influent water was gradually increased. Because maintaining a high organic load can provide sufficient carbon source for wetland microorganisms, intermittent aeration could moderately increase DO, which is conducive to microbial growth and pollutant removal in the system.

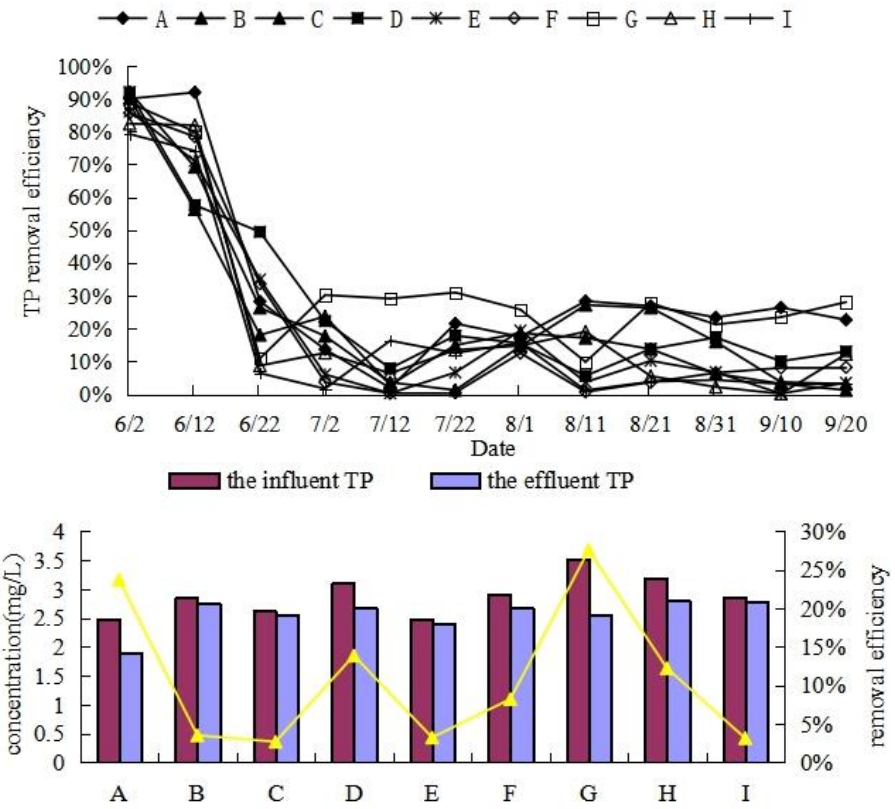

Figure 3. *Removal efficiency of TP in systems*

Analysis of influence factor range of orthogonal test

The main purpose of optimizing orthogonal experiment was to find the best values of the significant factors. Through the direct analysis of the orthogonal experiment of TN removal rate (*Table 2*), it can be seen that the influence size of the influence factor was: organic load > aeration level > hydraulic load, the optimal combination was $H_1O_3A_1$; Through the direct analysis of the orthogonal test of TP removal rate (*Table 3*), it can be seen that the influence size of the influence factor was: aeration level > organic load > hydraulic load, the optimal combination is $H_1O_3A_3$; Through the direct analysis of the COD removal rate orthogonal experiment (*Table 4*), it can be seen that the influence size of the influence factor was the organic load > aeration level > hydraulic load, and the optimal combination is $H_1O_3A_1$. Combined with the orthogonal experiment of nitrogen, phosphorus and COD removal, the comprehensive and intuitive

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DOI: http://dx.doi.org/10.15666/aeer/1806_79417951

analysis showed that under the conditions of low hydraulic load, high organic load and aeration, the pollutant removal performance was outstanding. Taking economic factors into consideration, we chose low hydraulic load of 20 cm/d, high organic load of 60 g/m^2·d, and intermittent aeration, as the optimal scheme, which was G ($H_1O_3A_3$). It was suggested that the design of low hydraulic load, proper increase of organic load and auxiliary aeration could improve the decontamination capacity in vertical-flow constructed wetlands.

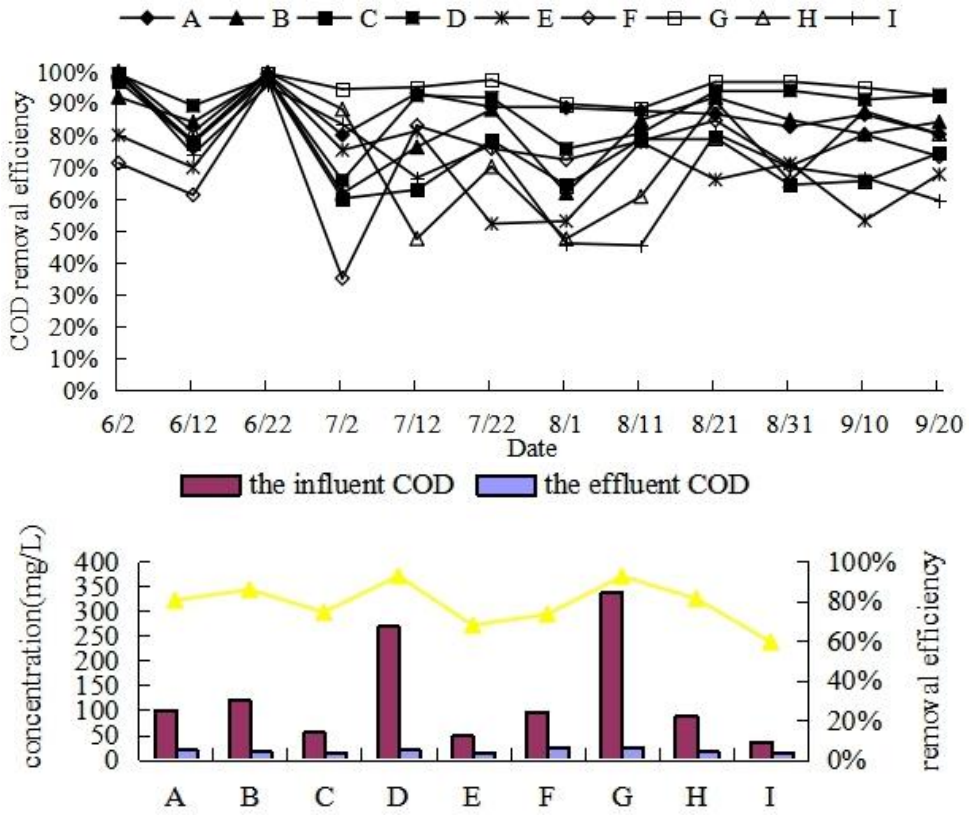

Figure 4. *Removal efficiency of COD in systems*

Table 2. *Orthogonal experiment visual analysis of TN removal rates*

	Hydraulic load	**Organic load**	**Aeration level**
K1	1.68	1.38	1.66
K2	1.45	1.43	1.24
K3	1.18	1.50	1.41
R	1.18	1.63	1.41

Table 3. *Orthogonal experiment visual analysis of TP removal rates*

	Hydraulic load	Organic load	Aeration level
K1	0.64	0.29	0.27
K2	0.19	0.26	0.25
K3	0.13	0.39	0.43
R	0.13	0.49	0.60

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Table 4. *Orthogonal experiment visual analysis of COD removal rates*

	Hydraulic load	Organic load	Aeration level
K1	2.65	2.07	2.39
K2	2.32	2.47	2.34
K3	2.07	2.50	2.32
R	2.07	2.92	2.32

K1, K2 and K3 represent the average of the different factors in the same level results, the level of the factor influence size; R value is the difference between the maximum and minimum values and it represents the influence of this factor on the index. The greater the difference, the greater the impact

The change of pH and ORP

From *Figure 5* it can be seen that at the end of the test, the pH value of inlet and outlet water of these treatments did not change much, but the ORP value changed significantly. Artificially assisted aeration helped to maintain a relatively high ORP value. Among these systems, G had the maximum ORP value at the end of the test. It indicated that intermittent aeration was more conducive to the reoxygenation in VFCW.

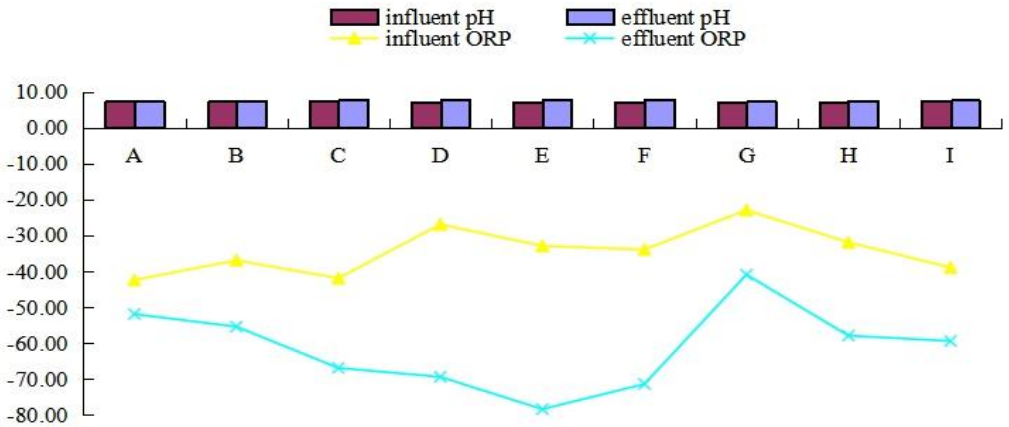

Figure 5. *Changes in pH and ORP*

Correlation analysis of substrate enzyme and sewage purification efficiency

Correlation analysis of matrix enzyme and pollutant removal is showed in *Table 5*. It was showed that there was no significant correlation between phosphatase and TP removal rate, because the removal of phosphorus mainly depended on the adsorption of matrix, while microorganisms and plants only played a certain role (Dinges, 1982; Wu et al., 2001; Yan et al., 2007). The urease and the removal rate of TN have a significant correlation ($p < 0.01$) (Wu et al., 2001; Huang et al., 2008). Catalase activity was also significantly correlated with TN removal rate ($p < 0.01$). It indicated that there may be some relationship between catalase and nitrogen degradation. In addition, catalase and COD removal rate also showed a very significant correlation ($p < 0.01$). It may be associated with the characteristics of catalase, which was the enzyme that breaks down hydrogen peroxide in living organisms and substrates, converting it into water and oxygen. It could directly result in changes of ORP of matrix, and TN removal was mainly done by nitrification and denitrification, oxygen produced for the degradation of

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COD removal provided a good environment. In the future, advantages of high molecular biology methods can help an in-depth study on substrate enzyme contact with the inner mechanism of the pollutants degradation, and establish an efficient fast measurement of wetland pollutant removal ability.

Table 5. *Correlation analysis of matrix enzyme and pollutant removal*

		TN (%)	TP (%)	COD (%)	Urease	Phosphatase	Catalase
Urease	Pearson correlation	.536**	-.202	.308	1	.071	-.025
	Significance (bilateral)	.004	.312	.350		.723	.902
Phosphatase	Pearson correlation	.154	.286	.327	.071	1	.306
	Significance (bilateral)	.443	.147	.026	.723		.121
Catalase	Pearson correlation	.791**	.223	.523**	-.025	.306	1
	Significance (bilateral)	.000	.263	.005	.902	.121	

*Significantly correlated at the level of 0.05 (bilateral). **Significantly correlated at the level of 0.01 (bilateral)

Conclusion

(1) In the VFCW, the order of influence on the removal effect of TN and COD were as follows: organic load > aeration method > hydraulic load; Based on the comprehensive analysis, it was concluded that for the VFCW system, maintaining a reasonable concentration of organic matter inflow, relatively low hydraulic load and auxiliary aeration are not only achieve the best removal effect, but also extend the life of wetland. It could provide theoretical support for the construction and management of VFCW in the future.

(2) Urease and catalase showed a very significant correlation with TN removal. Catalase may provide a good degradation environment for COD removal and it provide basic data for further study on the relationship between matrix enzyme and pollutant removal.

Acknowledgements. This study was supported by Guizhou Normal Colleges and Technology Top-Notch Talent Support Program Project (qianjiaohe KY [2016]097), Guizhou Provincial Science and Technology Plan Project (qiankeheLH [2016]7283), Doctoral Fund Project (asubsjj201607). The authors would like to thank for their financial support.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79417951

QUANTITATIVE ANALYSIS OF ETHNOBOTANY AND COMMON REMEDIES ASSOCIATED WITH THE THREATENED FLORA OF GUJRANWALA REGION, PUNJAB, PAKISTAN

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(Received 12th Apr 2020; accepted 29th Jul 2020)

Abstract. Current studies revealed the ethnobotanical importance of the threatened flora of Gujranwala region, Punjab, Pakistan including Gujranwala, Kamoki and Wazirabad Townships. This region is facing rapid expansion of industrialization and urbanization, therefore it is prime time to document and conserve it before it is lost. 100 different species belonging to 52 families were recorded through questionnaire and interviews. Steps for preservation, classification, quantitative indices and phytochemical composition were performed. Asteraceae was the dominant family with ten species. 55% of these represent herbs, 27% shrubs, 15% trees, 2% grasses and 1% weeds whereas 85% plant species were wild and 15% were cultivated. Leaves are the most frequently used parts 77%, followed by stem 12%, roots 12%, flowers 20%, rhizome 17%, seed oil 18%, and so on. As common remedies they are used as diuretics 26%, against fever 25%, laxatives 23%, emollients 22%, against constipation 20%, blood purifiers 20%, and against cough and cold 17% etc. RFC was recorded from 0.001 to 0.78. Informant consensus factor (FCI) ranged from 10-40, with the lowest value belonging to *Cucumus melo* which is used for treating eczema, dysuria, leucorrhea and as laxative whereas the highest 37 for *Indigofera heterantha* and *Quercus incana* reported to be used for hemorrhagic septicemia and joint pain. Concrete efforts are required to conserve traditional flora and to provide awareness for possible benefits of threatened species.
Keywords: *ethnobotanical knowledge, documentation, urbanization, economic benefits, Gujranwala, Pakistan*

Introduction

Ethnobotany and drugs derived from plants

The importance of plant based drugs is increasing day by day and their use spreads out of rural areas (Brandão et al., 2006; Shanley and Luz, 2003). These drugs are cheap, have minimum side effects and are usually more effective. Samie et al. (2005) reported that plant essential oils are used for treating sexually transmitted diseases, diarrhea, and dysentery like leaves of *Acacia senegalensis*. Both primary and secondary metabolites are major sources. Secondary metabolites are mostly used as phytomedicines that can be extracted from different plant parts including roots, stem, leaves, flowers, seeds and fruits etc. These plant based drugs include aspirin from willow bark, digoxin from foxglove, quinine from cinchona bark, and morphine from the opium poppy. Plants are natural source of remedies including cough, sneezing, head ache and even are successfully used against cancer. Plants are diverse in nature effective against more than one disease at a time (Khan et al., 2012). Pakistan has more than six to seven thousand species of plants that are wild in nature and about six hundred are known to have medicinally uses. Traditional knowledge is being transfer through Hakims who are Tabib (Traditional Medicine Practioner). Most of traditional knowledge is transferred through verbal

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DOI: http://dx.doi.org/10.15666/aeer/1806_79537979

communication and no comprehensive documentation is available to plants of particular area or this region and moreover comparison of topology, genetic variation for specific efficacy (Amiri and Joharchi, 2016). Ethnobotanical studies although have been carried out in different parts of Pakistan yet they are scarce and not properly documented.

It is known that plants of any type and species are suitable for medicines due to diverse biochemical composition. For example, an important genus of the family Moringaceae is Moringa and the species i.e. *Moringa oleifera* is an important medicinal plant that is native to the tropical areas and grows in all type of soils (Fahey, 2005). *Moringa* tree is regarded as one of the world's most reliable tree for medicines, as the whole plant can be utilized for food purposes or has some other advantageous characteristics (Gupta et al., 2010). Ethnobotanical account of 40 species relating to 26 families from Township Dargai, District Malakand Pakistan has been documented by Zaman et al. (2013). Likewise Wazir et al. (2007) recorded 20 medicinal salt tolerant plants found in the neighboring areas of District Karak. Akhtar and Begum (2009) reported that 55 species of plants relating to 38 families of plants were utilized for about 42 diseases in Jalala area District Mardan. Ahmed et al. (2013) identified 100 ethno medicinal plant species from Madyan valley in District Swat, Pakistan. Most of them were used as tonic, stimulant, narcotics, laxative and diuretic. Important knowledge about medicinal plants and their utilization from Dera Ghazi Khan, Punjab, Pakistan were documented by Gulshan et al. (2012). Zereen and Sardar (2013) enlisted the ethno botanical data of natives on wild trees in 08 Districts of Central Punjab that were Narowal, Sialkot, Sahiwal, Nankana Sahib, Faisalabad, Lahore, Pakpattan and Vehari. There were about 48 species of plants belonging to 23 families were gathered, including their utilization by people of particular districts for various activities i.e. fruits, vegetables, timber, medicine, fuel, fodder, etc. About 161 plant species from 57 different families involving 22 trees, 104 herbs, 23 shrubs, 3 parasitic and 9 grasses species from Township Takht-e-Nasrati, District Karak, and Pakistan were examined by Khan and Hussain (2013). The people of this region utilized 118 species (73.3%) as traditional remedial plants, 114 species as animal food (70.8%), 47 species as fuel (26.7%), 16 as timber (9.94%), 23 as vegetables (14.3%), 50 as veterinary medicines (31.06%) and 90 species are recognized as honey bee attractive species (55.9%).

Uses of plant parts and selection of the area

Seventy-one species of medicinal plants belonging to 38 families have been reported that were collected through different people. Mostly the favored part of plants used as traditional medicine were leaves (38%) followed by the seed (13%), whole plant (11%), flower (9%), fruit (8%), root and bark (6%). These were obtained through wild herbs (54%) followed by the wild shrubs and wild trees (13%), cultivated herbs (10%), cultivated trees (5%), cultivated shrubs (3%) and wild grasses (2%). The medicines or the herbal products are usually recommended by oral means to the patients (Mahmood et al., 2013).

Current studies focused on Gujranwala region that constitutes three Townships viz., Gujranwala, Kamoki and Wazirabad. Gujranwala is known as one of the most important industrial cities of Pakistan. Due to various anthropogenic activities especially urbanization, industrial waste and different types of pollution plants are losing their habitats and are at the verge of endangerment. So it is the need of the hour to cut short our all sources that cause pollution or to collect and preserve the plants that are being destroyed. For that purpose, ethnobotanical studies are the best approach for obtaining data, sample collection and preservation. This will ensure availability of enlisted species

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and documentation related to their significance, traditional uses, phytochemical composition and status of flora of different type of species/habitat within the District by both urban and rural people. This study aims to systematically document local flora of selected sites, traditional uses and their quantitative significance for illustrating importance of data collected for future application. This report would also be important to devise future strategy to plan survey, site selection, collection of sampling, interaction with local community and conservation efforts to ensure proper preservation for not only coming generations but also to earn revenue from herbal and pharmaceutical sector, along with comprehensive mode of action to resolve issues raised through anthropogenic activities.

Materials and methods

Geological distribution and sites for survey

Gujranwala region comprises of three Townships viz., Gujranwala, Kamonki and Wazirabad, which were selected for documenting traditional knowledge (*Fig. 1*). Gujranwala is an industrial city situated in the largest province of Punjab. Gujranwala is 226 m above sea level spreading over an area of 3198 km^2 with a population of 1,960,136. Gujranwala is Pakistan's 7th largest populous and metropolitan area and the fifth largest city. The months May, June and July are the hottest months with the temperature ranging from 36-42 °C (97-108 °F) while it may drop below zero sometimes in winter. Therefore, climate of the district is usually hot and semiarid. Soil is mainly fertile due to its plain and simple topology that supports plant biodiversity. Across Gujranwala, mostly people utilize plants as medicine for therapeutic purposes residing both in rural and urban areas. Plants are generally used for food, paper, clothing, dying, timber, fodder for livestock, medicines, cosmetics, and other domestic purposes. Many species are linked to cultural heritages. Due to little information Gujranwala region is selected to document local flora with ethnobotanical significance. Native people including male, female, farmers and herbalists were contacted for interview and questionnaire completion.

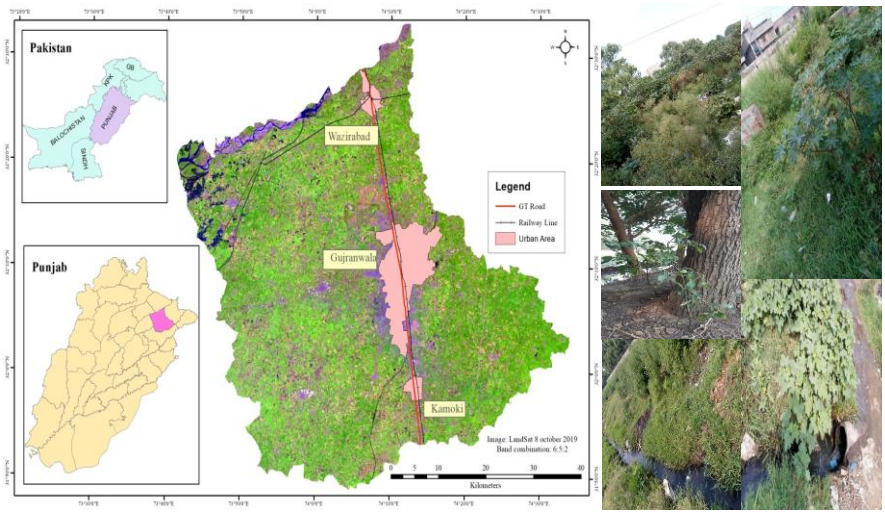

Figure 1. *Map of Gujranwala region showing selected sites and habitats for survey (Gujranwala, Wazirabad, and Kamonki townships)*

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The following materials were used in the survey: pencil, notebook, gloves, polythene bags and knife. The study of the selected sites were carried out from winter (November) 2018 to summer (July) 2019. Information regarding ethnobotanical uses were collected and noted in the form of questionnaires by the informants of the areas of different ages. For ethnobotanical knowledge herbalists, native people of old ages and hakims were also consulted. The method of classification was based upon the botanical rules and regulations.

Collection, preservation and quantitative indices

Collection was made during November 2018 to July 2019 through regular visits and field trips by selecting sites one by one. Plants were then dried under shade and converted to powder by grinding. Plants after their collection were washed by water, thoroughly cleaned and leaves were stripped by hands for better results. Questionnaire method was employed to note data for informants of the areas of all ages. For proper identification herbarium collections were consulted, many were matched with online databases and Flora of Pakistan. Herbarium specimen were prepared and deposited in Herbarium, Department of Botany, University of Gujrat, Gujrat, Pakistan. Herbarium specimen preparation was achieved by the following steps: (a) Voucher Number, (b) Common Names, (c) Botanical Names, (d) Habitats/Locality, (e) Family Names, and (f) The region etc. Quantitative indices were performed by employing various formulae as presented below.

Informant consensus factor (FCI/ICF)

FCI values were calculated as the following formula (Heinrich et al., 2009).

$$FCI = N_{ur} - N_t / N_{ur} - 1$$

where, N_{ur} is the total number of use reports for each disease category. N_t is the number of species used in the said category.

Relative frequency of citation (RFC)

The local importance of the species was calculated with the help of the relative frequency of citation. To find the RFC, number of responder's had provided useful information about species (FC) was divided by the total number of responder's in the field survey (N) as calculated previously (Tardío et al., 2008).

$$RFC = FC / N$$

Relative importance level (RIL)

To find the RIL, number of responder's had provided useful information about species (FC) was divided by the total number of responders of all species (FC_t) as described by (Friedman, 1986).

$$RIL = FC / FC_{tn}$$

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Use value (UV)

Use value can be calculated by:

$$UV = \Sigma U_i / n_i$$

ΣU_i is the use value of the species. n_i is the number of responders.

Fidelity level (FL)

It is the ratio of the respondents showing the uses of a specific plant to treat a particular disease. FL is calculated by following Alexiades (1996).

$$FC\ (\%) = FC_P / FC \times 100$$

where FC_p is the frequency of citation of particular disease. FC is the total frequency of citation for a particular disease.

Corrected fidelity level (CFL)

It is used for a correction factor to rank the plant with different RIL and FL values. It can be calculated by Ali-Shtayeh (2000).

$$CFL = FL \times RIL$$

Use reports (UR)

It is the total number of uses reported by the user.

At room temperature, the plants were dried and grinded in to powder form. Then 200 g of each powdered plant material was soaked in 200 ml ethanol for 24 h. Then this was filtered using a filter paper i.e. Whatmann's filter paper No.42. This plant extract and the filtrate was then placed in vacuum at about 30 °C and stored at 4 °C. The following procedures were applied for preliminary screening of phytochemicals:

Tests for alkaloids

0.2 g powdered sample of each plant was mixed with 10 ml of 1% HCl. Then this sample was transferred to water bath for a few minutes and 1 ml of this extract was treated with 2-4 drops of Dragendroff's reagent. The orange reddish precipitates confirmed the presence of alkaloids (Aiyegoro and Okoh, 2010).

Tests for flavonoids

For flavonoids presence two solutions were made. Solution A was prepared by the help of 5 ml ethanol extract that was prepared earlier. Solution B contained 5 ml of ethanol solvent and 5 ml of KOH. Both these solutions were mixed together and the presence of yellow color indicated the flavonoids (Jaffer et al., 1983).

Tests for tannins

10 ml of the solution in extract form was taken and about 2 drops of ferric chloride solution was put into the extract. The blue colored solution detected the presence of tannins (Aiyegoro and Okoh, 2010).

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Tests for terpenoids

1 ml of acid anhydride plus concentrated sulphuric acid was treated with 1 ml of the extract. Reddish Brown color appeared which confirmed the presence of terpenoids (Aiyegoro and Okoh, 2010).

Tests for saponins

The extract was added in 2 ml distilled water shaken well. Foam was the indication that the sample contains saponins in it (Shihata, 1951).

Tests for phenols

5 ml of the extracts of the plants were taken in a test tube and 1 ml of 1% solution of ferric chloride 1% solution of potassium ferrocyanide was added. Reddish blue color indicated the presence of phenols in it (Farhan et al., 2012).

Tests for resins

20 ml of HCL was added in the 10 ml extract of each plant. Turbidity indicated the presence of resins in the sample (Alsaidy, 2013).

Tests for steroids

2 ml of acetic anhydride plus 2 ml of sulphuric acid was added in the 0.5 g aqueous extracts of the plants. The change in the color from violet to blue or green indicated the presence of steroids (Sofowara, 1993).

Results

Ethnobotanical elaboration and therapeutic uses

Questionnaire was filled by different age group of people with varying types of qualification, educational background, gender, informant category (*Table 1*). Data were collected from natives of the selected sites i.e. Gujranwala, Kamonki and Wazairabad Townships through surveys. Total natives that provide useful information in this survey were 300, out of which 185 were females (61.67%) and 115 were males (38.33%). Traditional health practitioners included 45 persons and 245 were indigenous people in which people below 20 years of age were 30, people between 21 and 30 years old were 27, people in the category of 31 to 40 years were 73, people of age between 41 and 50 were 70, people of age between 51 and 60 were 66 and above 61 were 34. Regarding their educational background 165 illiterate persons were included, people of matriculation level were 70, 30 people were of intermediate level, 20 people were of bachelors level and 15 people were of masters level. Therefore, educational background also reflects understanding towards importance and application of natural resources among different age groups.

Collected plant species can be divided into different life forms/types viz., herbs (55%), shrubs (27%), trees (15%), grasses (2%) and weeds (1%). Wild and cultivated plants are also found in the study area which is 85% and 15% respectively (*Fig. 2*). Different plant parts such as roots, shoots, stem, leaves and flowers were used for various preparations. Leaves were the most frequently used parts (77%) followed by

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stem (12%), roots (12%), flower (20%), rhizome (17%), seed oil (18%), shoots (27%), gel (8%) and whole plant (24%) (*Fig. 3*). These parts were found to be used in the form of different preparations such as extract (67%), juice (40%), powder (58%), poultices (30%), decoction (57%), ash (25%), mixture with sugar (3%), infusion (48%) (*Fig. 4*).

Family Asteraceae contributes the highest number of medicinal plant species that included 10 species, followed by Malvaceae with 6, Solanaceae 5, Mimosaceae, Cucurbitaceae with 4, Poaceae, Euphorbiaceae and Amaranthaceae with 3 each, Fabaceae, Rosaceae, Tiliaceae, and Portulaceae with 2 each and Asparagaceae, Brassicaceae, Rutaceae have 1 species each and so on. The species found were whether monocot or dicot at particular location are shown in *Table 2*.

Table 1. *Demographic data of the respondents from the study area*

S. No.	Variable	Categories	Number of persons	% age
1.	Gender	Male	185	61.67
		Female	115	38.33
2.	Informant category	Traditional health practitioners	45	15
		Indigenous people	255	85
3.	Age	≤ 20 years	30	10
		21 - 30 years	27	9
		31 - 40 years	73	24.33
		41 - 50 years	70	23.33
		51 - 60 years	66	22
		≥ 61 years	34	11.33
4.	Educational background	Illiterate	165	55
		Matric	70	23.33
		Intermediate	30	10
		Bachelors	20	6.66
		Masters	15	5

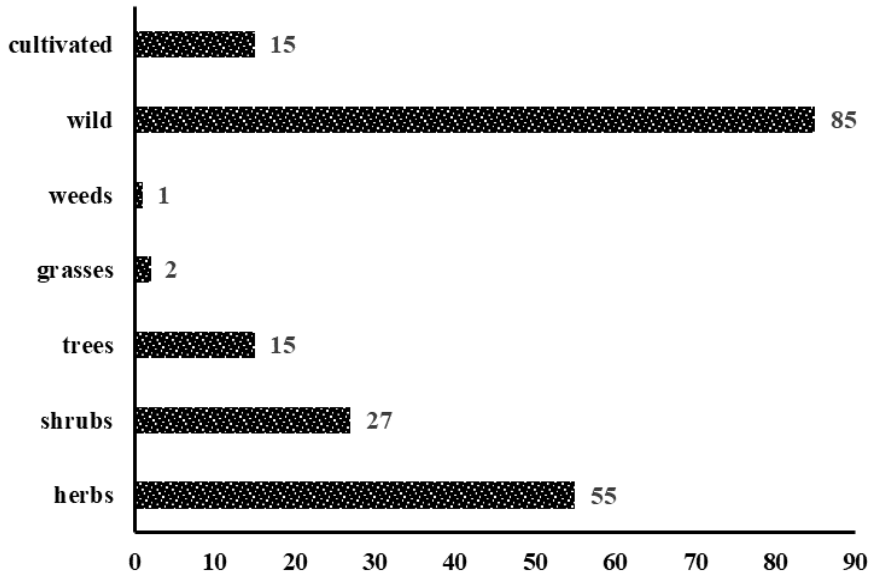

Figure 2. *Different plant types studied in 3 selected sites of Gujranwala region*

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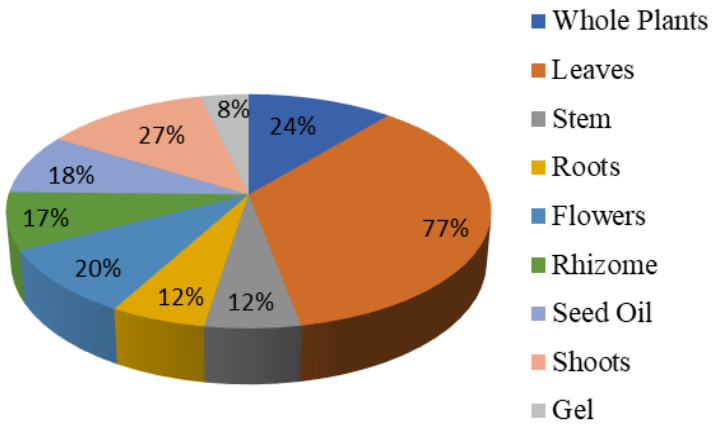

Figure 3. *Plant parts used for different therapeutic purposes studied in 3 selected sites of Gujranwala region*

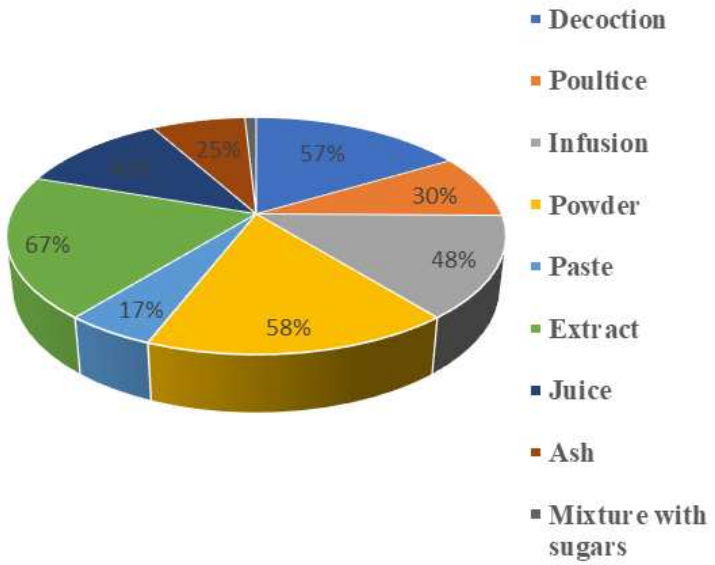

Figure 4. *Graphical presentation of plant preparations used for different therapeutic purposes from 3 selected sites of Gujranwala region*

Table 2. *Families count present in the selected sites of Gujranwala*

S.No.	Family	Genus	Species	Dicot/Monocot	Wazirabad	Gujranwala	Kamonki
1.	Astereace	*Xanthium*	*strumarium*	Eudicot	+	+	+
2.		*Parthenium*	*hysterophorus*	Eudicot	+	+	+
3.		*Artemisia*	*scoparia*	Eudicot	+	-	+
4.		*Eclipta*	*prostrate*	Dicot	+	-	+
5.		*Carthamus*	*oxyacantha*	Dicot	+	+	-
6.		*Taraxacum*	*officinale*	Dicot	+	+	-
7.		*Calendula*	*arvensis*	Dicot	-	+	+
8.		*Conyza*	*aegyptiaca*	Dicot	+	+	+
9.		*Bombax*	*ceiba*	Dicot	+	+	+
10.		*Cichorium*	*intybus*	Dicot	+	-	+

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11.	Malvaceae	*Hibiscus*	rosasinensis	Dicot	+	+	+
12.		*Abutilon*	*indicum*	Dicot	+	+	-
13.		*Marva*	*parviflora*	Dicot	+	+	+
14.		*Melia*	*azedarach*	Dicot	+	+	+
15.		*Althea*	*rosea*	Dicot	+	+	+
16.		*Malvastrum*	*coromandelianum*	Dicot	-	+	+
17.	Solanaceae	*Withania*	*somnifera*	Dicot	+	-	+
18.		*Solanum*	*surattense*	Dicot	+	+	-
19.		*Solanum*	*nigrum*	Dicot	+	+	+
20.		*Capsicum*	*frutescens*	Dicot	+	+	+
21.		*Datura*	*innoxia*	Dicot	+	+	+
22.	Papilionaceae	*Indigofera*	*linifolia*	Dicot	-	-	+
23.		*Dalbergia*	*sissoo*	Dicot	+	+	+
24.		*Pongamia*	*pinnata*	Dicot	+	-	+
25.		*Crotolaria*	*burhia*	Dicot	+	-	+
26.		*Alhagi*	*maurorum*	Monocot	+	-	-
27.	Cucurbitceae	*Citrullus*	*colocynthis*	Dicot	+	+	+
28.		*Cucurbita*	*Pepo*	Dicot	+	+	+
29.		*Cucumus*	*Melo*	Dicot	+	+	+
30.		*Benincasa*	*hispida*	Dicot	-	+	+
31.	Mimosaceae	*Albizia*	*Lebbek*	Dicot	+	+	+
32.		*Cassia*	*fistula*	Dicot	+	+	+
33.		*Acacia*	*modesta*	Dicot	+	+	+
34.		*Acacia*	*nilotica*	Dicot	+	+	+
35.	Poaceae	*Cynodon*	*dactylon*	Monocot	+	+	+
36.		*Avena*	*sativa*	Monocot	+	-	+
37.		*Bumbusa*	*arundinacea*	Monocot	-	+	+
38.	Euphorbiaceae	*Euphorbia*	*hirta*	Dicot	+	+	+
39.		*Euphorbia*	*heliscopia*	Dicot	+	+	+
40.		*Ricinus*	*communis*	Dicot	+	+	-
41.	Amaranthaceae	*Amaranthus*	*viridis*	Dicot	+	+	+
42.		*Achyranthes*	*aspera*	Dicot	+	+	+
43.		*Scandix*	*pectenveneris*	Dicot	-	-	+
44.	Fabaceae	*Melilotus*	*indica*	Dicot	-	+	+
45.		*Indigofera*	*heterantha*	Dicot	+	-	+
46.	Rosaceae	*Duchesnea*	*indica*	Dicot	+	+	-
47.		*Rosa*	*indica*	Dicot	+	+	+
48.	Tiliaceae	*Grewia*	*asiatica*	Monocot	-	+	+
49.		*Corchorus*	*aestuans*	Dicot	+	+	+
50.	Lamiaceae	*Micromeria*	*biflora*	Dicot	-	+	-
51.		*Mentha*	spicata	Dicot	+	+	+
52.	Papaveraceae	*Fumaria*	*indica*	Dicot	+	+	+
53.		*Argemone*	*mexicana*	Dicot	+	-	+
54.	Apocynaceae	*Vinca*	*major*	Dicot	+	+	+
55.		*Nerium*	*oleander*	Dicot	+	+	+
56.		*Rhazya*	*stricta*	Monocot	+	-	+
57.	Amaryllidaceae	*Allium*	*cepa*	Monocot	+	+	+
58.		*Allium*	*sativum*	Monocot	+	+	+
59.	Asclepiadaceae	*Calotropis*	*procera*	Eudicot	+	-	+
60.		*Caralluma*	*edulis*	Eudicot	+	+	-
61.	Moraceae	*Ficus*	*benghalensis*	Dicot	+	+	+
62.		*Morus.*	*nigra*	Dicot	+	+	+

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DOI: http://dx.doi.org/10.15666/aeer/1806_79537979

63.	Myrtaceae	*Syzygium*	*cumini*	Dicot	+	-	+
64.		*Eucalyptus*	*globulus*	Dicot		+	+
65.	Portulacaceae	*Portulaca*	*oleracea*	Dicot	+	+	-
66.		*Portulaca*	*quadrifida*	Dicot	+	+	-
67.	Boraginaceae	*Trichodesma*	*indicum*	Dicot	-	+	+
68.		*Heliotropium*	*strigosum*	Dicot	+	+	+
69.	Cuscutaceae	*Cuscuta*	*reflexa*	Dicot	+	+	+
70.	Cannabinaceae	*Cannabis*	*sativa*	Dicot	+	+	+
71.	Crassulaceae	*Bryophyllum*	*pinnatum*	Dicot	+	+	-
72.	Convolvulaceae	*Convolvulus*	*album*	Dicot	+	+	+
73.	Chenopodiaceae	*Chenopodium*	*album*	Dicot	+	+	+
74.	Cyperaceae	*Cyperus*	*rotundus*	Monocot	-	+	+
75.	Punicaceae	*Punica*	*granatum*	Dicot	+	+	+
76.	Nyctaginaceae	*Mirabilis*	*jalapa*	Dicot	+	-	-
77.	Rhamnaceae	*Zizyphus*	*nummularia*	Dicot	+	-	+
78.	Oxalidaceae	*Oxalis*	*corniculata*	Dicot	-	+	+
79.	Apiaceae	*Foeniculum*	*vulgare*	Dicot	+	+	+
80.	Aizoaceae	*Trianthema*	*portulacastrum*	Dicot	-	-	+
81.	Acanthaceae	*Justicia*	*adhatoda*	Dicot	+	+	-
82.	Salvadoraceae	*Salvadora*	*oleoides*	Dicot	+	+	+
83.	Tamaricaceae	*Tamarix*	*dioica*	Dicot	+	-	+
84.	Zygophyllaceae	*Tribulus*	*camaldulensis*	Dicot	+	+	-
85.	Arecaceae	*Phoenix*	*dactylifera*	Monocot	+	-	+
86.	Meliaceae	*Azadirachta*	*indica*	Monocot	+	+	+
87.	Asphodelaceae	*Aloe*	*vera*	Monocot	+	+	+
88.	Pontederiaceae	*Eichhornia*	*crassipes*	Monocot	-	+	+
89.	Asparagaceae	*Asparagus*	*racemosus*	Monocot	+	+	+
90.	Polygonaceae	*Polygonum*	*plebium*	Dicot	+	-	+
91.	Urticaceae	*Debregeasia*	*salicifolia*	Dicot	+	+	-
92	Brassicaceae	*Lepidium*	*didymium*	Dicot	+	+	+
93	Sapindaceae	*Dodonaea*	*viscosa*	Dicot	-	+	+
94.	Hypericaceae	*Hypericum*	*oblongifolium*	Dicot	+	-	+
95.	Oleaceae	*Jasminum*	*officinale*	Dicot	+	+	-
96.	Rananculaceae	*Ranunculus*	*muricatus*	Dicot	-	-	+
97.	Caprifoliaceae	*Lonicera*	*quinquelocularis*	Dicot	+	+	-
98.	Fagaceae	*Quercus*	*incana*	Dicot	+	-	+
99.	Berberidaceae	*Berberis*	*lyceum*	Dicot	+	+	-
100.	Rutaceae	*Zanthoxylum*	*armatum*	Dicot	-	+	+

Medicinal values of 100 species of plants belonging to different families were recorded, that was used for different purposes such as stimulant, anthelmintic, cardiovascular diseases, febrifuge, diuretic, vermifuge, sedative and even antidote. Many diseases were treated such as hemorrhagic septicemia, jaundice, wound healing, severe burns and skin treatments of various types. Important medicinal uses of the plants include cold, flu, fever, dysentery, male diseases like gonorrhea, sores on genitals and female diseases such as amenorrhea, leucorrhea, and diarrhea, tooth aches, diabetes etc. Plants associated with different diseases for their treatment found in the study area were classified as diuretic (26%), febrifuge (25%), laxative (23%), emollient (22%), constipation (20%), blood purifier (20%), tonics (19%), diarrhea curing (19%),

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carminatives (18%), cough and cold curing medicines (17%), dysentery treating (15%), jaundice pain relievers (15%), and anti-diabetics (14%) (*Table 3; Fig. 5*).

Twenty different plants were shortlisted for phytochemical analysis based on usefulness against common diseases through questionnaire method. Phytochemicals especially secondary metabolites are of supreme importance against number of ailments. The detection for presence/absence of these compounds is provided as a graphical presentation in *Figure 6*.

Table 3. *Scientific names, parts used, preparation used as therapeutic uses and their habit/habitat studied in the selected sites of Gujranwala*

S. No.	Scientific name	English names	Parts used	Preparation	Habit/habitat
1.	*Convolvulus album*	Field bindweed	Whole Plant, Mostly Leaves	Powder or Juice	Shrub, Wild/Cultivated
2.	*Bryophyllum pinnatum*	Air plant/Cathedral bells	Leaves	Extract, Paste	Herb, Cultivated
3.	*Cannabis sativa*	Cannabis	Shoot, Leaves, Seeds	Powder, Juice, Decoction	Herb, Wild
4.	*Chenopodium album*	Wild spinach/manure weed	Whole Plant	Powder, Cooked, Extract	Herb, Wild
5.	*Heliotropium strigosum*	Bristly Heliotrope	Whole Plant, Leaves	Powder, Extract	Herb, Wild
6.	*Xanthium strumarium*	Rough cocklebur	Leaves, Seeds, Fruit	Paste, Extract	Herb, Wild
7.	*Trichodesma indicum*	Indian borage	Leaves	Paste, Extract, Decoction	Herb, Wild
8.	*Citrullus colocynthis*	Bitter cucumber/wild gourd	Seed, Fruit, Seed oil	Paste, Extract, Oil	Herb, Wild
9.	*Benincasa hispida*	Wax gourd/ash gourd	Fruit, Seed oil	Oil, Juice	Herb, Cultivated
10.	*Cucumus melo*	Muskmelon	Fruit, Leaves	Decoction, Paste	Herb, Wild
11.	*Cucurbita pepo*	Winter squash/pumpkin	Fruit, Leaves, Seeds	Paste	Herb, Wild/Cultivated
12.	*Cuscuta reflexa*	Giant dodder	Whole Plant	Powder, Extract	Herb, Wild/Cultivated
13.	*Euphorbia hirta*	Asthma plant	Leaves, Milky Latex	Extract	Herb, Wild
14.	*Euphorbia heliscopia*	Sun spurge	Seeds, Milky latex	Roasted, Extract	Herb, Wild
15.	*Ricinus communis*	Castor bean	Seeds, Stem, Leaves	Powder, Oil	Shrub, Wild
16.	*Cyperus rotundus*	Nut sedge	Whole Plant, Leaves, Rhizome	Decoction, Paste	Herb, Wild
17.	*Indigofera linifolia*	Indigo	Whole Plant, Leaves	Decoction, Extract	Herb, Wild
18.	*Dalbergia sissoo*	North Indian rosewood	Bark, Leaves	Decoction, Juice, Infusion, Powder	Tree, Wild
19.	*Pongamia pinnata*	Indian beech/Pongam oiltree	Young Branches, Leaves, Seeds, Bark	Decoction, oil, Powder	Tree, Wild
20.	*Crotalaria burhia*	Ethiopian rattlebox	Flowers, Leaves, Whole Plant	Extract, Juice	Herb, Wild
21.	*Alhagi maurorum*	Camelthorn bush	Flowers, Leaves	Decoction, Powder	Herb, Wild
22.	*Argemone Mexicana*	Mexican poppy	Flowers, Milky Latex	Powder, Extract	Herb, Wild
23.	*Cynodon dactylon*	Bermuda grass/Dhoob	Roots, Flowers, Leaves	Powder, Juice	Grass, Wild
24.	*Avena sativa*	Common oat	Leaves, Whole Plant	Powder, Infusion	Grass, Wild
25.	*Bumbusa arundinacea*	Bamboo	Leaves, Young Shoots, Roots	Powder, Infusion, Ash	Shrub, Wild
26.	*Rosa indica*	Rose	Leaves, Flower, Roots	Powder, Extract, Mixture with sugar	Shrub, Wild/Cultivated
27.	*Zizyphus nummularia*	Wild jujube	Leaves, Fruit	Decoction, Paste	Shrub, Wild
28.	*Punica granatum*	Pomegranate	Bark, Fruit	Powder, Ash	Tree, Cultivated

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29.	*Portulaca oleracea*	Common purslane	Whole plant, Leaves	Powder, Infusion	Herb, Wild
30.	*Portulaca quadrifida*	Duckweed/little hogweed	Seeds, Leaves	Powder, Infusion, Paste	Herb, Wild
31.	*Eichhornia crassipes*	Common water hyacinth	Leaves	Infusion, Paste	Herb, Wild
32.	*Polygonum plebium*	Common knotweed	Leaves, Whole Plant	Extract, Oil, Decoction, Ash	Herb, Wild
33.	*Hibiscus rosasinensis*	Shoe flower/Chinese hibiscus	Fruit	Extract, Juice	Shrub, Wild/Cultivated
34.	*Abutilon indicum*	Mallow	Seeds, Leaves	Decoction	Herb, Wild
35.	*Marva parviflora*	Cheeseweed/marshmallow	Leaves	Decoction, Extract	Herb, Wild
36.	*Melia azedarach*	Chinaberry tree	Leaves	Paste, Infusion	Tree, Wild
37.	*Mentha spicata*	Bush mint/spearmint	Leaves, Shoot	Decoction, Paste	Herb, Wild/Cultivated
38.	*Mirabilis jalapa*	Marvel of peru/four o'clock flower	Leaves, Flower	Decoction, Paste, Juice	Herb, Wild/Cultivated
39.	*Syzygium cumini*	Black plum/Java plum	Seeds	Powder	Tree, Cultivated
40.	*Eucalyptus globulus*	Southern blue gum	Leaves	Decoction, Extract, Fumes	Tree, Cultivated
41.	*Acacia nilotica*	Gum Arabic tree	Flowers, Bark, Pod	Decoction, Powder, Juice	Tree, Wild
42.	*Albizia lebbeck*	Lebbek tree/flea tree	Flowers, Seeds, Bark	Decoction	Tree, Wild
43.	*Cassia fistula*	Indian laburnum/golden shower	Flowers, Seeds, Fruits	Powder	Tree, Wild
44.	*Acacia modesta*	Phulai	Bark, Stem, Gum	Powder	Tree, Wild
45.	*Ficus benghalensis*	Banyan	Milky Latex, Young Branches	Powder, Gum	Tree, Wild
46.	*Morus nigra*	Black mulberry	Leaves, Roots, Fruit	Juice, Infusion, Powder	Tree, Wild
47.	*Oxalis corniculata*	Creeping woodsorrel	Leaves, Whole Plant	Extract, Powder	Herb, Wild
48.	*Foeniculum vulgare*	Sweat fennel	Leaves, Seeds	Extract, Decoction	Herb, Cultivated
49.	*Trianthema portulacastrum*	Desert horsepurslane	Whole Plant, Leaves, Roots	Powder, Decoction, Extract	Herb, Wild
50.	*Justicia adhatoda*	Malabar nut/adhatoda	Flower, Leaves, Roots	Juice, Decoction	Herb, Wild
51.	*Allium sativum*	Garlic	Bulbs	Juice	Herb, Wild
52.	*Nerium oleander*	Oleander	Roots, Stem, Leaves	Powder, Extract	Shrub, Wild
53.	*Amaranthus viridis*	Slender amaranth	Leaves	Powder, Paste	Herb, Wild
54.	*Achyranthes aspera*	Chaff flower	Leaves, Roots, Seeds	Powder, Extract, Decoction	Herb, Wild
55.	*Calotropis procera*	Apple of Sodom/roostertree	Stem, Milky Latex, Branches, Leaves, Flowers	Poultices, Ash, Powder	Herb, Wild
56.	*Caralluma edulis*	Caralluma	Whole plant, Leaves	Juice, Extract, Powder	Shrub, Wild
57.	*Parthenium hysterophorus*	Santa Maria feverfew/whitetop weed	Flowers, Leaves	Extract, Powder	Herb, Wild
58.	*Artemisia scoparia*	Virgate wormwood	Flowers, Leaves, Whole Plant, Shoot	Extract, Powder, Decoction	Herb, Wild
59.	*Eclipta prostrate*	False daisy	Roots, Whole Plant	Extract, Juice, Oil	Herb, Wild
60.	*Carthamus oxyacantha*	Wild safflower	Seeds	Oil	Herb, Wild
61.	*Taraxacum officinale*	Common dandelion	Leaves, Roots	Paste, Powder, Decoction	Herb, Wild
62.	*Salvadora oleoides*	Salvadora	Branches, Fruits	Decoction	Shurb, Wild
63.	*Datura innoxia*	Pricklyburr	Leaves, Seeds	Extract	Herb, Wild
64.	*Capsicum frutescens*	Chili pepper	Fruits	Powder, Paste	Herb, Cultivated

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65.	*Solanum nigrum*	Black night shade	Fruit, Leaves, Whole Plant	Poultice, Infusion, Extract	Herb, Wild
66.	*Solanum surattense*	Surattense nightshade	Fruit, Leaves, Roots	Decoction	Herb, Wild
67.	*Withania somnifera*	Ashwagandha	Whole Plant, Roots	Decoction	Herb, Wild
68.	*Tamarix dioica*	Saltcedar	Branches, Leaves	Powder	Shurb, Wild
69.	*Grewia asiatica*	Phalsa	Branches, Fruit	Decoction, Juice	Tree, Wild/Cultivated
70.	*Corchorus aestuans*	Jute mallow	Seeds, Whole Plant	Powder	Herb, Wild
71.	*Tribulus camaldulensis*	River red gum	Seeds, Fruits, Leaves	Powder, Infusion	Herb, Wild
72.	*Scandix pectenveneris*	Shepherd's needle	Stems, Leaves, Roots	Powder, Decoction	Herb, Wild
73.	*Rhazya stricta*	Rhazya	Stems, Leaves, Fruits	Extract	Shrub, Wild
74.	*Phoenix dactylifera*	Date palm	Leaves, Fruits	Extract, Decoction	Tree, Cultivated
75.	*Calendula arvensis*	Field marigold	Leaves, Flowers	Extract	Herb, Wild
76.	*Conyza aegyptiaca*	Canadian horseweed	Leaves, Flowers	Extract	Herb, Wild
77.	*Bombax ceiba*	Cotton tree/red silk cotton	Leaves, Stem, Seeds	Extract, Decoction	Herb, Wild
78.	*Aloe vera*	Indian aloe/Chinese aloe	Leaves, Gel	Extract, Decoction, Gel	Shrub, Wild/Cultivated
79.	*Allium cepa*	Onion	Leaves, Stem, Bulbs	Extract	Herb, Cultivated
80.	*Azadirachta indica*	Neem	Leaves	Extract, Decoction	Herb, Wild/Cultivated
81.	*Althea rosea*	Holyoke	Leaves, Flowers, Stems	Extract, Decoction	Herb, Wild/Cultivated
82.	*Asparagus racemosus*	Satawari	Leaves, Flowers, Stems	Extract, Decoction	Shrub, Wild
83.	*Vinca major*	Bigleaf periwinkle	Leaves, Seeds	Extract, Decoction	Shrub, Wild
84.	*Cichorium intybus*	Chicory	Leaves, Stem, Seeds	Extract, Decoction	Weed, Wild/Cultivated
85.	*Fumaria indica*	Fumitory	Leaves, Whole Plant	Extract	Weed, Wild
86.	*Lepidium didymium*	Swine cress	Leaves, Whole Plant	Extract	Herb, Wild
87.	*Malvastrum coromandelianum*	Red malvastrum	Leaves, Flowers	Decoction	Shrub, Wild
88.	*Melilotus indica*	Sweet clover	Leaves, Whole Plant	Decoction	Herb, Wild
89.	*Micromeria biflora*	Lemon scented thyme	Whole plant	Decoction	Herb, Wild/Cultivated
90.	*Ranunculus muricatus*	Scilly buttercup	Whole plant, Fruit, Leaves	Decoction	Herb, Wild
91.	*Quercus incana*	Bluejack oak	Leaves	Extract	Tree, Cultivated
92.	*Berberis lyceum*	Barberry	Leaves, Roots, Stem	Extract, Decoction	Shrub, Wild/Cultivated
93.	*Debregeasia salicifolia*	Debregeasia	Leaves	Powder	Shrub, Wild
94.	*Dodonaea viscosa purpurea*	Broad leaf hopbush/candle wood	Leaves, Flowers, Stem	Powder, Decoction, Oil, Extract	Shrub, Wild
95.	*Hypericum oblongifolium*	Pendant St. John's wort	Leaves, Fruits	Decoction, Extract	Shrub, Wild/Cultivated
96.	*Indigofera heterantha*	Himalayan indigo	Leaves, Fruits	Powder, Extract	Shrub, Wild
97.	*Jasminum officinale*	Common jasmine	Leaves, Flowers	Decoction, Extract	Shrub, Wild/Cultivated
98	*Lonicera quinquelocularis*	Translucent honeysuckle	Leaves, Flowers	Extract	Shrub, Wild
99.	*Zanthoxylum armatum*	Winged prickly ash	Whole Plant, Leaves	Extract, Decoction, Powder	Shrub, Cultivated
100	*Duchesnea indica*	Indian strawberry	Whole plant	Extract, Decoction,	Herb, Wild

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DOI: http://dx.doi.org/10.15666/aeer/1806_79537979

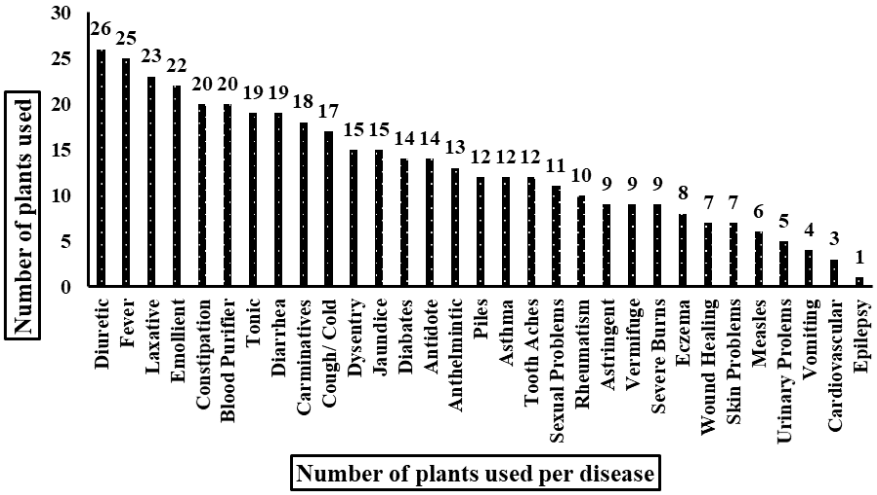

Figure 5. *Number of plants used for treatment of diseases studied from 3 selected sites of Gujranwala region*

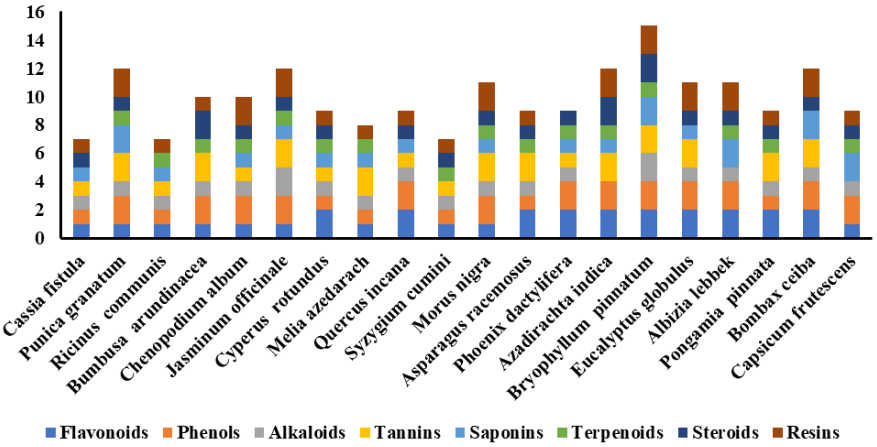

Figure 6. *Comparison of phytochemicals present in selected species studied in 3 selected sites of Gujranwala region*

Quantitative indices

Medicinal values of different plants belonging to different families are present in *Table 4,* along with their common name, scientific name, families, quantitative indices viz., UV, FC, RFC, UR, RIL, FL, CFL and therapeutic uses. Plants of different types were found in the study area that has different therapeutic uses. They were quantitatively analyzed by using different formulas. Factor of informant consensus can be particularly useful to select the categories of diseases for which the species can traditionally are used. The relative importance level (RIL) presents the level of prominence of each species in a study site. Fidelity level (FL) of species uses, i.e. the ratio between the number of informants who independently cite the use of a species for the same major purposes use reports (URs) and the total number of informants who mentioned the plant for any use. Corrected fidelity level (CFL) of plant species were used as correction factor to accurately rank the plant species with different FL and RIL values. CFL was derived from FL, by multiplying FL with RIL values.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 4. *Inventory of plant species, their quantitative analysis, therapeutic uses and reports of Gujranwala area*

S.No	Common name	Scientific name and voucher specimen	Family	UV	FCI	RFC	UR	RIL	FL	CFL	Therapeutic uses
1.	Bahar Bail	*Convolvulus album* L. UOG-001005	Convolvulaceae	0.89	27	0.11	15	0.89	50	25	Emollient, anthelminthic, urinary tract infections, increasing bile production
2.	Zakhm-e-hayat	*Bryophyllum pinnatum* L. UOG-001006	Crassulaceae	0.71	11	0.02	15	0.78	70	35	Emollient, analgesic, carminative, vomiting, diarrhea
3.	Bhung	*Cannabis sativa* L. UOG-001007	Cannabinaceae	0.76	22	0.13	18	0.81	89.2	47	Tonic, relaxing purposes
4.	Bathu	*Chenopodium album* L. UOG-001008	Chenopodiaceae	0.67	14	0.03	19	0.56	77	38	Laxative, urinary problems, sexual diseases
5.	Gorakh Paan	*Heliotropium strigosum* L. UOG-001009	Boraginaceae	0.87	12	0.01	19	0.98	34	26	Diuretic, blood purifier, liver tonic
6.	Chota Dathoora	*Xanthium strumarium* L. UOG-001010	Asteraceae	0.65	16	0.15	17	0.89	64	34	Sedative, diuretic, eczema
7.	Kulfa	*Trichodesma indicum* L. R. Br. UOG-001011	Boraginaceae	0.98	15	0.16	11	0.67	78	44	Antidote, diuretic, dysentery, fever, diarrhea
8.	Tumma	*Citrullus colocynthis* L. UOG-001012	Cucurbitaceae	0.10	22	0.12	13	0.78	45	24	Purgative, amenorrhea, constipation, stomach aches, jaundice
9.	Bhtha	*Benincasa hispida* L. UOG-001013	Cucurbitaceae	0.45	25	0.16	15	0.65	46.35	20	Laxative, diuretic, tonic, anthelminthic, epilepsy
10.	Jangli Khaboz	*Cucumus melo* L. UOG-001014	Cucurbitaceae	0.76	10	0.14	12	0.78	78	40	Laxative, dysuria, leucorrhea, eczema
11.	Kaddu	*Cucurbita pepo* L. UOG-001015	Cucurbitaceae	0.56	20	0.16	12	0.56	25	10	Anthelminthic, emollient
12.	Neeli Taar	*Cuscuta reflexa* R. UOG-001016	Cuscutaceae	0.67	27	0.17	15	0.89	28	13	Rheumatism, anti-diabetic, toothache
13.	Spodhodal	*Euphorbia hirta* L. UOG-001017	Euphorbiaceae	0.45	17	0.19	18	0.87	30	18	Asthma, applied on injuries
14.	Dhodal	*Euphorbia heliscopia* L. UOG-001018	Euphorbiaceae	0.87	27	0.023	19	0.67	40	39	Purgative, skin eruptions
15.	Arnd	*Ricinus communis* L. UOG-001019	Euphorbiaceae	0.89		0.07	21	0.87	60	35	Regulate menses, scabies, obesity
16.	Deela	*Cyperus rotundus* L. UOG-001020	Cyperaceae	0.76	19	0.30	22	0.78	40	19	Diuretic, vermifuge, tonic for animals, diarrhea, pneumonia, children dysentery, antidote

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S.No	Common name	Scientific name and voucher specimen	Family	UV	FCI	RFC	UR	RIL	FL	CFL	Therapeutic uses
17.	Turkhrai	*Indigofera linifolia* L. UOG-001021	Papilionaceae	0.89	10	0.078	10	0.89	56	30	Emollient, febrile skin problems
18.	Shishum	*Dalbergia sissoo* L. UOG-001022	Papilionaceae	0.13	29	0.54	20	0.99	64	34	Emollient, emetic, dysentery, fever, pyorrhea
19.	Sukhchain	*Pongamia pinnata* L. UOG-001023	Papilionaceae	0.24	10	0.67	22	0.78	84	67	Carminative, vermifuge, rheumatism, toothache
20.	Sann	*Crotolaria burhia* L. UOG-001024	Papilionaceae	0.65	15	0.12	52	0.87	37	20	Diuretic, blood purifier
21.	Phuwa	*Alhagi maurorum* L. UOG-001025	Papilionaceae	0.23	25	0.15	26	0.78	90	65	Piles, constipation
22.	Stianasi	*Argemone mexicana* L. UOG-001026	Papaveraceae	0.67	32	0.12	25	0.98	56	37	Laxative, effective against premature ejaculation, spermatorrhea
23.	Khbal Kha	*Cynodon dactylon* L. UOG-001027	Poaceae	0.87	27	0.16	15	0.56	73	49	Purgative, epistasis, dysentery, blood purifier
24.	Javi	*Avena sativa* L. UOG-001028	Poaceae	0.89	29	0.16	13	0.89	72	50	Antispasmodic, liver tonic, diuretic
25.	Baans	*Bumbusa arundinacea* L. UOG-001029	Poaceae	0.67	28	0.16	17	0.76	40	10	Carminative, cold, flu, fever, effective against, ringworms burns
26.	Gulab	*Rosa indica* L. UOG-001030	Rosaceae	0.77	21	0.19	16	0.56	37	14	Emollient, laxative, anthelminthic, diuretic, constipation
27.	Bair	*Zizyphus nummularia* L. UOG-001031	Rhamnaceae	0.60	27	0.20	29	0.78	48	27	Purgative, emollient, antiseptic, measles, constipation
28.	Anaar	*Punica granatum* L. UOG-001032	Punicaceae	0.83	26	0.23	17	0.87	47	19	Dysentery, pyorrhea, blood purifier
29.	Kulfa	*Portulaca oleracea* L. UOG-001033	Portulacaceae	0.34	25	0.34	19	0.450	45	13	Jaundice, liver and spleen disorders
30.	Desi Kulfa	*Portulaca quadrifida* L. UOG-001034	Portulacaceae	0.78	21	0.13	29	0.78	47	15	Vermifuge, piles, cold, flu, constipation
31.	Water Hyacinth	*Eichhornia crassipes* L. UOG-001035	Pontederiaceae	0.56	26	0.167	22	0.98	26	12	Emollient, blood purifier
32.	Droonk	*Polygonum plebium* L. UOG-001036	Polygonaceae	0.87	12	0.121	10	0.78	30	16	Eczema, heartburns
33.	Sura	*Hibiscus rosasinensis* L. UOG-001037	Malvaceae	0.56	23	0.321	15	0.98	38	26	Laxative, effective against burning sensation in the urine, constipation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79537979

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34.	Pelae	*Abutilon indicum* L. UOG-001038	Malvaceae	0.78	20	0.176	170	0.89	67	56	Purgative, piles
35.	Sunchal	*Marva parviflora* L. UOG-001039	Malvaceae	0.64	27	0.321	190	0.99	98	78	Laxative, cold, flu, cough, fever
36.	Dhreek	*Melia azedarach* L. UOG-001040	Malvaceae	0.76	26	0.165	130	0.66	36	29	Emollient, laxative, antidote, blood purifier
37.	Podeena	*Mentha spicata* L. UOG-001041	Lamiaceae	0.54	25	0.178	190	0.78	48	20	Carminative, diarrhea
38.	Gul-e-Asar	*Mirabilis jalapa* L. UOG-001042	Nyctaginaceae	0.44	24	0.154	280	0.88	40	10	Purgative, Skin eruptions, constipation, hepatitis
39.	Jamun	*Syzygium cumini* L. R. UOG-001043	Myrtaceae	0.57	19	0.12	380	0.67	20	10	Diabetes
40.	Safaida	*Eucalyptus globulus* L. R. UOG-001044	Myrtaceae	0.89	17	0.16	170	0.98	89	56	Cold, flu, fever, sore throat
41.	Kikar	*Acacia nilotica* L. UOG-001045	Mimosaceae	0.98	14	0.156	270	0.78	54	22	Stops premature ejaculation, helps in digestion
42.	Shirin	*Albizia lebbek* L. UOG-001046	Mimosaceae	0.97	21	0.12	28	0.98	39	18	Diuretic, asthma, blood purifier
43.	Amaltas	*Cassia fistula* L. UOG-001047	Mimosaceae	0.79	24	0.21	26	0.67	56	27	Diarrhea
44.	Phulae	*Acacia modesta* L. UOG-001048	Mimosaceae	0.94	26	0.16	37	0.66	54	26	Carminative
45.	Bohrh	*Ficus benghalensis* L. UOG-001049	Moraceae	0.56	20	0.16	34	0.67	38	21	Stop premature ejaculation, syphilis, gonorrhea, provides sexual strength
46.	Kala Toot	*Morus nigra* L. UOG-001050	Moraceae	0.87	30	0.21	30	0.87	30	16	Carminative, blood purifier, sore throat, cold, cough
47.	Khktl	*Oxalis corniculata* L. UOG-001051	Oxalidaceae	0.37	20	0.71	29	0.67	40	20	Emollient, spermatorrhea
48.	Saunf	*Foeniculum vulgare* L. UOG-001052	Apiaceae	0.44	18	0.13	37	0.87	89	65	Carminative, purgative, diuretic, laxative, gas trouble, enhances eye sight
49.	Itst	*Trianthema portulacastrum* L. UOG-001053	Aizoaceae	0.76	19	0.34	37	0.56	65	47	Anthelmintic, diuretic, asthma, liver infection, jaundice
50.	Baykr	*Justicia adhatoda* L. UOG-001054	Acanthaceae	0.67	15	0.122	35	0.23	98	88	Abortifacient, cold, cough, flu, toothache, fever

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51.	Thoom	*Allium sativum* L. UOG-001055	Amaryllidaceae	0.19	16	0.33	28	0.65	70	45	Lowering blood pressure, cardiac problems. Respiratory tract infection, hypertension
52.	Kanair	*Nerium oleander* L. UOG-001056	Apocynaceae	0.18	35	0.78	45	0.67	54	30	Abortions, toothache, earache
53.	Ghunar	*Amaranthus viridis* L. R. Br. UOG-001057	Amaranthaceae	0.67	34	0.67	26	0.78	89	67	Carminative, emollient, rheumatism
54.	Puthkanda	*Achyranthes aspera* L. R. Br. UOG-001058	Amaranthaceae	0.76	27	0.76	19	0.98	87	56	Pyorrhea, cold, flu, fever, pneumonia leprosy
55.	Desi Aak	*Calotropis procera* L. R. Br. UOG-001059	Asclepiadaceae	0.18	24	0.007	27	0.67	67	35	Expectorant, antidote, scabies, rheumatism, dysentery
56.	Chonga	*Caralluma edulis* L. R. Br. UOG-001060	Asclepiadaceae	0.18	18	0.0675	29	0.54	50	25	Anthelmintic, diuretic, diabetes
57.	Partha	*Parthenium hysterophorus* L. R. Br. UOG-001061	Asteraceae	1.05	17	0.67	26	0.67	78	44	Laxatives, diabetes, regulate bowl movements
58.	Chaou	*Artemisia scoparia* L. UOG-001062	Asteraceae	0.37	27	0.76	17	0.87	56	35	Purgatives, antidote, malaria
59.	Bhangra	*Eclipta prostrate* L. UOG-001063	Asteraceae	0.80	28	0.89	14	0.78	78	45	Emetic, jaundice
60.	Pholi	*Carthamus oxyacantha* L. UOG-001064	Asteraceae	0.90	35	0.003	2019	0.76	45	20	Ulcer, jaundice
61.	Hund	*Taraxacum officinale* L. R. UOG-001065	Asteraceae	0.79	12	0.330	22	0.89	47	26	Constipation, diabetes, antidote
62.	Pelo	*Salvadora oleoides* L. UOG-001066	Salvadoraceae	0.76	29	0.012	21	0.87	30.78	12	Toothache, tonic
63.	Siah Dahtora	*Datura innoxia* L. UOG-001067	Solanaceae	0.29	12	0.0456	25	0.90	67	33	Sedative, stops premature ejaculation
64.	Surkh Mirch	*Capsicum frutescens* L. UOG-001068	Solanaceae	1.08	22	0.054	40	0.87	56	31	Dyspepsia
65.	Kaanch Mainch	*Solanum nigrum* L. UOG-001069	Solanaceae	1.02	24	0.067	45	0.99	49	28	Laxative, jaundice, hepatitis, obesity
66.	Mookri	*Solanum surattense* L. UOG-001070	Solanaceae	0.67	25	0.05	15	0.34	36	29	Rheumatism, fever, asthma
67.	Aksn	*Withania somnifera* L. R. UOG-001071	Solanaceae	1.0	21	0.60	19	0.76	38	29	Dysentery, diarrhea

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68.	Rukh	*Tamarix dioica* L. UOG-001072	Tamaricaceae	0.56	26	0.19	16	0.99	98	78	Piles, diarrhea
69.	Falsa	*Grewia asiatica* L. UOG-001073	Tiliaceae	0.98	27	0.19	15	0.47	36	25	Diuretic, constipation emetic, blood purifier
70.	Jute	*Corchorus aestuans* L. UOG-001074	Tiliaceae	0.76	19	0.19	13	0.36	40	20	Pneumonia
71.	Pakhra	*Tribulus camaldulensis* L. UOG-001075	Zygophyllaceae	0.67	19	0.167	18	0.98	93	67	Remove gall bladder and kidney stones
72.	Ziri	*Scandix pectenveneris* L. UOG-001076	Amaranthaceae	0.54	24	0.7	14	0.67	27	12	Astringent, palpitations, blood coagulant
73.	Rangobul	*Rhazya stricta* L. UOG-001077	Apocynaceae	0.23	23	0.778	17	0.56	39	22	Antihypertensive
74.	Khajoor	*Phoenix dactylifer* L. UOG-001078	Arecaceae	0.46	18	0.56	15	0.87	20	06	Astringent, gonorrhea, abdominal pains
75.	Gul-e-Sharf	*Calendula arvensis* L. UOG-001079	Asteraceae	0.65	27	0.655	20	0.67	48	20	Severe pains
76.	Gul-e-Hozah	*Conyza aegyptiaca* L. UOG-001080	Asteraceae	0.66	15	0.006	22	0.87	49	29	Diarrhea, fever, toothache, earache
77.	Sumbal	*Bombax ceiba* L. UOG-001081	Asteraceae	0.78	27	0.45	25	0.78	46	28	Sericulture
78.	Kawar Gandal	*Aloe vera* L. UOG-001082	Asphodelaceae	0.53	26	0.65	27	0.89	45	20	Acne, pimples
79.	Ganda	*Allium cepa* L. UOG-001083	Amaryllidaceae	0.83	14	0.34	29	0.87	90	67	Disinfectant, earache, cardiovascular disorders
80.	Neem	*Azadirachta indica* L. UOG-001084	Meliaceae	0.67	20	0.56	27	0.78	87	50	Joints pain, malaria
81.	Gul-e-Khairu	*Althea rosea* L. R. UOG-001085	Malvaceae	0.91	18	0.87	24	0.76	45	27	Laxative, expectorant, demulcent, emollient, chest pain
82.	Moosli Safaid	*Asparagus racemosus* L. UOG-001086	Asparagaceae	0.35	27	0.16	37	0.89	47	28	Measles, liver tonic, heal wounds, asthma
83.	Periwinkle	*Vinca major* L. UOG-001087	Apocynaceae	0.87	22	0.02	20	0.65	49	20	Diarrhea, cancer, diabetes
84.	Kasni	*Cichorium intybus* L. UOG-001088	Asteraceae	0.17	25	0.34	34	0.78	29	13	Diuretic, stimulant

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85.	Paprra	*Fumaria indica* L. UOG-001089	Papaveraceae	0.82	27	0.65	24	0.87	39	17	Fever, asthma
86.	Kurly Cress	*Lepidium didymium* L. UOG-001090	Brassicaceae	0.27	12	0.034	27	0.89	38	17	Antipyretic
87.	Bariar	*Malvastrum coromandelianum* L. UOG-001091	Malvaceae	0.2	34	0.560	27	0.98	30	19	Aphrodisiac, wound healing, fever
88.	Singi	*Melilotus indica* L. UOG-001092	Fabaceae	0.67	26	0.006	27	0.88	37	29	Abdominal pain, diabetes, dysentery, diarrhea
89.	Boine	*Micromeria biflora* L. UOG-001093	Lamiaceae	0.83	18	0.007	5	0.98	38	20	Diuretic, vomiting, constipation, headache
90.	Kor-Kandoli	*Ranunculus muricatus* L. UOG-001094	Rananculaceae	0.18	27	0.070	18	0.78	37	27	Laxative, antidote, cough, remove tumors and bursts
91.	Erian	*Quercus incana* L. UOG-001095	Fagaceae	0.67	37	0.54	10	0.65	38	20	Hemorrhagic septicemia, joint pain
92.	Komal	*Berberis lyceum* L. UOG-001096	Berberidaceae	0.54	24	0.006	2938	0.78	49	29	bone fractures, jaundice, wound healing, dyspepsia, hypertension
93.	Sindari	*Debregeasia salicifolia* L. UOG-001097	Urticaceae	0.48	18	0.654	30	0.87	50	25	Febrifuge, jaundice
94.	Sanatha	*Dodonaea viscosa purpurea* L. UOG-001098	Sapindaceae	0.95	10	0.67	29	0.76	48	30	Anthelmintic, healing burns, wounds and bruises, astringent, blood purifier, paralysis
95.	Pinli	*Hypericum oblongifolium* L. UOG-001099	Hypericaceae	0.67	20	0.76	16	0.89	37	20	Blood pressure, ulcer
96.	Hiran Charri	*Indigofera heterantha* L. UOG-001100	Fabaceae	0.9	37	0.34	10	0.98	40	15	Vermifuge, hepatitis
97.	Chambeli	*Jasminum officinale* L. UOG-001101	Oleaceae	0.36	22	0.543	20	0.76	57	28	Anti-inflammatory, ringworms
98.	Phutt	*Lonicera quinquelocularis* L. UOG-001102	Caprifoliaceae	0.87	20	0.001	37	0.99	69	37	Improve vision, remove cataract, ophthalmic agent, wound healing
99.	Timbar	*Zanthoxylum armatum* L. UOG-001103	Rutaceae	0.10	12	0.004	19	0.99	46	21	Carminative, dyspepsia, stomach pains, piles
100.	Surkh Akhra	*Duchesnea indica* L. UOG-001104	Rosaceae	0.45	23	0.760	12	0.45	48	23	Diuretic, astringent, laxative, sore throat, nerve tonic

Informant consensus factor (FCI), Fidelity Level (FL), Corrected Fidelity Level (CFL), User Reported (UR), Relative frequency of citation (RFC), Relative Importance Level (RIL), Use Value (UV)

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Discussion

Ethnobotanical knowledge of threatened species and therapeutic uses

Utilization of plants by humans as medicines was almost started in the Middle of Paleolithic era some 60,000 years back (Shipley and Kindscher, 2016). Plants can also be used as herbal medicines and the quantity of their usage in daily life varies because these plants are selected randomly and screened for different type of ailments (Gaoue et al., 2017). Traditional knowledge as per practice transferred from generation to generation through verbal and oral means of communication that is why evaluation on scientific basis got prime importance (Aziz et al., 2017; Polat et al., 2017). Data collected represent different life forms as herbs, shrubs and trees indicated presence at various localities of the selected sites (Mahmood et al., 2013).

Plants were generally used as stimulant, anthelmintic, cardiovascular diseases, febrifuge, diuretics, vermifuge, sedative and even antidote as this was previously reported by Mahmood et al. (2013) with few new species noticed in the current study. *Cichorium intybus* L. belongs to family Asteraceae used as a stimulant (Nandagopal and Kumari, 2007). Various plant species like *Convolvulus album* L., *Benincasa hispida* L., *Cucurbita pepo* L., *Rosa indica* L., *Trianthema portulacastrum* L., *Caralluma edulis* L. and *Dodonaea viscosa purpurea* L. were used as anthelmintic purposes and anti-parasitic properties (Amjad et al., 2017). Current studies reported plants such as *Trianthema portulacastrum* L., and *Allium cepa* L. are the best known for cardiovascular problems as it is good for the blood flow and pressure (Ugulu, 2011). *Debregeasia salicifolia* L. was considered the best against febrifuge.

Number of researchers reported the importance of different plants such as *Heliotropium strigosum* L., *Xanthium strumarium* L., *Trichodesma indicum* L., *Benincasa hispida* L., *Cyperus rotundus* L., *Crotolaria burhia* L., *Avena sativa* L., *Rosa indica* L., *Albizia lebbek* L., *Trianthema portulacastrum* L., *Caralluma edulis* L., *Grewia asiatica* L., *Micromeria biflora* L. and *Duchesnea indica* L. as diuretic (Mahmood et al., 2013; Bhakshu et al., 2008; Verma and Kumar, 2011; Zia-Ul-Haq et al., 2012; Amjad et al., 2017). The plants that were used for vermifuge purposes includes *Indigofera heterantha* L., *Portulaca quadrifida* L., *Pongamia pinnata* L. and *Cyperus rotundus* L. (Ghumare et al., 2014). *Xanthium strumarium* L. and *Datura innoxia* L., were used for sedative purpose. Current studies revealed few plants as antidote such as *Taraxacum officinale* L., *Artemisia scoparia* L., *Calotropis procera* L., *Ranunculus muricatus* L., *Melia azedarach* L. and *Trichodesma indicum* L.

One of the most noticed diseases from the study area were rheumatism and many plants were used for curing this disease like *Cuscuta reflexa* L., *Pongamia pinnata* L., *Amaranthus viridis* L., *Calotropis procera* L. and *Solanum surattense* L. Ghumare et al. (2014) noticed the first two species and rest of all are reported in this study. For blood purification species like *Dodonaea viscosa purpurea* L., *Scandix pectenveneris* L., *Grewia asiatica* L., *Morus nigra* L., *Albizia lebbek* L., *Melia azedarach* L., *Eichhornia crassipes* L., *Punica granatum* L., *Cynodon dactylon* L., *Crotolaria burhia* L. and *Heliotropium strigosum* L. (Bhakshu et al., 2008).

Many diseases such as hemorrhagic septicemia, jaundice, wound healing, severe burns and skin treatments of various types were also found to be treated with different plant species like *Quercus incana* L. Jaundice was also treated by different plants such as *Debregeasia salicifolia* L., *Berberis lyceum* L., *Solanum nigrum* L., *Carthamus oxyacantha* L., *Eclipta prostrate* L., *Trianthema portulacastrum* L., *Portulaca oleracea*

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L. *Citrullus colocynthis* L. *Indigofera linifolia* L. while *Mirabilis jalapa* L. for skin treatment. Similarly, this study also signifies that *Lonicera quinquelocularis* L., *Berberis lyceum* L. and *Malvastrum coromandelianum* L. were observed to be useful in wound healing (Amjad et al., 2017).

Some plants were also reported for treating various diseases like piles, dysentery, stomach problems, vomiting, measles, diarrhea, asthma, diabetes, liver tonic, tooth aches, urinary problems and sexual diseases like *Zanthoxylum armatum* L., *Tamarix dioica* L., *Abutilon indicum* L., *Portulaca quadrifida* L., *Alhagi maurorum* L., and *Convolvulus album* L., are used for the treatment of piles (Amjad et al., 2017; Mahmood et al., 2013). For dysentery, some plants were useful such as *Trichodesma indicum* L., *Cyperus rotundus* L., *Dalbergia sissoo* L., *Cynodon dactylon* L., *Punica granatum* L., *Calotropis procera* L., *Cynodon dactylon* L. In current studies, *Citrullus colocynthis* L. and *Zanthoxylum armatum* L. were shown to be used for releasing stomach pain while *Micromeria biflora* L. and *Bryophyllum pinnatum* L. are used against vomiting that were present in both Gujranwala and Wazirabad. For curing measles *Zizyphus nummularia* L. and *Asparagus racemosus* L. were found efficient. Amjad et al. (2017) reported many plants present in this region were used for treatment of diarrhea which were *Melilotus indica* L., *Vinca major* L., *Conyza aegyptiaca* L., *Tamarix dioica* L., *Withania somnifera* L., *Cassia fistula* L., *Mentha spicata* L., *Cyperus rotundus* L., *Trichodesma indicum* L. and *Bryophyllum pinnatum* L. and these all are present in the study area. For asthma *Euphorbia hirta, Albizia lebbek, Trianthema portulacastrum* L., *Solanum surattense* L., *Asparagus racemosus* L. and *Fumaria indica* L. were beneficial plants found in the study area. Plants which were found associated with the treatment of diabetes were *Melilotus indica* L., *Vinca major* L., *Taraxacum officinale* L., *Parthenium hysterophorus* L., *Caralluma edulis* L., *Syzygium cumini* L. and *Cuscuta reflexa* L. grown at various places in Kamonki and Wazirabad. *Heliotropium strigosum* L., *Avena sativa* L., *Portulaca oleracea* L., *Trianthema portulacastrum* L., *Asparagus racemosus* L. and *Duchesnea indica* L. acts as liver tonic. For tooth aches *Conyza aegyptiaca* L., *Salvadora oleoides* L., *Nerium oleander* L., *Justicia adhatoda* L., *Pongamia pinnata* L. and *Cuscuta reflexa* L. were important which are also present in Wazirabad, Kamonki and Gujranwala. For urinary infection some plants such as *Chenopodium album* L. and *Hibiscus rosasinensis* L. were found in the study area.

Plants used as anti-inflammatory purposes included *Trichodesma indicum* L., *Lepidium didymium* L. and *Jasminum officinale* L., (Tareen et al., 2016). Most of the plants were linked with treatment of common diseases like fever and constipation. For fever, *Convolvulus album* L., *Trichodesma indicum* L., *Dalbergia sissoo* L., *Bumbusa arundinacea* L., *Marva parviflora* L., *Eucalyptus globulus* L., *Morus nigra* L., *Justicia adhatoda* L., *Achyranthes aspera* L., *Artemisia scoparia* L., *Solanum surattense* L., *Conyza aegyptiaca* L., *Fumaria indica* L., *Malvastrum coromandelianum* L. *Debregeasia salicifolia* L were used found at various places of selected sites and for constipation the beneficial plants found are *Micromeria biflora* L., *Grewia asiatica* L., *Taraxacum officinale* L., *Mirabilis jalapa* L., *Hibiscus rosasinensis* L., *Zizyphus nummularia* L., *Rosa indica* L., *Alhagi maurorum* L., *Citrullus colocynthis* L. and *Convolvulus album* L. Interestingly a unique species and only one was also recorded for treating epilepsy from this region that was *Benincasa hispida* L. that belongs to the family Cucurbitaceae.

Plants were also reported to be used for various other aspects especially for aesthetic beauty, decorative purposes and as ornamentals etc., along as food, shelter, clothing, dyes, coloring etc. *Artemisia scoparia* L. a member of the family Asteraceae was used

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DOI: http://dx.doi.org/10.15666/aeer/1806_79537979

in preventing hair loss. For this, the plant extract should be mixed with coconut oil and then boiled. It can be applied on the head to strengthen the hairs and to prevent them from becoming white. *Calendula arvensis* L., another member of the same family was used in weddings for decorative purposes. *Melia azedarach* L. and *Malvastrum coromandelianum* L. are the members of family Malvaceae used as ornamentals in various places of Gujranwala. Similarly, *Cassia fistula* L. and *Acacia modesta* L. members of family Mimosaceae are grown at various places in the selected sites of the Gujranwala region. Different plants have been used differently by the people such as *Chenopodium* is used as fodder crop for animals and it is used in the case of constipation. *Cucurbeta peppo* L. the favorite vegetable of the summer was found in the study area. It is the member of family Cucerbitaceae, other members of the same family were *Cucumus melo* L. and *Benincasa hispada* L. that were used as vegetables. *Acacia modesta* L. and *Acacia nilotica* L. members of family Mimosaceae were wildly found in the study area and are used in making and as additive for gums.

Quantitative indices, phytochemical diversity and their role for conservation

The relative frequency of citation (RFC) varies from 1 (means the number of useful plants responded by the respondents) to 0 (i.e. no one reported the plants uses). The lowest value for RFC calculated by the gathered data is 0.001 of plant *Lonicera quinquelocularis* which is used for the treatment of improving vision, removing cataract, as ophthalmic agent, wound healing purpose and the highest value is 0.78 of plant *Nerium oleander* used in the treatment for abortions, toothache and earache. Informants consensus factor (FCI) also describes the number of used reports for a said disease category and in this study it ranged from 10 to 40. The lowest value of FCI was 10 for *Cucumus melo* which was used in the treatment of eczema, dysuria, leucorrhea and also acts as laxative. The highest value was 37 of plants *Indigofera heterantha and Quercus incana* used in treatment of hemorrhagic septicemia and joint pain.

Relative importance level (RIL) indicated the eminence of the species in the selected sites of study area. RIL varies from 0 (not much importance) to 1.0 (medicinal importance). The lowest value recoded by the data was 0.34 of plant *Solanum surattense* used for rheumatism, fever, asthma and the highest value was 0.99 of different plants like *Marva parviflora*, *Solanum nigrum*, *Tamarix dioica*, *Lonicera quinquelocularis* and *Zanthoxylum armatum* were being used of carminative, dyspepsia, stomach pains and piles (Umair et al., 2019). Fidelity level (FL) showed uses of specific plants to treat particular diseases. The fidelity count of plants in the study area ranges up to 90 that mark their importance in the field of medicines. *Benincasa hispida* have the lowest value calculated through gathered data i.e. 46.35 used for different problems laxative, diuretic, tonic, anthelminthic, epilepsy and the highest value is 98 for *Marva parviflora, Justicia adhatoda* and *Tamarix dioica* used in piles and diarrhea. Corrected fidelity level (CFL) is a correction factor that links RIL with FL and shows relative importance of different plants found in the study area. Different respondents give useful information which is an important aspect of the study. The lower value of CFL was 12 for *Eichhornia crassipes,* having different uses such as emollient, blood purifier and the highest value was 88 of *Justicia adhatoda* plant that was used as abortifacient, for cold, cough, flu and toothache.

User reports (UR) is the total number of users reported about a particular plant and this ranges between 1 and 20 mostly i.e. up to 20 users reported about a particular disease to be treated by the particular plant. The lowest numbers of users reported were

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11, for plant *Trichodesma indicum*, which was used as antidote, diuretic, against dysentery and fever and the highest numbers of users reported were 380, for plant *Syzygium cumini* which was helpful in diabetes. The significance of each species was calculated by UV (use value) especially in the case of herbal drinks (Etongo et al., 2017). Use value (UV) of the plants was also beneficial for the studying of medicinal plants and in this study it ranges from 0 to 1 i.e. little to more importance in the field of medicines. The value of UV of plant *Citrullus colocynthis* was 0.1 which is the lowest value used in the treatment of amenorrhea, constipation, stomach aches, jaundice, also used as purgative, and the highest value of UV was 0.98 of plant *Trichodesma indicum* used for dysentery, fever and diarrhea.

Secondary metabolites are vital ingredients present in plants were quantified qualitatively using standard procedures. The severity of chemicals present in the selected sites of plants indicates their importance. About Twenty different plants were analyzed for phytochemical analysis. *Punica granatum, Chenopodium album, Jasminum officinale, Cyperus rotundus, Morus nigra, Bryophyllum pinnatum* and many others showed presence/indication of alkaloids, flavonoids, tannins, steroids, terpenoids, saponins, phenols and resins that are the major source of efficacy of various bioactive compounds effective against number of ailments. Further, these plants rich in biochemical constituents recommended for further characterization through GCMS or HPLC to gather more information about biochemical structure and their ingredients ultimate impact against ailments which are still considered as complex to treat completely. But in some plants such as *Quercus incana, Bambusa arundinacea* and *Syzygium cumini* compounds like terpenoids, saponins and steroids did not show any indication that might be due to variation in geographical area, topology, soil physical and chemical factors, climatic factors, and/or species or genetic variation. One of the major reasons might be industrialization that causes accumulation of toxic chemicals, industrial waste and heavy metals that needs thorough examination.

Conclusion

In conclusion, the anthropocentric activities due to industry and urbanization may alter or degrade phytochemical diversity that ultimately change efficacy of particular extracts. Current studies revealed 100 different species of 52 families, where Asteraceae was dominant. Different life forms recorded i.e., 55% herbs, 27% shrubs, 15% trees, 2% grasses and 1% weeds, 85% wild and 15% cultivated. Leaves were predominantly used as 77%. Plants were found effective against fever, as laxatives, emollients, against constipation, as blood purifiers, and against cough and cold etc. Informant consensus factor for *Cucumus melo* showed the lowest value while *Indigofera heterantha* and *Quercus incana* represent the highest. Involvement of local community with public sector for implementing policies and guidelines according to Biodiversity action plan of Pakistan is required to be practiced to achieve environmental sustainability.

Acknowledgements. Authors are thankful to Prof. Dr. Mushahid Anwar, Dean FS/Chairperson, Department of Geography, University of Gujrat, Gujrat, Pakistan for Gujranwala map cartography and Ms. Tayyaba Khalil, Incharge Herbarium, Department of Botany, University of Gujrat, Gujrat, Pakistan for identification and herbarium specimen preparation.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79537979

RESEARCH ON THE EFFECT OF SOIL HEAVY METAL ELEMENTS IN THE POTENTIAL HABITATS OF GIANT PANDAS (*AILUROPODA MELANOLEUCA*)

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(Received 20th Apr 2020; accepted 29th Jul 2020)

Abstract. By building a geochemical (heavy metal) niche model for giant pandas, this paper researched and analyzed the restriction of Cr, Hg and Pb in the soil in Pingwu County, Sichuan Province on the giant pandas' selection of habitats. According to the model analysis, in this study area, Cr has the largest impact on the giant pandas' selection of habitats. In this habitat, the maximum bearing value of Cr is 70×10^{-6} mg/kg; the maximum bearing value of Hg is 462.8×10^{-9} mg/kg; and the maximum bearing value of Pb is 37.94×10^{-6} mg/kg. If the content of the above three heavy metal elements exceeds their respective maximum bearing value, the encounter rate of the giant pandas in this area will decrease to 0, and the giant pandas will no longer choose this area as a habitat.
Keywords: *endangered species, ecological niche model, habitat selection, pollutant, Pingwu County*

Introduction

As a symbol of China, an endangered species and a world-class protected animal, the giant panda, *Ailuropoda melanoleuca* (David, 1869) receives much attention from the global scientific research circles. The heavy metal pollutants in a giant panda habitat enter and accumulate in the bodies of the giant pandas and other animals along the food chain, and thus pose a threat to their health and even life. To better select habitats for pandas and thus better protect them, we carried out this study. With an ecological niche model, we studied and analyzed how the three elements of Cr, Hg and Pb in soil in Pingwu County of Sichuan restricted the habitat selection of pandas, to find out the influence of the content of the three heavy metallic elements in soil on the habitat selection of pandas under natural conditions, and finally determine an area in Pingwu County with a minimum influence of the three heavy metallic elements on pandas, as an effective reference for the habitat selection of pandas.

General Situation of the Study Region

The number of giant pandas in Sichuan Province takes up over 70% of the global wild pandas, while Pingwu County in Sichuan Province is honored as the number one county in the world for pandas. This location also appears to be an important transition zone between panda species A and B (Hu, 2005). The area belongs to the Himalayan-Hengduan Mountains, one of the core global biodiversity areas. The reserve maintains typical natural

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79817988

ecological systems and this one in particular is the most intact ecosystem within its latitude region. It has representation and typicality that is outstanding on a global scale.

Review of Literature

The influence of heavy metal elements on the giant pandas has been studied for a long time. For instance, Liu and Wang (1988) took 43 panda samples from Minshan Mountains and 10 from Qionglai Mountains, and then tested the normal value range of 10 trace elements, including Pb, by analyzing the trace elements in the panda hair. A direct path for pollutants, including heavy metals, to enter the bodies of the giant pandas and other animals is their food bamboo (de Almeida Curi et al., 2012), thus many researches have been carried out based on the content of heavy metals in the food. For example, Wang (2011) tested how much different concentrations of Pb and Cd were absorbed and accumulated in 5 kinds of bamboos, including potted bamboo in laboratory and simulated black bamboo. Liu (2015) from Chinese Academy of Sciences analyzed the exposure of the endangered animals in Qinling Mountains, including giant pandas, to heavy metals (Cr, Pb, Hg and As), and made a comparison in heavy metals between the habitats of the wild giant pandas and the giant pandas in captivity. Through research, Zheng et al. (2016) found that traffic could be an important source of heavy metallic pollution in soil in habitats of pandas, as expressways increased the concentration of toxic metal in these places. Tian et al. (2019) observed high potential risks to pandas that were exposed to Pb, As and Hg in a study. Zhao (2019) found that concentrations of heavy metal in feces and bamboo samples were positively correlated, which meant that polluted bamboo could be a major direct source of pollutants to which pandas were exposed.

As mentioned above, some scholars have noticed the harmful impacts of heavy metals on the giant panda and other relevant endangered animals. In addition, to carry out an efficient analysis on the potential habitats of giant pandas, we have to consider the distribution of heavy metal elements in the study area. But at present, there are very few reports on the effect of heavy metal elements on the giant pandas' selection of habitats. So, this paper built a niche model of heavy metals, researching and analyzing the restriction of Cr, Hg and Pb in soil on the giant pandas' selection of habitats, in hope of providing reference for analyses of giant panda habitats in future.

Materials and Methods

Maximum Entropy and Niche Theory

Niche theory can be used to explain and predict the distribution range of a giant panda habitat. The person who first proposed niche theory is Grinnell (1917), while the one who first treated niche theory mathematically is Hutchinson and Macarthur (1959). The core idea of mathematical treatment is that every organism is restricted by different factors in the natural environment, there is a suitable quantizing interval for all these factors, and the composite factors in the interval are concrete indexes for this organism's existence and multiplication. Species niche is a collection of the composite factors of all the intraspecific organisms. The concept of maximum entropy was proposed by Jaynes and Cummings (1963). Maximum entropy is derived from information science, and at present, it has been applied to economics, ecology and the relevant inter-disciplines. Maximum entropy has a good confinement effect on incomplete information-based probability distribution. Its purpose is to infer unknown information by full use of incomplete

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DOI: http://dx.doi.org/10.15666/aeer/1806_79817988

information, and take known information as a constraint value, with the probability distribution meeting the condition for entropy value maximization. Incomplete information comes from entropy theory, which was proposed by Shannon (1948). As the amount of information increases, entropy value decreases. When it is used for forecasting, no tendentious hypothesis will be made, but on the premise that the existing information is indeed contained, when the entropy value is maximized, redundant information will be completely excluded, so that the uncertainty of unknown information should be reduced. Set a random variable ξ, which may have n possibilities, including A1, A2, A3,…An, and for the appearance of each possibility, there may be a probability, which equals to p1, p2, p3,…, pn, so the information entropy H (*Eq.1*) is:

$$H(\xi) = \sum_{i=1}^{n} p_1 \log \frac{1}{p_i} = -\sum_{i=1}^{n} p_i \log p_i \quad \text{(Eq.1)}$$

MaxEnt Model

Phillips et al. (2004) developed MaxEnt model based on the above maximum entropy and niche theory. According to the known distribution range of a species based on environmental factors, this model calculates the maximum entropy value corresponding to the distribution rule of this species, and predicts the potential distribution area of this species. That is, a constraint condition needs to be determined first in accordance with the environmental characteristics of the target species' habitat, and then the maximum entropy can be calculated in line with this constraint condition. The predicted distribution range of this species is the probability distribution of this species when entropy reaches its maximum.

Since its development by Phillips et al. (2004) MaxEnt model has gone on a good run and been used by many scholars to predict habitats of animals and plants. Prates-Clark et al. (2008) based on a maximum entropy model, researched the distribution law of three tree species in the Amazon Basin and predicted their distribution using MODIS data and other relevant remotely sensed data. Hernandez et al. (2006) discovered in their research that despite the limited number of biologic points, the result would also be reliable, and the precision could meet the requirements, suggesting that it is applicable despite a small number of sample points. With the constant improvement of the model, more and more people have applied it to the analysis of habitats. Thus, this paper constructed a niche for the giant pandas in Pingwu County using this mode.

Niche Model for the Heavy Metals in the Habitat of Giant Pandas in Pingwu County

Data Source

This paper primarily built a niche model for heavy metals with Cr, Hg and Pb in the soil as analysis objects. The map data of heavy metals (*Fig. 1*) comes from a WWF topic: Follow-up Study of Evaluations on the Ecological Environment in the Habitat of Giant Pandas in Pingwu County; the data of giant panda distribution come from the routine monitoring of Xiaohegou Nature Reserve in Pingwu County.

Attribute of heavy metal element data is extracted from the geochemical element map of this region, and the comparison in grade between these three elements is shown in *Fig. 2*.

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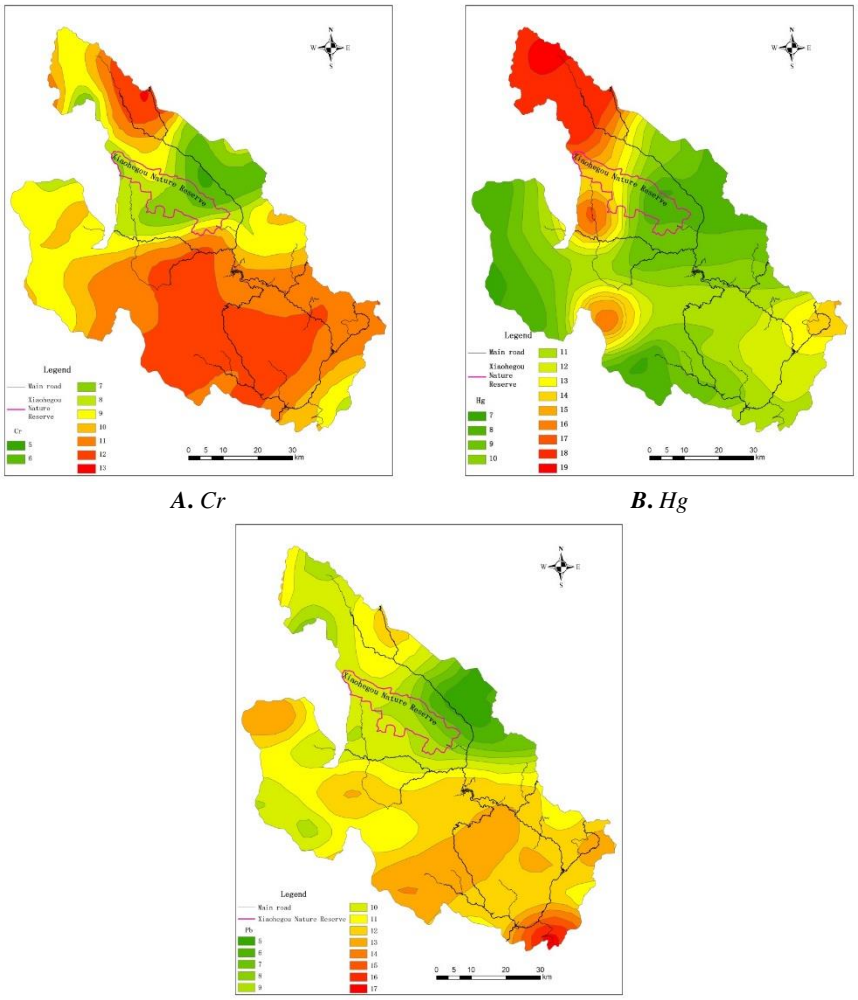

A. *Cr* ***B.*** *Hg*

C. *Pb*

Figure 1. *Distributionof Heavy metal in Pingwu*

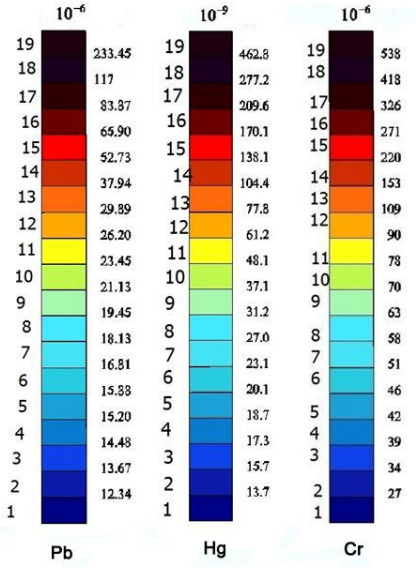

Figure 2. *Element Grade and Content Comparison (element unit mg/kg)*

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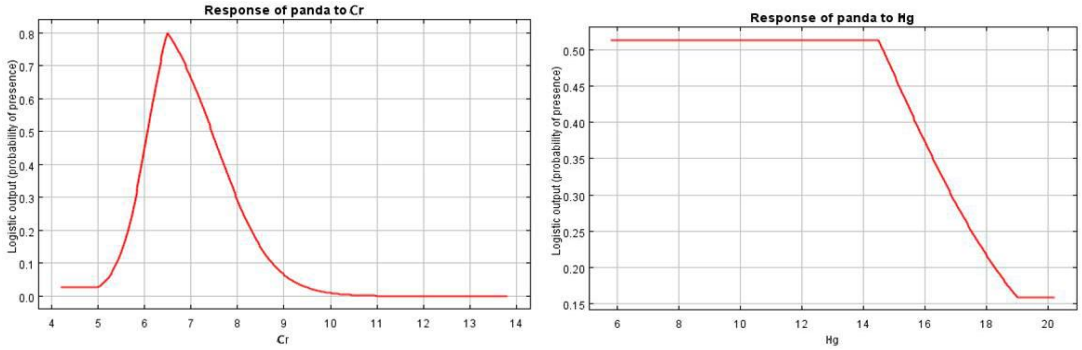
DOI: http://dx.doi.org/10.15666/aeer/1806_79817988

Result

In *Fig. 3*, the horizontal ordinate indicates the variation range of heavy metal value, and the vertical coordinate shows a natural logarithm of heavy metal value's contribution to the suitability of the giant panda habitat. The value is directly proportional to habitat suitability, and for the giant pandas, the more suitable the habitat is, the higher their encounter rate is.



A. *Response Curve of Cr to the Giant Panda Encounter Rate* **B.** *Response Curve of Hg to the Giant Panda Encounter Rate*

Figure 3. *Response Curve of Cr and Hg to the Giant Panda Encounter Rate*

According to *Fig. 3A*, as Cr content increases to Grade 10 (70×10^{-6} mg/kg), the giant panda encounter rate decreases to 0, suggesting that the maximum bearing value of Cr (*Fig. 3B*) in the habitat of giant pandas in this area is 70×10^{-6} mg/kg; before Hg content increases to Grade 15 (138.1×10^{-9} mg/kg), the probability of giant pandas' appearance is always stable, but as Hg content increases to 19 (462.8×10^{-9} mg/kg), this probability keeps decreasing to 0 from time to time, suggesting that Hg has a great effect on giant pandas. This effect is not obvious when Hg content is lower than 138.1×10^{-9} mg/kg, but if it's higher than 138.1×10^{-9} mg/kg, great harm will be done to the giant pandas. Maximum bearing value of Hg in the habitat soil in this area equals to 462.8×10^{-9} mg/kg.

As Pb content (*Fig. 4*) increases to Grade 14 (37.94×10^{-6} mg/kg), the giant panda encounter rate decreases to 0. So, the maximum bearing value of Pb in the habitat soil in this area is 37.94×10^{-6} mg/kg. The contribution of these three elements is shown in *Table 1*.

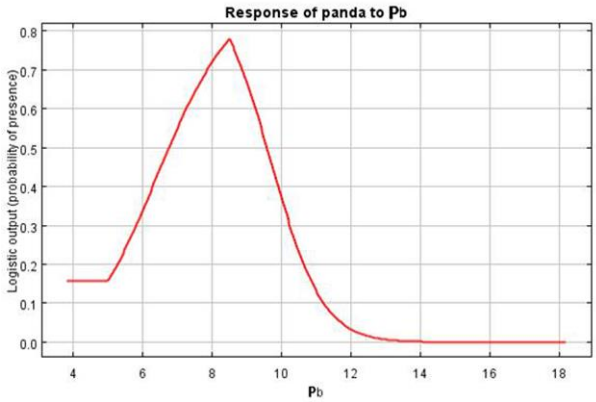


Figure 4. *Response Curve of Pb to the Giant Panda Encounter Rate*

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Table 1. *Contribution of Heavy Metal Elements*

Cr contribution	89.413
Hg contribution	2.033
Pb contribution	8.554

It can be seen from *Table 1* that among these three heavy metal elements, Cr has the largest impact on the giant panda habitat, accounting for 89.413%, followed by Pb, accounting for 8.554%; Hg has the least impact on the giant panda habitat in Pingwu County, accounting for 2.033%. The result of the analysis by MaxEnt model is shown in *Fig. 5*.

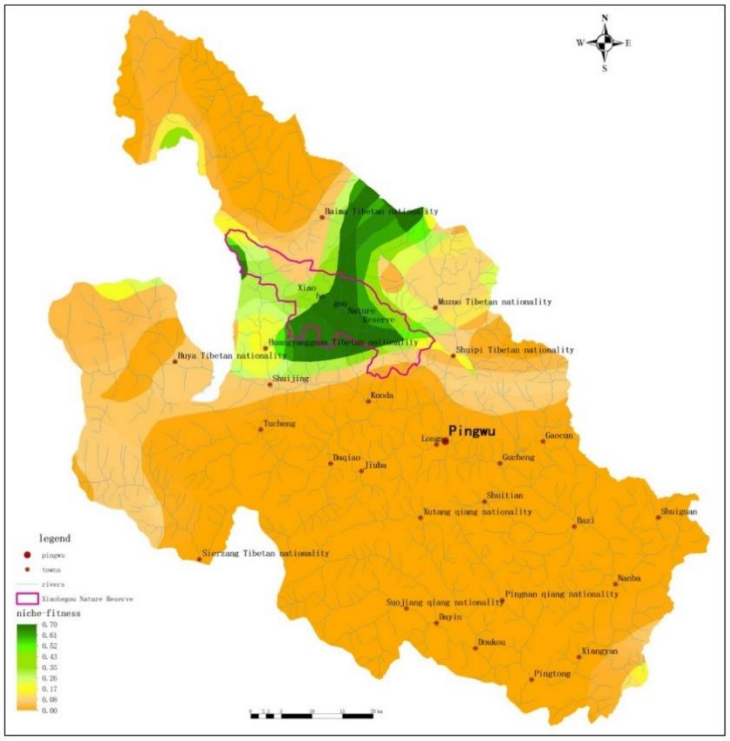

Figure 5. *Suitable Region for MaxEnt Model*

Precision Evaluation of Operation Results

For the giant panda habitat niche model, ROC (Receiver Operator Characteristic Curves) and AUC (the area under curves) were used to test the reliability of predicting outcomes. The larger the value is, the more strongly environment variables are correlated with the geographical distribution model of the predicted species, and the higher the predicting reliability is. AUC ranges from 0 to 1, and its interval division is shown as follows: when AUC ranges between 0.5 and 0.7, model evaluation is less reliable; when AUC ranges between 0.7 and 0.9, model evaluation is reliable; when AUC is greater than 0.9, model evaluation is highly reliable. AUC is insusceptible to the threshold value, and the evaluation result is objective and has a wide application (Duan, 2015). In the heavy metal niche model, the AUC of the simulation result training set is 0.965 (*Fig. 6*), suggesting that the predictive effect is good, and the result is available.

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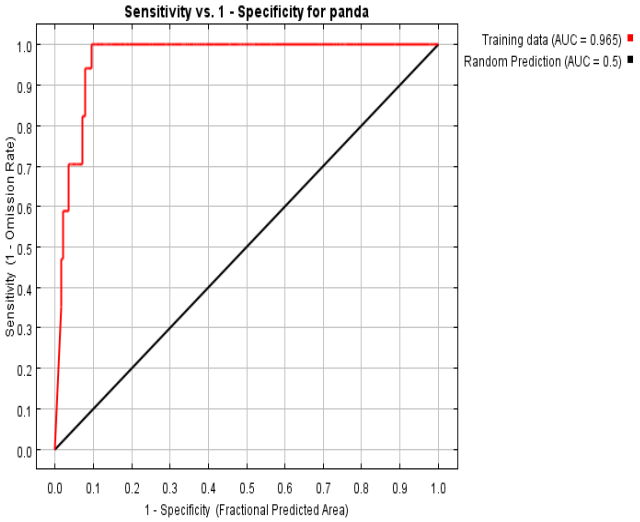

Figure 6. *Predicting Result of Heavy Metal Niche Model ROC & AUC*

Discussion

Many scholars have their own opinions on wildlife conservation (Liu et al., 2018; Kija et al., 2020; Pédarros et al., 2020). Some of the contents and conclusions of this paper have been described in the author's previous Chinese articles (Zhang, 2016). But at present, there are very few reports on the effect of geochemical (heavy metal) elements on the giant pandas' selection of habitats. In order to provide reference for more researchers, we tried to extract part of the information, by building a geochemical (heavy metal) niche model, this paper researched and analyzed the restriction of Cr, Hg and Pb in the soil in Pingwu County, Sichuan Province on the giant pandas' selection of habitats, and calculated the maximum bearing value of three elements in the giant panda habitat in the study area, in hope of providing reference for analyses of giant panda habitats in future. But due to the immaturity of the existing research on the mechanization of heavy metals' effect on the giant pandas, this paper suggests just taking it as an appropriate reference for the selection of potential habitats, rather than taking it as the main judgment basis for predictions of potential habitats in order to avoid ignoring the areas containing over-high bearing capacity. This paper studied the influence of the content of the three heavy metallic elements in soil on the habitat selection of pandas. The analysis shows that the content of heavy metal in soil will finally influence the habitat selection of pandas, which coincides with the research findings of Tian et al. (2019) that "we should attach greater importance to regional heavy metal pollution to protect this animal species". Major limitations of this paper lie in that the study fails to include other factors in this region such as the content of heavy metal in plants and the feces or hair samples of this targeted species, which will be further discussed in follow-up research.

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DOI: http://dx.doi.org/10.15666/aeer/1806_79817988

ALLEVIATION OF LITHIUM TOXICITY IN SORGHUM (*SORGHUM VULGARE* PERS.) BY INOCULATION WITH LITHIUM RESISTANT BACTERIA

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(Received 26th Apr 2020; accepted 7th Oct 2020)

Abstract. The present study was carried out to assess the impact of lithium and the alleviation of its influence on the growth and physiological performance of *Sorghum vulgare* using lithium resistant bacteria. Various growth and physiological parameters were measured at 15 days intervals and plants were harvested after 150 days to assess the lithium up-take in both the above-and belowground parts. A significant reduction in the number of leaves, plant height, panicle number, and grain yield, was observed among the plants treated with lithium. While, these growth parameters exhibited a significant increase among plants inoculated with bacteria. There was not a significant difference on gas exchange rate in the lithium treated plants as compared to the plants treated with bacterial inoculation. A strong significant relationship was observed between lithium concentrations in roots and biomass accumulation factor. Furthermore, the concentration of lithium was significantly lower in above and below-ground parts of plants with bacteria as compared to those treated with lithium only. Our findings demonstrate that lithium can cause significant reduction in growth and yield of sorghum, although reduction can be compensated by applying lithium resistant bacteria. Such data can be useful for lithium remediation in agricultural crops.
Keywords: *plant growth promoting bacteria, vegetative, yield, physiology, pollution*

Introduction

Pollution, specifically that of soil and water has formulated the crisis for life on earth, wide efforts have been taken to explore solution for this disorder (Brown and Chaney, 2016). Lithium is extremely mobile element geochemically, so, the environmental and occupational health and safety risks are high due to its mobility (Aral and Vecchio-Sadus, 2008). Lithium is toxic to plants, and only a few plant species are also lithium tolerant. Lithium contents concentration may reach up to 1000 $\mu g g^{-1}$ of plant mass (Shahzad et al., 2016). In fact, it is not required for the growth and development of plant. Symptoms of lithium toxicity in plants are mostly chlorosis. Prime uses of lithium in today's daily life is for manufacturing of batteries (39%) ceramics and glass (30%) and rest in some other uses. Australia, Chile, China and Argentina are the largest lithium-producing countries in the world respectively (Jaskula, 2019). At present Lithium-ion batteries recycling is paying attention only on collection of economically viable elements like Cobalt and Nickel. Lithium is neglected even in sophisticated recycling systems because it is cheap and easily available for mining of virgin material. Currently lithium pollution is present in the countries where it is extracted and where finally the products of lithium are disposed off without any treatment (Ferreira et al., 2009; Dewulf et al., 2010; Wanger, 2011; Gu et al., 2017; Winslow et al., 2018).

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Currently, there are not much studies about the phytotoxic effects of lithium, hence the metabolic role of lithium in plants is not fully documented (Hawrylak-Nowak et al., 2012; Franzaring et al., 2016). Being soluble in water it forms ions absorbed by plants (Aral and Vecchio-Sadus, 2008). Tolerance and uptake of lithium varies among plant families even in species according to their physiology for the ion up-take. Members of family leguminosae are most affected by lithium (Kabata-Pendias and Mukherjee, 2007). Plant and bacterial interaction has great importance, bacteria are helpful for plants to absorb nutrients and cope with different stresses faced by the plants. Bacteria synthesize plant hormones, which can cause direct effect on plant physiology. Bacteria also increase plant access to soil nutrients, and enhance plant growth. Such interaction result in the form of better growth and yield in plants (Hayat et al., 2010; Nadeem et al., 2014; Theka-Kutumela et al., 2020).

Sorghum (*Sorghum vulgare* Pers.) is an important crop cultivated in most of tropical, subtropical, and temperate regions. It is used worldwide for both cereal grain and fodder purposes. It belongs to C4 group of plants and have ability to face wide range of stresses (Gill et al., 2014; Zancheta et al., 2015; Jia et al., 2016). To the best of our knowledge the sorghum-bacterial interaction under lithium stress has not been much investigated previously. Major objectives of present study were to: (1) determine the effects of different concentrations of lithium and lithium resistant bacteria inoculation on the growth and yield of sorghum; (2) explore the role of different concentrations of lithium and inoculation of lithium resistant bacteria on the rate of photosynthesis, transpiration and stomatal conductance; (3) assess the uptake patterns (root, shoot and grains) of lithium in terms of bioaccumulation and translocation. For these objectives, we hypothesized that the significant reduction in plant growth and their physiology will be caused by the lithium, further the inoculation of lithium resistant bacteria will compensate the lithium effects. It was also hypothesized that there may be the relationship of lithium concentrations in plant parts and biomass accumulation factor. Findings of this study will add to understand tolerance of lithium toxicity in sorghum, and will provide eco-friendly approach to implement in the field.

Materials and Methods

Study site and experimentation

The pot experiment was conducted in the wire house of Botanic Garden GC University Lahore, Pakistan, containing *Sorghum vulgare* Pers. (sorghum) following the factorial experiment in a completely randomized design. Earthen pots of 35.56 cm diameter were used in this experiment, at start three seeds were sown in each pot, when plants were established, thinning was carried out and only one plant per pot was remained. There were six replicate pots per treatment and total of five treatments for lithium (0, 50, 100, 150 and 200 ppm and were labelled as T0, T1, T2, T3, T4) and five treatments for lithium and bacterial inoculations were applied (labelled as T0SB, T1SB, T2SB, T3SB, T4SB). Inoculum of lithium resistant bacteria (isolated from soil and identified as *Bacillus velezensis*) was prepared by dissolving eight gram of nutrient broth in some amount of distilled water and final volume was raised to one liter with the help of distilled water. This broth was added to flasks in which isolated lithium resistant bacterial colonies were added and kept at 37ºC for 24 hours. Then 10 mL of liquid nutrient broth containing lithium resistant bacteria (10^7 CFU ml^{-1}) was mixed with 40 mL distilled water and was

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poured into pots. The experiment was conducted for 150 days, and plants were destructively harvested.

Growth, yield and physiological assessment

During experiment biomass production was monitored through both physical and physiological parameters (Aslam et al., 2007). The parameters for growth included, number of leaves per plant, and total plant height of plant (cm), which were measured every 15 days. While the physiological parameters included the leaf area based maximum rate of photosynthesis, maximum stomatal conductance, rate of maximum transpiration using IRGA LCA4, in the ambient conditions in the morning time from 10:30 am to 11:30 am. These measurements were taken three times of study duration (first week of start of experiment, at the time of flowering and before the final harvest with mature seeds). The chlorophyll contents were measured using spectrophotometer (Spectroscan 80D). After final harvest, length of roots and shoots, fresh and dry mass of roots and shoots, number of panicle and grain yield were estimated in the Plant Eco-physiology Laboratory of GC University Lahore.

Determination of lithium

Plant material was placed in a porcelain crucible, which were placed in a muffle furnace for 5 hours at 550ºC for the preparation of ash. Ash of plant material was taken and dissolved in 5 mL of 2N HCl solution and mixed with a stirrer. After fifteen minutes, the final volume was raised to 50 mL with the help of distilled water. The solution was mixed with the help of stirrer and then it was filtered by using Whattman No. 42 filter paper. The extract was collected in a flask and lithium was measured by flame photometer (S20 Spectrolab).

Translocation factor was calculated by following the method of Marchiol et al. (2004). Bioaccumulation factor (BAF) was computed according to Ruus et al. (2005).

Statistical analysis

The results obtained in experiment were expressed in terms of means and standard deviations. Data was statistically analyzed using one way ANOVA (*Duncan's Multiple Range test* (DMRT)) involving the comparison of the means of each treatment having the lithium application with the means of each treatment having the lithium application but also with the bacterial inoculation for each studied parameters. The statistical analyses, correlations between parameters and graphics were carried out using software Sigma Plot 12.5 version.

Results

Effect of lithium and bacterial inoculation on growth and yield of sorghum

A significant decreasing trend of number of leaves was observed from the T0 to T4 during the experiment duration of 150 days, but the significant increase in the number of leaves was observed in the treatment of T0SB as compared to T0 in all the observations (*Fig. 1*). There was also the significant increase in the number of leaves in T1SB as compared to T1 in 30 days observation and the similar significant difference was observed in 75 days, and 105 days duration. The significant increase in the number of leaves was also observed in T3SB as compared to T3 in 60, 90 135 and 150 days duration (*Fig. 1*).

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The significant increase in leaves number was observed in T3SB in 135 and 150 days duration. In the T4SB the significant increase in the number of leaves was only observed in 150 days duration (*Fig. 1*). Overall, among all the treatments, significant increase in the total number of leaves was observed, except, in the means of T2, T2SB during 30, 45 and 90 days duration. The similar non-significant difference was observed between the means of T1, T1SB in 45 and 120 days duration. The non-significant difference was also observed between T4, T4SB in 45, 60 and 90 days duration (*Table S1*).

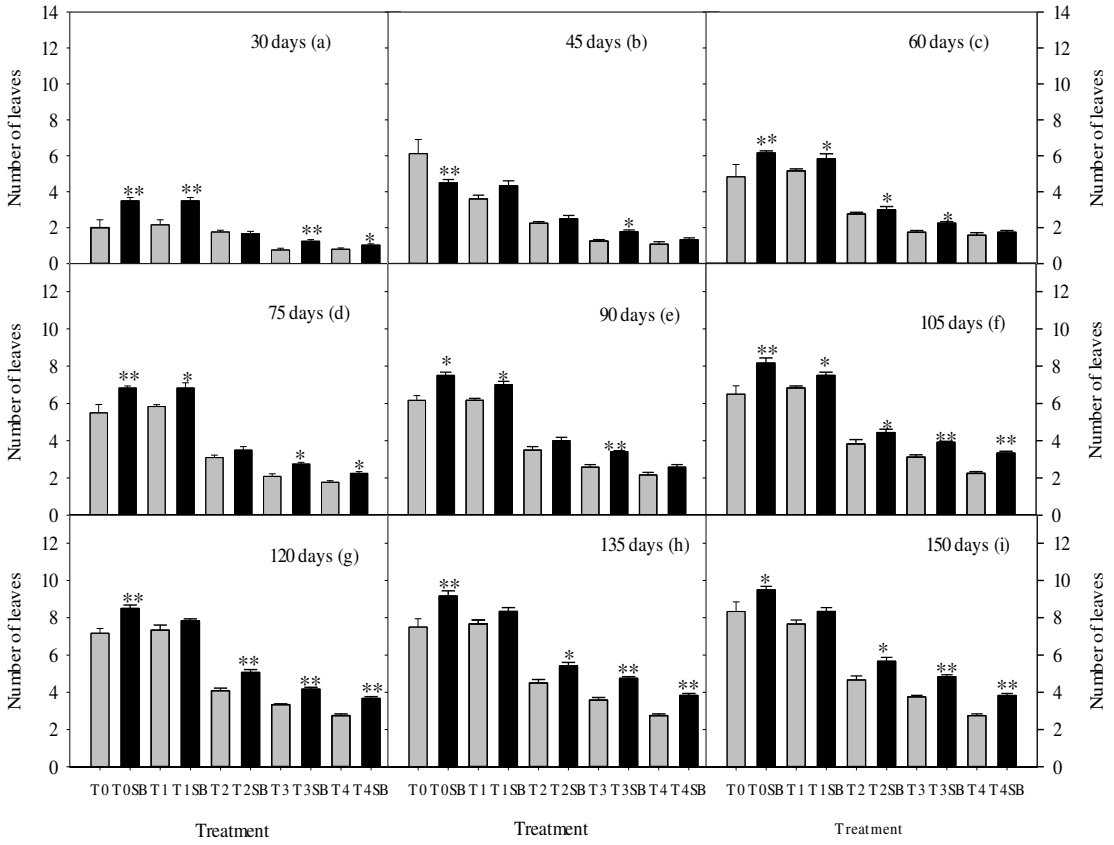

Figure 1. *Mean of the total number of leaves per plant for each treatment with the number of days having 15 days intervals from 30-150 days. Where *** indicate P< 0.0001, ** P< 0.001 and * P < 0.05. Grey bars indicate the soil with Lithium treatment, while black bars indicate the soil with Lithium treatment and also application of bacteria. Error bars indicate SE (±)*

A significant decrease in the total height of sorghum plants was observed from T0 to T4 during the 150 days of experiment, but the significant increase in the plant height was observed in all the treatments having the soil with lithium but also inoculated with bacteria as compared to those having the lithium application only (*Fig. 2*). The significant increase in plant height was observed in T0SB as compared to T0 in 30 and 150 days duration only (*Fig. 2*). The similar significant increase in the plant height was also observed in T1SB, T2SB and T3SB as compared to T1, T2 and T3 in all the studied observations. While there was the consistent significant increase in plant height for T4SB as compared to T4 in all durations except the 30 days duration (*Fig. 2*). The non-significant increase in plant height was only observed between the mean of T0, T0SB at 60 days duration (*Table S2*).

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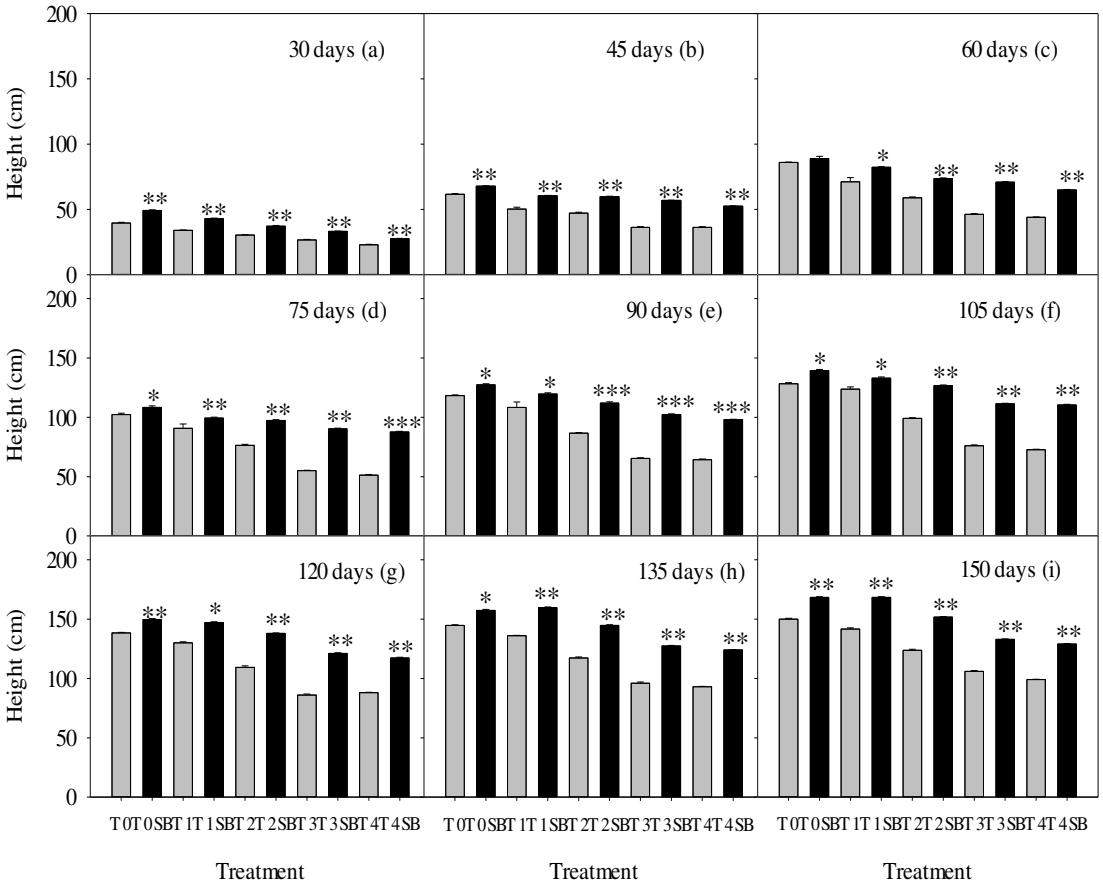

Figure 2. *Mean total height of plants for each treatment with the number of days having 15 days intervals from 30-150 days. Where *** indicate P < 0.0001, ** P< 0.001 and * P < 0.05. Grey bars indicate the soil with Lithium treatment, while black bars indicate the soil with Lithium treatment and also application of bacteria. Error bars indicate SE (±)*

The consistent decrease in the panicle number per plant, panicle length, number of grains and grains yield was also observed across all treatments T0 to T4, while the significant increase in panicle number per plant, panicle length, number of grains and grains yield was observed in all treatments T0SB to T4SB (*Fig. 3*). While there was not the significant difference in the number of panicles for T1 and T1SB, T4 and T4SB, this non-significant difference was observed panicle length for T1 and T1SB (*Table S3*).

Effect of lithium and bacterial inoculation on gas exchange, biomass and lithium contents in sorghum

A significant increase in the rate of maximum photosynthesis, stomatal conductance and rate of transpiration was observed from the initial week to the flowering time till the measurements taken during the seed maturation, the flowering time measurements were also significantly larger than the measurements of seed maturation time in the T0, T0SB to T2, T2SB (*Fig. 4a-i*). While there was a significant increase in the rate of maximum photosynthesis, stomatal conductance and rate of transpiration from the initial week to the flowering time till seed maturation (*Fig. 4j-o, Table S4*).

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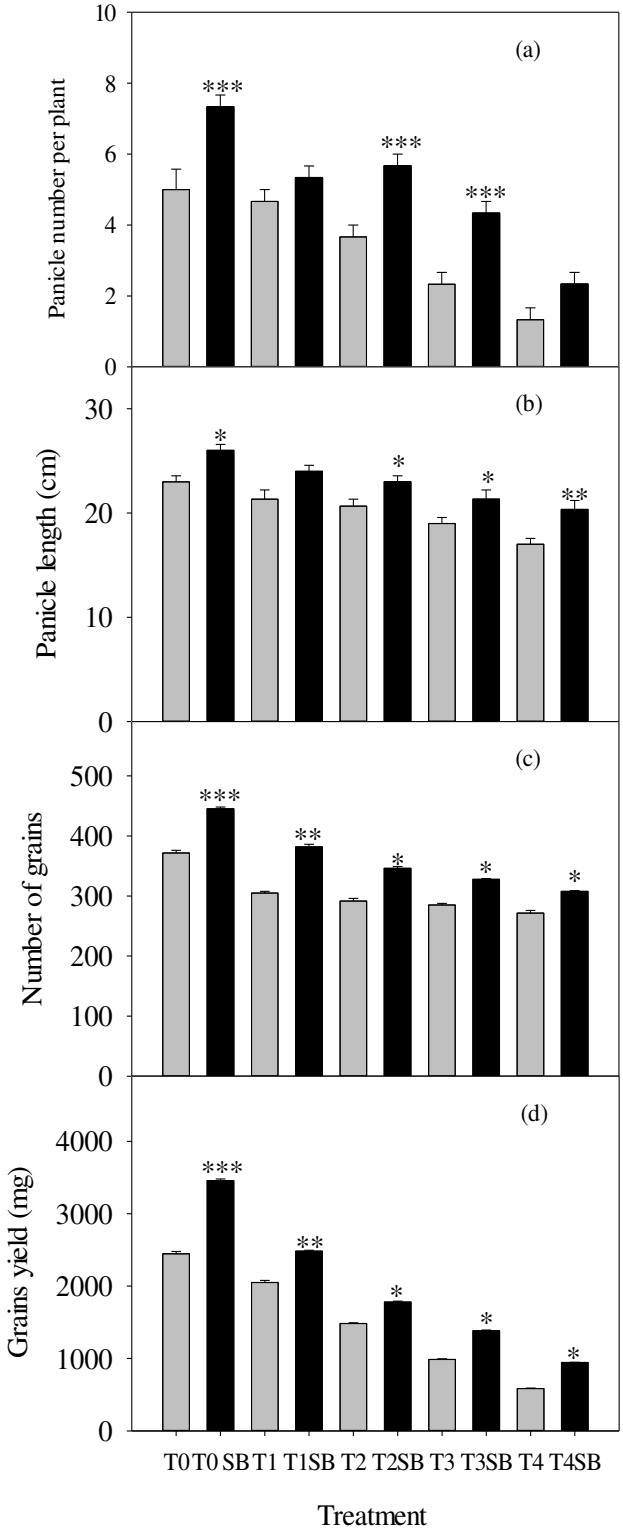

Figure 3. *Mean panicle number per plant (a), panicle length (b), total number of grains per plant (c), and grain yield (d) for each treatment. Where *** indicate P< 0.0001, ** P< 0.001 and * P < 0.05. Grey bars indicate the soil with Lithium treatment, while black bars indicate the soil with Lithium treatment and also application of bacteria. Error bars indicate SE (±)*

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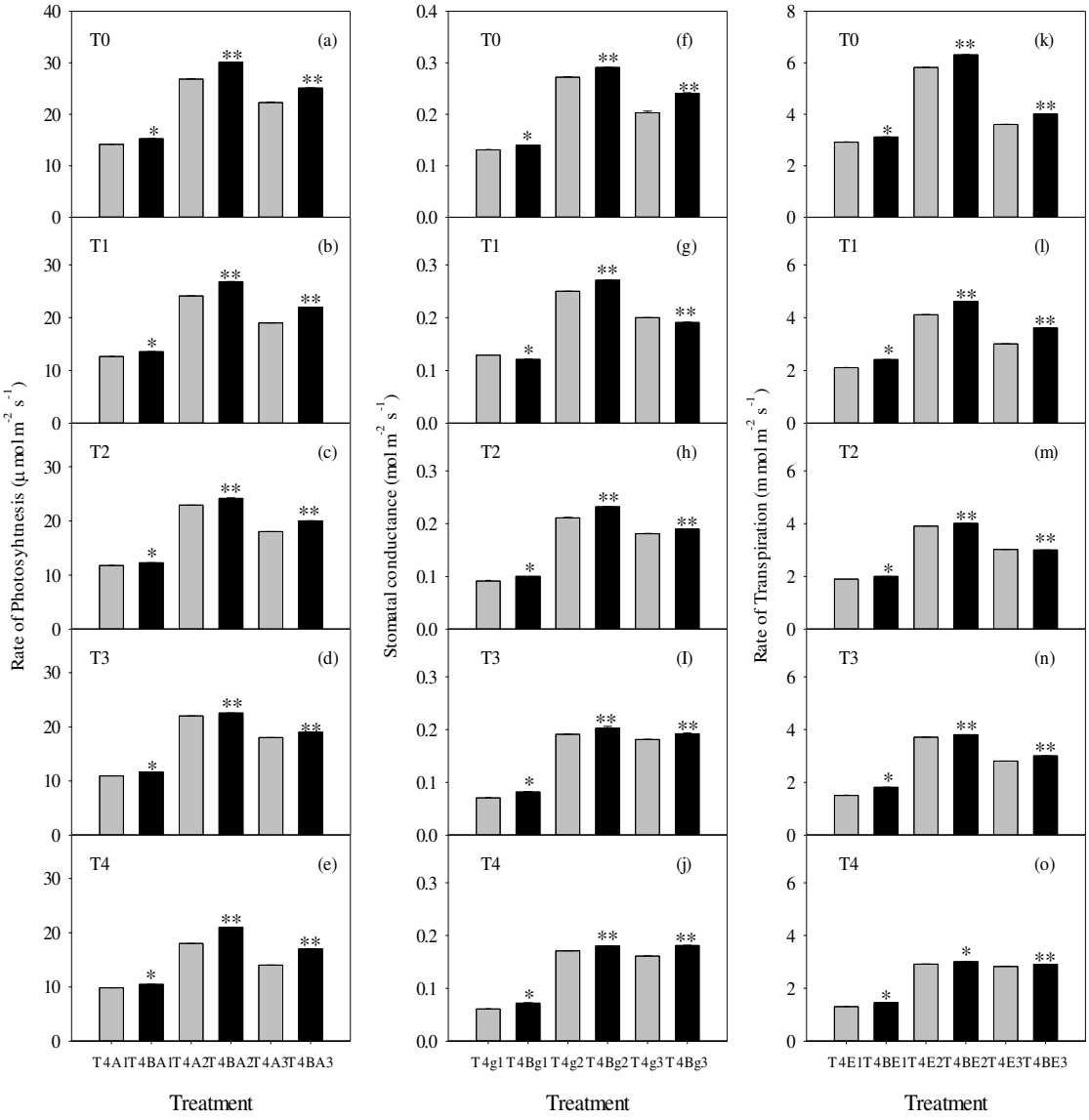

Figure 4. *Mean of the maximum rate of photosynthesis, maximum stomatal conductance, rate of maximum transpiration at the first week of start of experiment, at the time of flowering and before the final harvest with mature seeds. Where *** indicate P < 0.0001, ** P< 0.001 and * P < 0.05. Grey bars indicate the soil with Lithium treatment, while black bars indicate the soil with Lithium treatment and also application of bacteria. Error bars indicate SE (±)*

There was not a significant increase in the shoot and root fresh weight, shoot and root dry weight, and root fresh and dry weight in T0 and T0SB and T1SB (*Fig. 5*). The non-significant difference in root fresh weight was also observed between T1 and T1SB, and T3 and T3SB, as well as between root dry weight of T3 and T3SB and T4 and T4SB. Specific root length and specific shoot length was observed across all the treatments from T0, T0SB to T4, T4SB (*Fig. 5e,f*). The comparison all the remaining treatments indicated the significant differences between the lithium treatments and the bacterial inoculations (*Fig. 5, Table S5*).

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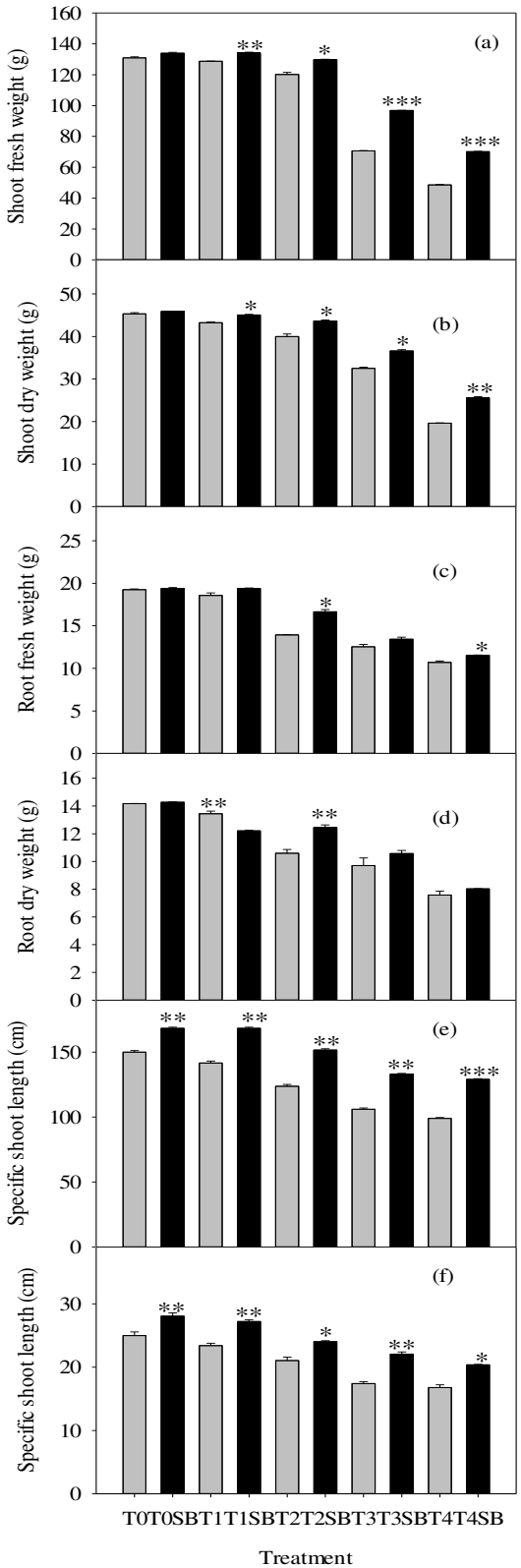

Figure 5. *Mean fresh and dry weight of shoot (a,b) mean fresh and dry weight of root (c,d) mean specific shoot length and root length (e,f) for each treatment. Where *** indicate P < 0.0001, ** P< 0.001 and * P < 0.05. Grey bars indicate the soil with Lithium treatment and black bars indicate the soil with Lithium treatment and also application of bacteria. Error bars indicate SE (±)*

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Similarly, the non-significant difference in chlorophyll a and chlorophyll b was observed between T1 and T1SB, and T0 and T0SB, respectively (*Fig. 6a,c*). For the rest of treatments there was a significant difference in the studied chlorophyll contents (*Table S6*).

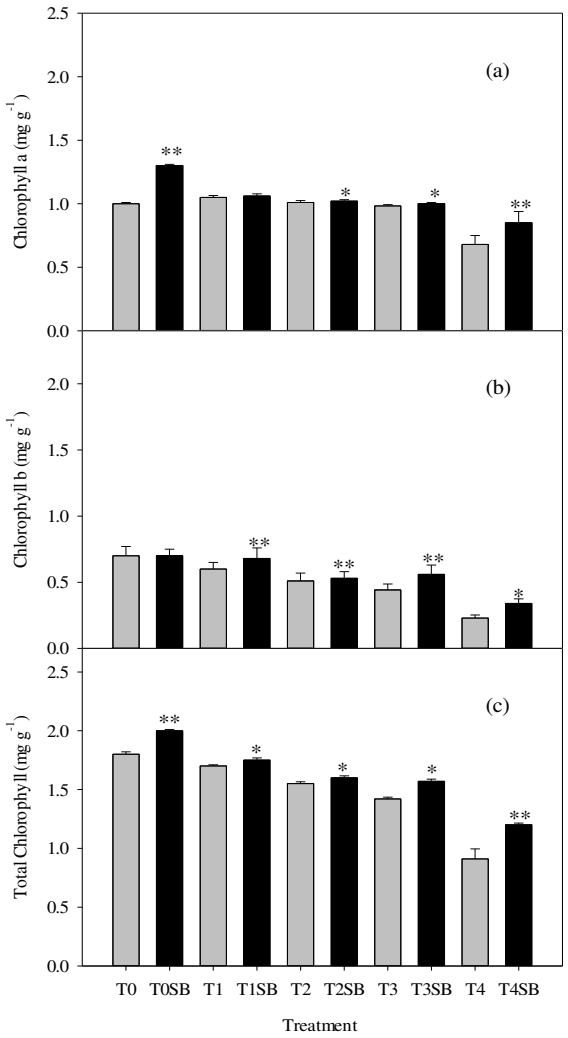

Figure 6. *Mean chlorophyll a (a), chlorophyll b (b) and total chlorophyll (c) for each treatment. Where *** indicate P < 0.0001, ** P< 0.001 and * P < 0.05. Grey bars indicate the soil with Lithium treatment, while black bars indicate soil with Lithium treatment and also application of bacteria. Error bars indicate SE (±)*

A strong significant and positive relationship was observed between lithium concentration in roots with biomass accumulation factor (*Fig. 7a*). But, the slope of relationship was significantly different for the roots of plants with lithium concentration and application bacterial inoculation. Similarly, the lithium concentration in roots was also significantly and positively related to lithium concentration in grains (*Fig. 7b*). The translocation factor was significantly and positively associated with biomass accumulation factor across all the treatments of lithium, while translocation factor was significantly and negatively associated with biomass accumulation factor for the plants having lithium with bacterial inoculation (*Fig. 8a,b*).

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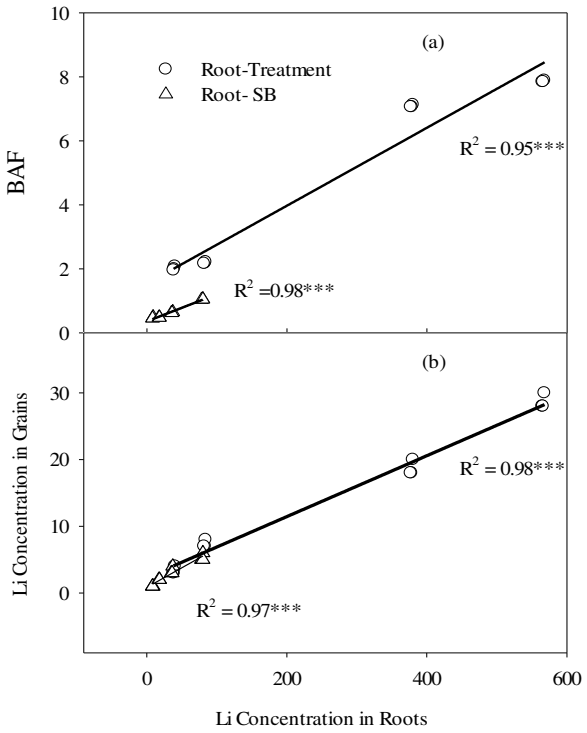

Figure 7. *Relationship of Lithium concentration in roots with Biomass Accumulation Factor (a) and the Lithium concentration in grains (b). Where *** indicate P ≺ 0.0001, ** P≺ 0.001 and * P ≺ 0.05*

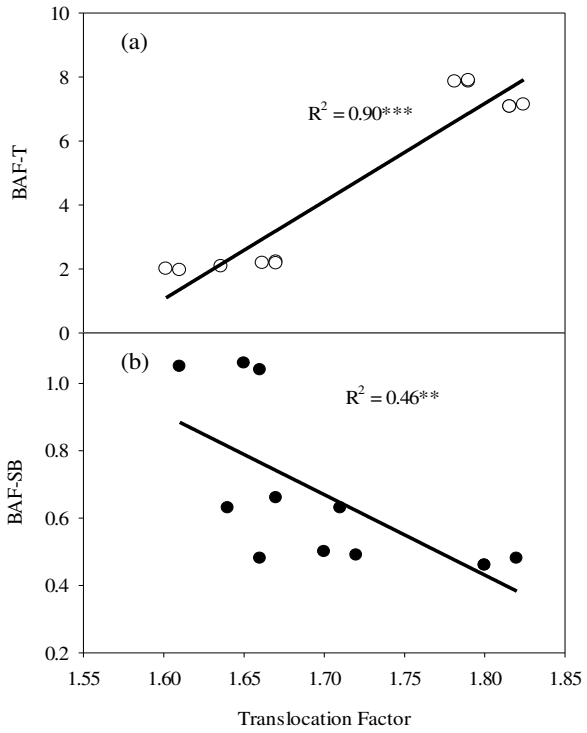

Figure 8. *Relationship of translocation factor with Biomass Accumulation Factor having Lithium treatment (a) and with Biomass Accumulation Factor inoculated with Bacteria (b). Where *** indicate P ≺ 0.0001, ** P≺ 0.001 and * P ≺ 0.05*

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In all the plant parts, roots, shoots and grains the lithium was significantly lower in the plant parts which were inoculated with bacteria as compared to the plant parts having the application of various lithium concentrations (*Fig. 9a-c*). The lithium concentration was maximum in shoot, followed by the root and the minimum lithium was up-taken by the seeds (*Table S7*).

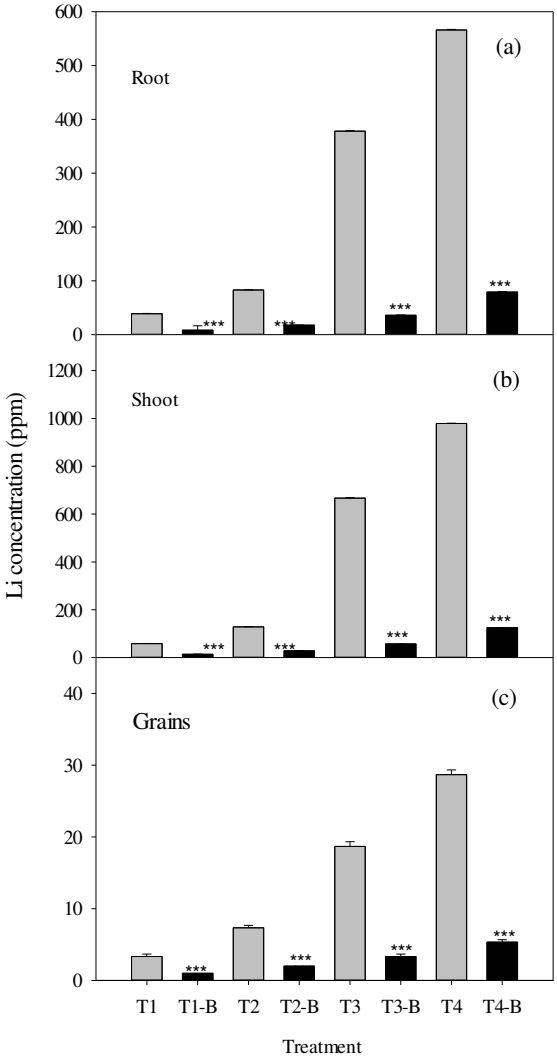

Figure 9. *Lithium concentration in roots (a), shoots (b) and grains (c) for each treatment, where *** indicate P< 0.0001. Grey bars indicate the soil with Li treatment and black bars indicate the soil with Li treatment and also application of bacteria. Error bars indicate SE (±)*

Discussion

Our study demonstrates that the lithium can impact the growth and yield of sorghum, but the application of bacteria can alleviate the impact. This supports our hypothesis and also highlights the importance of lithium resistant bacteria application in the remediating the lithium pollution for the studied species. Our results demonstrate that lithium significantly reduced the growth of plants, but the growth was compensated through the lithium resistant bacteria. As hypothesized, we found the strong relationship of lithium concentration roots and grains. In most of lithium treatments and the observations made

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during the experiment, significant reduction in the number of leaves and plant height, panicle number, number of grains and grain yield indicating the overall reduction in plants growth and yield. This pattern was also reflected in the fresh and dry weight of above and below ground parts of plants. Such reductions indicate that the applied lithium concentration was toxic to sorghum. Our findings support the findings of Aral and Vecchio-Sadus (2008) who found the reduction in Citrus with lithium concentration. While, contradict with Schrauzer (2002), Kabata-Pendias and Mukherjee (2007) reporting the less impact on the growth of plants from Asteraceae, and Solanaceae when the lithium was applied in their soil.

We did not find the significant reduction in the area based rate of maximum photosynthesis, stomatal conductance, and transpiration which could be because of the reduction of number of leaves. The plant can have the adjustments in its gas exchange while exposed to some abiotic stress. The similar pattern was observed for most of treatment on chlorophyll contents, except for the T4, where all the chlorophyll a, b and total chlorophyll showed the significant increase when the bacterial inoculation was applied. Indicating the compensatory effect of bacterial inoculation.

We found that the significant and positive relationship between the lithium concentration in roots and biomass accumulation factor, but the inoculation of bacteria resulted in a significantly reduced slope for the relationship of BAF and lithium concentration in the roots. Indicating a mechanism in which bacteria would have work to reduce the effect of lithium. The similar pattern was observed for the lithium concentration in roots and lithium concentration in grains (Anjum et al., 2015). Such interaction between plant and microbes result in the formation of biofilm around the roots of plant. Successful colonization of microbes in the form of biofilm provides efficient shield to the plants against various stresses. There are different mechanisms involve to cope abiotic and biotic stresses via biofilm formation one of them is the production of exopolysaccharides. These exopolysaccharides are not only helpful against high ionic contents but also retain water and nutrients in these stressful conditions (Qurashi and Sabri, 2012; Kasim et al., 2016). This was supported by the results of our *Fig. 9*, where we observed highly significant reduction in lithium concentration in the plant parts, i.e., root, shoot, and grains. We found that the maximum concentration of lithium was found in shoot, followed by root and the minimum concentration was found in grains. This could be due to the plant mass accumulation to different organs. But in general, our results support the findings of (Jurkowska et al., 1998, 2003) in terms of lithium concentration to different parts of the plant.

Conclusion

Our study concludes that sorghum is susceptible to the lithium, when applied in the soil. As the significant reduction in the growth and yield were observed. Although, there was compensation for the reduction of growth by introducing the lithium resistant bacteria. The findings are useful for the cultivation of sorghum in particular and other cultivated crops in general, especially with reference to the lithium. Mechanisms of tolerance for Li are not yet clear and much work is underway to elucidate mechanisms of tolerance because of the consequences of Li is still poorly represented in many ecosystems. Li-pollution becoming a big environmental issue and having adverse implications for animals and plants like human update to these problems are crucial and require more detailed research.

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DOI: http://dx.doi.org/10.15666/aeer/1806_79898008

Acknowledgements. We gratefully acknowledge the generous support of GC University Lahore Pakistan to finance the present study. The help of gardeners at Botanic Garden is also acknowledged.

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

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SUPPLEMENTARY INFORMATION

Table S1. *One Way ANOVA showing the comparison of mean values number of leaves for each treatment, where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Total number of leaves in different durations					
Treatment	**Duration (Days)**	**Mean (±SE)**	**MS**	**F**	***P***
T0, T0SB	30	2.00 (0.18) - 3.5 (0.18)	6.75	33.75	<0.001
T1 , T1SB	30	2.16 (0.27) - 3.5 (0.18)	5.33	16.0	0.003
T2 , T2SB	30	1.76 (0.09) - 1.6 (0.31)	0.030	0.402	0.54
T3 , T3SB	30	0.76 (0.09) - 1.25 (0.09)	0.701	13.92	0.004
T4 , T4SB	30	0.80 (0.07) - 1.03 (0.07)	0.163	5.05	0.048
T0 , T0SB	45	3.5 (0.18) - 4.5 (0.18)	3.00	15.0	0.003
T1 , T1SB	45	3.66 (0.21) - 4.33 (0.27)	1.33	3.3	0.086
T2 , T2SB	45	2.50 (0.09) - 2.50 (0.18)	0.18	1.5	0.24
T3 , T3SB	45	1.25 (0.09) - 1.80 (0.09)	0.85	16.62	0.002
T4 , T4SB	45	1.08 0.13) - 1.33 (0.10)	0.188	2.04	0.183
T0 , T0SB	60	4.83 (0.27) - 6.16 (0.10)	5.33	20.00	0.001
T1 , T1SB	60	5.16 (0.10) - 5.83 (0.27)	1.33	5.00	0.049
T2 , T2SB	60	2.76 (0.09) - 3.0 (0.18)	0.163	1.30	0.28
T3 , T3SB	60	1.75 (0.09) - 2.25 (0.09)	0.75	15.00	0.003
T4 , T4SB	60	1.58 (0.13) - 1.75 (0.09)	0.083	1.0	0.34
T0 , T0SB	75	5.50 (0.18) - 6.83 (0.10)	5.33	40	< 0.001
T1 , T1SB	75	5.83 (0.10) - 6.83 (0.27)	3.00	11.25	0.007
T2 , T2SB	75	3.10 (0.13) - 3.50 (0.18)	0.48	3.15	0.11
T3 , T3SB	75	2.08 (0.13) - 2.75 (0.09)	1.33	16.0	0.003
T4 , T4SB	75	1.76 (0.09) - 2.75 (0.09)	2.90	57.63	< 0.001
T0 , T0SB	90	6.16 (0.10) - 7.50 (0.18)	5.33	40.0	< 0.001
T1 , T1SB	90	6.17 (0.15) - 7.0 (0.18)	2.08	15.62	0.003
T2 , T2SB	90	3.5 (0.18) - 4.00 (0.18)	0.75	3.75	0.082
T3 , T3SB	90	2.58 (0.13) - 3.41 (0.05)	2.08	31.25	< 0.001
T4 , T4SB	90	2.16 (0.13) - 2.58 (0.13)	0.52	4.46	0.061
T0 , T0SB	105	6.50 (0.18) - 8.16 (0.27)	8.33	25.0	< 0.001
T1 , T1SB	105	6.83 (0.10) - 7.50 (0.18)	1.33	10.0	0.01
T2 , T2SB	105	3.83 (0.23) - 4.43 (0.18)	1.08	4.09	0.071
T3 , T3SB	105	3.17 (0.12) - 4.0 (0.05)	1.92	35.12	< 0.001
T4 , T4SB	105	2.25 (0.09) - 3.33 (0.10)	3.52	60.35	< 0.001
T0 , T0SB	120	7.16 (0.10) - 8.50 (0.18)	5.33	40.00	< 0.001
T1 , T1SB	120	7.33 (0.27) - 7.83 (0.10)	0.75	2.81	0.12
T2 , T2SB	120	4.08 (0.13) -5.06 (0.14)	2.90	23.45	< 0.001
T3 , T3SB	120	3.33 (0.05) - 4.12 (0.10)	2.08	50.00	< 0.001
T4 , T4SB	120	7.66 (0.21) - 8.33 (0.21)	1.33	5.00	0.05
T0 , T0SB	135	7.5 (0.18) - 9.16 (0.27)	8.33	25.00	< 0.001
T1 , T1SB	135	7.66 (0.21) - 8.33 (0.21)	1.33	5.00	0.05
T2 , T2SB	135	4.50 (0.18) - 5.42 (0.19)	2.52	12.10	0.006
T3 , T3SB	135	3.58 (0.13) - 4.75 (0.09)	4.08	49.00	< 0.001
T4 , T4SB	135	2.75 (0.09) - 3.83 (0.10)	3.52	60.35	< 0.001
T0 , T0SB	150	8.33 (0.21) - 9.50 (0.18)	4.08	17.50	0.002
T1 , T1SB	150	7.66 (0.21) - 8.33 (0.21)	1.33	5.00	0.05
T2 , T2SB	150	4.66 (0.21) - 5.66 (0.21)	3.00	11.25	0.007
T3 , T3SB	150	3.75 (0.09) - 4.83 (0.10)	3.52	60.35	< 0.001
T4 , T4SB	150	2.75 (0.09) - 3.83 (0.10)	3.52	60.35	< 0.001

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DOI: http://dx.doi.org/10.15666/aeer/1806_79898008

Table S2. *One Way ANOVA showing the comparison of mean values for total plant height of each treatment, where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Total Plant height in different durations					
Treatment	**Duration (Days)**	**Mean (± SE)**	**MS**	**F**	***P***
T0 , T0SB	30	39.67 (0.55) , 49.33 (0.42)	280.33	191.1	< 0.001
T1 , T1SB	30	34.00 (0.36) , 43.00 (0.36)	243.0	303.75	< 0.001
T2 , T2SB	30	30.50 (0.18), 37.33 (0.42)	140.08	221.18	< 0.001
T3 , T3SB	30	26.66 0.42), 3.33 (0.21)	133.33	200.00	< 0.001
T4 , T4SB	30	23.00 (0.3), 28.00 (0.21)	65.33	122.50	< 0.001
T0 , T0SB	45	61.66 (0.55), 67.66 (0.5)	108.78	57.68	< 0.001
T1 , T1SB	45	50.33 (1.52), 60.33 (0.55)	300.0	38.16	< 0.001
T2 , T2SB	45	47.33 (0.55), 60.0 (0.55)	45.3	244.64	< 0.001
T3 , T3SB	45	36.33 (0.55), 56.66 (0.42)	1240	845	<0.0001
T4 , T4SB	45	36.43 (0.55), 52.11 (0.49)	768	411.6	< 0.001
T0 , T0SB	60	86.0 (0.36), 89.0 (1.67)	27.0	3.06	0.11
T1 , T1SB	60	71.33 (3.16), 82.3 (0.58)	363.0	12.1	0.006
T2 , T2SB	60	59.0 (0.73), 73.66 (0.55)	6455.3	254.7	< 0.001
T3 , T3SB	60	46.3 (0.55), 71.0 (0.36)	1825	1369	< 0.0001
T4 , T4SB	60	44.0 (0.36), 65.0 (0.36)	1323	1653	< 0.0001
T0 , T0SB	75	102.3 (1.11), 108.33 (1.28)	108.0	12.46	0.005
T1 , T1SB	75	90.66 (3.6), 99.3 (0.76)	225.3	5.28	0.04
T2 , T2SB	75	76.33 (0.91), 97.33 (0.76)	1323	310.07	< 0.0001
T3 , T3SB	75	55.0 (0.36), 90.33 (0.55)	3745	2809	< 0.0001
T4 , T4SB	75	51.33 (0.55), 87.66 (0.61)	3960	2121	< 0.0001
T0 , T0SB	90	118.33 (0.55), 127.3 (0.91)	243	70.1	< 0.01
T1 , T1SB	90	108.3 (4.5), 119.6 (0.91)	385.3	5.92	0.035
T2 , T2SB	90	86.66 (0.55), 112.0 (0.96)	1925	515.7	< 0.0001
T3 , T3SB	90	65.21 (0.35), 102.3 (0.85)	4107	2200	< 0.0001
T4 , T4SB	90	64.33 (0.41), 98.0 (0.36)	3400	2550	< 0.001
T0 , T0SB	105	128.3 (0.76), 139.3 (0.91)	363	85.0	< 0.001
T1 , T1SB	105	123.6 (2.0), 133.0 (0.96)	261.3	17.5	0.002
T2 , T2SB	105	99.0 (0.73), 126.7 0.61)	2296	906	< 0.0001
T3 , T3SB	105	76.0 (0.73), 111.3 (0.55)	3745	1478	< 0.0001
T4 , T4SB	105	73.0 (0.42), 110.3 (0.51)	4256	2902	< 0.0001
T0 , T0SB	120	138.3 (0.58), 149.6 (0.76)	385.3	144.5	< 0.001
T1 , T1SB	120	130.0 (0.96), 147.0 (0.73)	867.0	195.0	< 0.001
T2 , T2SB	120	109.3 (1.2), 138.0 (0.36)	2465	462	< 0.0001
T3 , T3SB	120	86.0 (0.96), 121.0 (0.63)	3675	918	< 0.001
T4 , T4SB	120	88.0 (0.36), 117.3 (0.55)	2581	1936	< 0.001
T0 , T0SB	135	144.6 (0.55), 157.3 (0.91)	481.3	138.8	< 0.001
T1 , T1SB	135	136.0 (0.36), 159.6 (0.55)	1680	1260	< 0.001
T2 , T2SB	135	117.3 (0.76), 144.6 (0.55)	2241	840.5	< 0.001
T3 , T3SB	135	96.0 (1.0), 127.3 (0.49)	2945	788	< 0.001
T4 , T4SB	135	93.0 (0.36), 124.0 (0.40)	2883	3603	< 0.001
T0 , T0SB	150	150.0 (0.73), 168.3 (0.58)	1008	398	< 0.001
T1 , T1SB	150	141.6 (0.91), 168.3 (0.50)	2133	615	< 0.001
T2 , T2SB	150	123.6 (0.91), 151.6 (0.41)	2352	6778.4	< 0.001
T3 , T3SB	150	106.0 (0.63), 133.0 (0.36)	2187	1366	< 0.0001
T4 , T4SB	150	99.0 (0.36), 129.0 (0.29)	2700	3375	< 0.0001

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Table S3. *One Way ANOVA showing the comparison of mean values of number of Panicles, Panicle length, number of grains per Panicle and total number of grains per plant. Where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Number of Panicles				
Treatment	**Mean (± SE)**	**MS**	**F**	***P***
T1 , T1SB	4.66 (0.33), 5.33 (0.33)	0.67	2.00	0.23
T2 , T2SB	3.66 (0.33), 5.66 (0.33)	6.00	188.00	0.013
T3 , T3SB	2.33 (0.33), 4.33 (0.33)	6.00	18.00	0.013
T4 , T4SB	1.33 (0.33), 2.33 0.33)	1.50	4.50	0.10
Panicle Length				
T0 , T0SB	23.0 (0.5), 26.0 (0.57)	13.50	13.5	0.021
T1 , T1SB	21.3 (0.88), 24.0 (0.57)	10.66	6.40	0.065
T2 , T2SB	20.66 (0.66), 23.00 (0.58)	8.16	7.00	0.057
T3 , T3SB	19.00 (0.6), 21.33 (0.88)	8.16	4.90	0.091
T4 , T4SB	17.0 (0.51), 20.33 (0.90)	16.7	10.00	0.034
Number of grains per Panicle				
T0 , T0SB	371.66 (4.41), 445.0 (2.88)	8066	193.6	< 0.001
T1 , T1SB	305.00 (2.88), 381.66 (4.41)	8816	211	< 0.001
T2 , T2SB	291.66 (4.41), 346.0 (3.05)	4428	102.5	< 0.001
T3 , T3SB	285.0 (2.88), 327.6 (1.45)	2730	174.2	< 0.001
T4 , T4SB	271.6 (4.41), 307.6 (1.46)	1944	60.12	0.001
Grains per plant				
T0 , T0SB	2446.6 (31.79), 3458.3 (22.04)	135204	683.57	< 0.001
T1 , T1SB	2050 (28.68), 2483.33 (12.09)	281666	192.04	< 0.001
T2 , T2SB	1483.3 (8.81),, 1780.0 (11.54)	132016	416.89	< 0.001
T3 , T3SB	986.67 (8.82), 1383 (8.89)	236016	1011	< 0.001
T4 , T4SB	583.33 (8.81), 945.0 (2.88)	196204	1519	< 0.001

Table S4. *One Way ANOVA showing the comparison of mean values for Photosynthetic rate, transpiration, and stomatal conductance for each treatment in three different durations. Where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Photosynthetic rate					
Treatment	**Duration (Days)**	**Mean (± SE)**	**MS**	**F**	***P***
T0 , T0SB	Initial Week	14.73 (0.01), 15.23 (0.01)	1.8	2317	< 0.001
T1 , T1SB	Initial Week	12.65 (0.02), 13.57 (0.02)	1.25	987	< 0.001
T2 , T2SB	Initial Week	11.7 (0.01), 12.28 (0.001)	0.385	888	< 0.001
T3 , T3SB	Initial Week	10.93 (0.03), 11.67 (0.01)	0.700	362.28	< 0.001
T4 , T4SB	Initial Week	9.83 (0.01), 10.43 (0.001)	0.634	1086	< 0.001

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79898008

Photosynthetic rate					
Treatment	**Duration (Days)**	**Mean (± SE)**	**MS**	**F**	***P***
T0 , T0SB	Flowering time	26.83 (0.08), 30.10 (0.01)	16.07	1369	< 0.001
T1 , T1SB	Flowering time	24.12 (0.01), 26.83 (0.01)	10.98	19392	< 0.001
T2 , T2SB	Flowering time	22.90 (0.01), 24.16 (0.01)	2.35	199.96	< 0.001
T3 , T3SB	Flowering time	22.01 (0.01), 22.57 (0.01)	0.48	810.47	< 0.001
T4 , T4SB	Flowering time	18.01 (0.01), 21.02 (0.01)	13.56	31292	< 0.001
T0 , T0SB	Seed formation	22.26 (0.14), 25.10 (0.06)	12.42	328.49	< 0.001
T1 , T1SB	Seed formation	19.0 (0.01), 22.0 (0.01)	13.37	50512	< 0.001
T2 , T2SB	Seed formation	18.0 (0.01), 20.0 (0.00)	5.94	23760	< 0.001
T3 , T3SB	Seed formation	18.0 (0.00), 19.01 (0.01)	1.54	11552	< 0.001
T4 , T4SB	Seed formation	14.0 (0.01), 17.02 (0.01)	13.44	26013	< 0.001
Transpiration					
T0 , T0SB	Initial Week	2.92 (0.01), 3.11 (0.00)	0.058	183.21	< 0.001
T1 , T1SB	Initial Week	2.11 (0.00), 2.41 (0.01)	0.138	828	< 0.001
T2 , T2SB	Initial Week	1.90 (0.00), 2.00 (0.00)	0.016	961.0	< 0.001
T3 , T3SB	Initial Week	1.50 (0.01), 1.81 (0.01)	0.138	637.0	< 0.001
T4 , T4SB	Initial Week	1.31 (0.00), 1.45 (0.01)	0.032	276.57	< 0.001
T0 , T0SB	Flowering time	5.81 (0.01), 6.31 (0.01)	0.365	1152	< 0.001
T1 , T1SB	Flowering time	4.11 (0.01), 4.61 (0.01)	0.3775	2045	< 0.001
T2 , T2SB	Flowering time	3.91 (0.00), 4.01 (0.00)	0.015	150.0	< 0.001
T3 , T3SB	Flowering time	3.71 (0.00), 3.80 (0.00)	0.0131	196.0	< 0.001
T4 , T4SB	Flowering time	2.91 (0.01), 3.01 (0.00)	0.014	84.10	< 0.001
T0 , T0SB	Seed formation	3.60 (0.01), 4.01 (0.01)	0.248	13353	< 0.001
T1 , T1SB	Seed formation	3.01 (0.00), 3.61 (0.00)	0.540	5400.0	< 0.001
T2 , T2SB	Seed formation	3.023 (0.00), 3.00 (0.00)	0.00	18.0	0.013
T3 , T3SB	Seed formation	2.80 (0.00), 3.00 (0.00)	0.06	1800	< 0.001
T4 , T4SB	Seed formation	2.82 (0.01), 2.91 (0.00)	0.0104	28.40	0.006
Stomatal Conductance					
T0 , T0SB	Initial Week	0.131 (0.00), 0.140 (0.00)	0.00	168.2	< 0.001
T1 , T1SB	Initial Week	0.130 (0.00), 0.121 (0.00)	0.00	81.0	< 0.001
T2 , T2SB	Initial Week	0.091 ((0.00), 0.10 (0.00)	0.00	42.5	0.003
T3 , T3SB	Initial Week	0.07 (0.00), 0.08 (0.00)	0.00	64.05	0.001
T4 , T4SB	Initial Week	0.061 (0.00), 0.072 (0.00)	0.00	90.75	< 0.001
T0 , T0SB	Flowering time	0.272 (0.001), 0.291 (0.001)	0.000	196.0	< 0.001
T1 , T1SB	Flowering time	0.250 (0.00), 0.271 (0.00)	0.000	496.12	< 0.001
T2 , T2SB	Flowering time	0.211 (0.00), 0.232 (0.00)	0.00	240.25	< 0.001
T3 , T3SB	Flowering time	0.91 (0.00), 0.203 (0.00)	0.00	13.29	0.022
T4 , T4SB	Flowering time	0.171 (0.00), 0.180 (0.00)	0.00	420.50	< 0.001
T0 , T0SB	Seed formation	0.20 (0.00), 0.241 (0.00)	0.00	121.45	< 0.001
T1 , T1SB	Seed formation	0.200 (0.00), 0.191(0.00)	0.00	168.20	< 0.001
T2 , T2SB	Seed formation	0.181 (0.00), 0.190 (0.00)	0.00	196.0	< 0.001
T3 , T3SB	Seed formation	0.181 (0.00), 0.192 (0.00)	0.00	72.25	0.001
T4 , T4SB	Seed formation	0.161 (0.00), 0.181 (0.00)	0.00	531.57	< 0.001

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79898008

Table S5. *One Way ANOVA showing the comparison of mean values of shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, specific shoot length, and specific root length across different treatments. Where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Shoot Fresh weight				
Treatment	**Mean (± SE)**	**MS**	**F**	***P***
T1 , T1SB	128.7 (0.17), 134.20 (0.41)	44.82	146.2	< 0.001
T2 , T2SB	120.21 (1.31), 129.6 (0.40)	133.95	47.13	0.002
T3 , T3SB	70.66 (0.20), 96.90 (0.057)	1032.0	14770	< 0.001
T4 , T4SB	48.56 (0.29), 70.13 (0.24)	697.68	3195.48	< 0.001
Root Fresh weight				
T0 , T0SB	19.25 (0.081), 19.40 (0.098)	0.0353	1.44	0.296
T1 , T1SB	18.57 (0.29), 19.39 (0.058)	1.009	7.52	0.052
T2 , T2SB	13.92 (0.03), 16.64 (0.26)	11.12	107.40	< 0.001
T3 , T3SB	12.53 (0.29), 13.43 (0.23)	1.21	5.83	0.073
T4 , T4SB	10.73 (0.17), 11.54 (0.02)	1.067	23.82	0.008
Shoot Dry weight				
T0 , T0SB	45.33 (0.26), 45.87 (0.057)	0.443	3.96	0.11
T1 , T1SB	43.25 (0.14), 45.06 (0.12)	4.95	93.55	< 0.001
T2 , T2SB	40.00 (0.57), 43.63 (0.27)	19.83	32.57	0.005
T3 , T3SB	32.50 (0.26), 36.60 (0.306)	25.21	102.91	< 0.001
T4 , T4SB	19.65 (0.038), 25.56 (0.29)	52.45	391.76	< 0.001
Root Dry weight				
T0 , T0SB	14.17 (0.00), 14.27 (0.024)	0.015	15.25	0.017
T1 , T1SB	13.44 (0.19), 12.20 (0.05)	2.29	37.0	0.004
T2 , T2SB	10.59 (0.26), 12.45 (0.18)	5.17	32.36	0.005
T3 , T3SB	9.70 (0.55), 10.57 (0.22)	1.127	2.11	0.22
T4 , T4SB	7.58 (0.28), 8.03 (0.01)	0.299	2.415	0.195
Specific Shoot length				
T0 , T0SB	150.00 (1.15), 168.3 (0.88)	504.16	159.21	< 0.001
T1 , T1SB	141.66 (1.45), 168.33 (0.88)	1066	246	< 0.001
T2 , T2SB	123.66 (1.45), 151.66 (0.88)	1176	271.38	< 0.001
T3 , T3SB	106.00 (1.00), 133.0 (0.57)	1093	546.75	< 0.001
T4 , T4SB	99.0 (0.57), 129.0 (0.57)	1350	1350	< 0.001
Specific Root length				
T0 , T0SB	25.0 (0.57), 28.11 (0.61)	14.10	15.55	0.02
T1 , T1SB	23.40 (0.37), 27.23 (0.29)	21.88	6.70	0.001
T2 , T2SB	21.05 (0.53), 24.40 (0.15)	13.38	29.17	0.006
T3 , T3SB	17.42 (0.29),, 22.08 (0.32)	32.48	111.336	< 0.001
T4 , T4SB	16.80 (0.41), 20.37 (0.06)	19.11	71.69	0.001

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79898008

Table S6. *One Way ANOVA showing the comparison of mean values of chlorophyll a, chlorophyll b, and total chlorophyll across different treatments. Where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Chlorophyll a				
Treatment	**Mean (± SE)**	**MS**	**F**	***P***
T1 , T1SB	1.20 (0.15), 1.06 (0.001)	0.028	0.855	0.408
T2 , T2SB	1.01 (0.001), 1.02 (0.001)	0.000	45.0	0.003
T3 , T3SB	0.98 (0.00), 1.009 (0.004)	0.001	30.48	0.005
T4 , T4SB	0.68 (0.00), 0.85 (0.00)	0.043	26112	< 0.001
Chlorophyll b				
T0 , T0SB	0.703 (0.00), 0.703 (0.00)			1.00
T1 , T1SB	0.60 (0.001), 0.682 0.001)	0.0096	1873	< 0.001
T2 , T2SB	0.512 (0.00), 0.531 (0.00)	0.000	336	< 0.001
T3 , T3SB	0.44 (0.00), 0.56 0.00)	0.021	9257	< 0.001
T4 , T4SB	0.23 (0.00), 0.34 (0.00)	0.0181	18150	< 0.001
Total Chlorophyll				
T0 , T0SB	1.80 (0.003), 2.03 (0.03)	0.079	47.13	0.002
T1 , T1SB	1.71 (0.005), 1.75 (0.00)	0.002	51.53	0.002
T2 , T2SB	1.55 (0.00), 1.61 (0.001)	0.0057	84.09	< 0.001
T3 , T3SB	1.41 (0.005), 1.57 (0.002)	0.039	705.37	< 0.001
T4 , T4SB	0.91 (0.00), 1.22 (0.01)	0.143	714.322	< 0.001

Table S7. *One Way ANOVA showing the comparison of mean values of lithium concentration in shoot, lithium concentration in root, and lithium concentration in grains. Where T0, indicates the soil without lithium and bacterial inoculation, T1, T2, T3, T4 indicate the application of different concentration of lithium and T0SB, indicates the soil without lithium application but with the inoculation of bacteria, T1SB, T2SB, T3SB and T4SB indicate the different concentrations of lithium with the application of bacterial inoculations*

Lithium Concentration (Shoot)				
Treatment	**Mean (± SE)**	**MS**	**F**	***P***
T1 , T1SB	1288.68 (0.70), 28.70 (0.43)	15000	18000	< 0.0001
T2 , T2SB	667.0 (1.52), 57.00 (0.61)	55815	139537	< 0.0001
T3 , T3SB	978.66 (1.2), 125.0 (0.57)	1093120	409920	< 0.0001
Lithium Concentration (Root)				
T0 , T0SB	39.0 (0.6), 8.33 (0.33)	1410	2116	< 0.0001
T1 , T1SB	83.0 (0.61), 17.66 (0.355)	6402	9604	< 0.001
T2 , T2SB	378.33 (0.88), 36.0 (0.60)	17755788	105472	< 0.0001
T3 , T3SB	566.35 (0.88), 79.35 (0.67)	355753	194047	< 0.0001
Lithium Concentration (Grains)				
T0 , T0SB	3.33 (0.25), 1.00 (0.00)	8.16	49.0	0.002
T1 , T1SB	7.33 (0.31), 2.00 (0.00)	42.66	256.0	< 0.001
T2 , T2SB	18.66 (0.71), 3.33 (0.21)	352.66	423.20	< 0.001
T3 , T3SB	28.66 (0.66), 5.33 (0.33)	816.67	980.00	< 0.001

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_79898008

META-ANALYSIS OF THE HYPOCHOLESTEROLEMIC POTENTIALS OF TURMERIC (*CURCUMA LONGA*) IN BROILER CHICKENS

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(Received 12th May 2020; accepted 16th Sep 2020)

Abstract. The purpose of this experiment was to use a meta-analysis approach to resolve conflict, identify knowledge gap and develop new insights on the effect of turmeric on plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) concentrations in broiler chickens. The authors found 64 studies following a systematic search conducted in 3 electronic databases and the World Wide Web using several search queries. Eighteen out of the 64 publications met the predefined eligibility criteria to assess the impact of turmeric on plasma TC, HDL-c and LDL-c concentrations in broiler chickens. Data collected were analysed in OpenMEE software and pooled estimation revealed that turmeric-based diets significantly reduced the concentrations of TC and LDL-c ($p<0.01$), and improved the concentrations of HDL-c ($p<0.01$) in chicken blood when compared to those on the control diets. Sub-analysis found significant associations between covariates (chicken strain, supplementation level, number of birds per treatment and duration of supplementation) and measured outcomes (TC, HDL-c and LDL-c). Significant heterogeneity (I^2) existed in the meta-analysis, and meta-regression analysis found that chicken strain and duration of supplementation were predictors of HDL-c and LDL-c, and explained 72% of the sources of heterogeneity among the studies used in the meta-analysis. The results of the present meta-analysis showed the potential of turmeric-based diets to modulate the production of blood cholesterol in broiler chickens.
Keywords: *turmeric, meat-typed broilers, cholesterol, data synthesis, meta-regression, funnel plot*

Introduction

Currently, herbs and spices such as ginger, garlic and turmeric are receiving attention in chicken production because of their positive health benefits. Turmeric (*Curcuma longa* L.), a member of Zingiberaceae family is used in preparations of human foods and native medicines. It is low in macro-nutrients, moderate in fibre and rich in essential oils. Turmeric is a potent source of curcuminoids and possesses antioxidative, anti-inflammatory, antihepatotoxic and anticarcinogenic properties and may be used as a phytoadditive in animal feed. The beneficial effects of turmeric in chickens include improvement of immune response, reduction of negative effects of heat stress via several mechanisms, enhancement of antioxidant activity, reduction in the number of harmful microbes in the gut, and improvement of blood indices which are linked to its rich phytochemicals (Jurenka, 2009; Rahimi and Kazemi-Oskuee, 2014). Addition of turmeric in animal diet has been found to enhance growth performance in broiler chickens (Emadi and Kermanshahi, 2006; Adegoke et al., 2018) and improve reproductive efficiency in rabbits (Ogbuewu et al., 2017). Emadi et al. (2007) have reported low plasma LDL-c value and improved plasma HDL-c value in broiler chickens fed turmeric at 7.5 g/kg feed. Furthermore, the incorporation of turmeric at 2 g/kg feed lowers plasma triglyceride, TC and LDL-c concentrations in laying hens (Kermanshahi and Riasi, 2006). Others have

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80098021

reported the influence of turmeric on blood cholesterol values of broiler chickens (Akbarian et al., 2012; Alagawany, 2015; Arslan et al., 2017; Choudhury et al., 2018). However, these results were at variance with one another and the variability may depend on factors such as dietary components, inclusion rate, feeding duration, age and stocking density among others. The use meta-analysis approach to resolve conflicting results among authors have been reported (Ressing et al., 2009). Meta-analysis is an inferential statistical method that enables the results of individual studies to be pooled and quantitatively analysed to reach conclusion that ordinarily could not be drawn from individual primary studies or narrative reviews. To our knowledge, no meta-analysis has investigated the effect of turmeric on health indices of broiler chickens. Therefore, this meta-analysis aimed to determine the effect of turmeric on plasma cholesterol characteristics in broiler chickens as to aid evidence-based decision-making.

Materials and Methods

The meta-analysis was conducted in the Department of Agriculture and Animal Health, University of South Africa, Florida Science Campus, South Africa. To realize the objectives of the current research, the authors searched Scopus, Google scholar and AGORA databases and the World Wide Web for studies that investigated the effect of turmeric on plasma TC, HDL-c and LDL-c concentrations in broiler chickens using combinations of different search queries. The search terms were turmeric, broiler chickens, plasma cholesterol, serum cholesterol and blood cholesterol. The search was conducted using Boolean logic operators (AND, NOT and OR), wildcat (* or $), alternate spellings (?) and proximity searching ("…" or "…."). Our search yielded 64 publications of which 18 met the apriori selection criteria. For an article to be included in the analysis, the experiment must have control treatment (without turmeric) and experimental treatments (with turmeric). The diet must be free of growth-promoting substances. The study is written in English and reported at least one of these outcomes (TC, LDL-c or HDL-c) in healthy chickens with their corresponding measures of dispersion such as standard deviation (SD) or standard error (SE). In a condition, where SE of the mean was reported instead of SD, the SD value was calculated from SE using standard method (Higgins and Deeks, 2011). Information on the surname of first author/year of publication, outcomes of interest (TC, HDL-c and LDL-c) with their corresponding SD as well as our chosen covariates (strain, supplementation level, duration of supplementation and the number of birds included in each treatment group) were extracted from each paper that fulfilled the eligibility criteria. This meta-analysis was conducted following standard protocols as described by Koricheva et al. (2013). Standardized effect sizes were estimated using standardized mean difference (SMD), and SMD was said to significant at $p < 0.01$ (Koricheva et al., 2013). We employed 2 random effect model in the study with Model I being a full model:

$$Y_i = \mu + T_i + e_i \quad \text{(Eq.1)}$$

where,
Y_i: Response variable (TC, LDL-c and HDL-c) for the i^{th} study (i = I, 2, ….., N), N is the number of study included in the analysis,
μ: Overall mean,
T_i: Treatment effect (dietary turmeric),

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80098021

e_i: Random error associated with observation *i*.

Model II = Model I + effects for potential of between-study variance across studies:

$$Y_{ijklm} = \mu + T_i + S_j + D_k + C_l + B_m + e_{ijklm} \quad \text{(Eq.2)}$$

where,
Y_{ijklm}: Measure for the i^{th} study (i = I, 2, ….., N),
μ: Overall mean,
S_j: Effect of j^{th} strain (j = 5; Hubbard, Ross, Cobb, Arbor acre and Anak),
D_k: Effect of k^{th} duration of supplementation (k = 1 to 56 days),
C_l: Effect of l^{th} supplementation level (l = 0.2 g/kg feed ≥ n ≤ 15 g/kg feed),
B_m: Effect of m^{th} number of broiler chickens included in each treatment group (m = 10 ≥ n ≤ 108),
e_{ijklm}: Random error.

Pooled effects estimate with 95% confidence interval (CI) for the TC, HDL-c and LDL-c were presented in forest plots using OpenMEE software (Wallace et al., 2016). Funnel plot method and Rosenberg's fail-safe number (Nfs) were used to identify the presence of publication bias. Jennions et al. (2013) have reported the robustness of meta-analysis results despite the presence of publication bias if Nfs is greater than (5n + 10), where, n = number of comparisons. We used Q statistics (Hedges and Olkin, 1985) and I^2 statistics (Higgins et al., 2003) to identify the presence of heterogeneity across studies, whereas sources of heterogeneity were quantified using meta-regression analysis.

Results

Overview of studies included in the analysis

Our search yielded 64 articles of which 18 papers with 2200 broiler chickens and 54 comparisons met the predefined inclusion criteria for the meta-analysis (*Table 1*). The articles used for the analysis span 12 years with the first study published in 2007 and the most recent published in 2018.

Meta-analysis and analysis of covariates

Pooled estimation revealed significantly ($p<0.01$) lower TC (SMD = − 0.332, I^2 = 90.74%; *Fig. 1*) in broiler chickens fed turmeric diets compared to those fed control diets. In addition, statistically increased ($p<0.01$) HDL-c (SMD = 0.581, I^2 = 90.29%; *Fig. 2*) were recorded for broiler chickens on treatment diets when compared with broiler chickens on control diets. LDL-c (SMD = − 0.331 mg/dl; *Fig. 3*) was lower in broiler chickens fed turmeric-based diets as compared to those fed control diets, however, the difference was not significant ($p>0.01$). There was presence large heterogeneity among the studies used in the analysis as presented in *Figs. 1, 2* and *3* (results from Model I). In an effort to determine the actual turmeric dose that optimizes blood cholesterol concentrations in broiler chickens, the authors chose 2 levels of supplementation (i.e. < 6 g/kg feed and > 6 g/kg feed) based on the mean inclusion levels reported in the studies included in the meta-analysis. The results of the effect of turmeric on plasma TC in broiler chickens are presented in *Table 2*. Chickens fed turmeric diet at > 6 g/kg feed and < 6 g/kg feed had significantly reduced TC. Similarly, there was no significant association between the plasma TC and the studied covariates.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80098021

There was a significant relationship between TC and duration of supplementation with chickens from studies that fed turmeric for 42 days recording lower value than studies that fed turmeric for 21, 35 and 56 days.

Table 1. *Studies used to evaluate the effect of turmeric on TC, HDL-c and LDL-c in broiler chickens*

References	Moderators				Outcomes
	Strain	SL (g/kg)	DOS (day)	No. of birds per treatment	
Adegoke et al 2018	Arbor acres	<6	42	<46	TC, HDL-c
Adegoke et al 2018	Arbor acres	<6	42	<46	TC
Hussein 2013	Ross	<6	42	>46	TC
Hussein 2013	Ross	>6	42	>46	TC
Hussein 2013	Ross	>6	42	>46	TC
Abou-Elkhair et al 2014	Cobb	<6	35	<46	TC
Fallah & Mirzaei 2016	Ross	<6	42	>46	TC, HDL-c, LDL-c
Nouzarian et al 2011	Ross	<6	42	>46	TC, HDL-c, LDL-c
Nouzarian et al 2011	Ross	>6	42	>46	TC, HDL-c, LDL-c
Nouzarian et al 2011	Ross	>6	42	>46	TC, HDL-c
Choudhury et al 2018	Arbor acres	<6	42	<46	TC, HDL-c
Choudhury et al 2018	Arbor acres	<6	42	<46	TC, HDL-c
Choudhury et al 2018	Arbor acres	>6	42	<46	TC
Ukoha and Onunkwo 2016	Anak	>6	56	<46	TC
Ukoha and Onunkwo 2016	Anak	>6	56	<46	TC
Ukoha and Onunkwo 2016	Anak	>6	56	<46	TC
Alagawany et al 2015	Hubbard	<6	35	<46	TC, LDL
Alagawany et al 2015	Hubbard	<6	35	<46	TC, HDL-c, LDL-c
Arslan et al 2017	Hubbard	<6	35	<46	TC, HDL-c, LDL-c
Arslan et al 2017	Hubbard	>6	35	<46	TC, HDL-c, LDL-c
Arslan et al 2017	Hubbard	>6	35	<46	TC, HDL-c, LDL-c
Emadi et al 2007	Ross	<6	21	>46	TC, HDL-c, LDL-c
Emadi et al 2007	Ross	<6	21	>46	TC, HDL-c, LDL-c
Emadi et al 2007	Ross	>6	21	>46	TC, HDL-c, LDL-c
Emadi et al 2007	Ross	<6	35	>46	TC, HDL-c, LDL-c
Emadi et al 2007	Ross	<6	35	>46	TC, HDL-c
Emadi et al 2007	Ross	>6	35	>46	TC, HDL-c
Emadi et al 2007	Ross	<6	42	>46	TC, HDL-c
Emadi et al 2007	Ross	<6	42	>46	HDL-c
Emadi et al 2007	Ross	>6	42	>46	HDL-c
Kamdev et al. 2016	Cobb	<6	21	<46	TC
Kamdev et al. 2016	Cobb	>6	42	<46	TC
Mehala & Moorthy 2008	Cobb	<6	42	<46	TC, HDL-c
Mehala & Moorthy 2008	Cobb	<6	42	<46	TC, HDL-c
Mehala & Moorthy 2008	Cobb	<6	42	<46	HDL-c
Mehala & Moorthy 2008	Cobb	<6	42	<46	HDL-c
Tirupati Reddy et.al. 2012	Cobb	<6	42	<46	TC
Tirupati Reddy et.al. 2012	Cobb	<6	42	<46	TC
Ratika et al. 2018	Ross	<6	21	<46	TC
Daneshyar et al. 2011	Ross	<6	21	>46	TC, HDL-c, LDL-c
Daneshyar et al. 2011	Ross	<6	21	>46	TC, HDL-c, LDL-c
Daneshyar et al. 2011	Ross	>6	21	>46	TC, HDL-c, LDL-c
Daneshyar et al. 2011	Ross	<6	42	>46	TC, HDL-c, LDL-c
Daneshyar et al. 2011	Ross	<6	42	>46	TC, LDL-c
Akbarian et al. 2012	Ross	<6	21	<46	TC, HDL-c, LDL-c
Al-Noori et al. 2011	Ross	<6	42	<46	TC
Al-Noori et al. 2011	Ross	>6	42	<46	TC
Saima et al. 2014	Cobb	<6	42	<46	TC

SL = supplementation level, DOS = duration of supplementation; TC = total cholesterol; HDL-c = high density lipoprotein cholesterol; LDL-c = low density lipoprotein cholesterol

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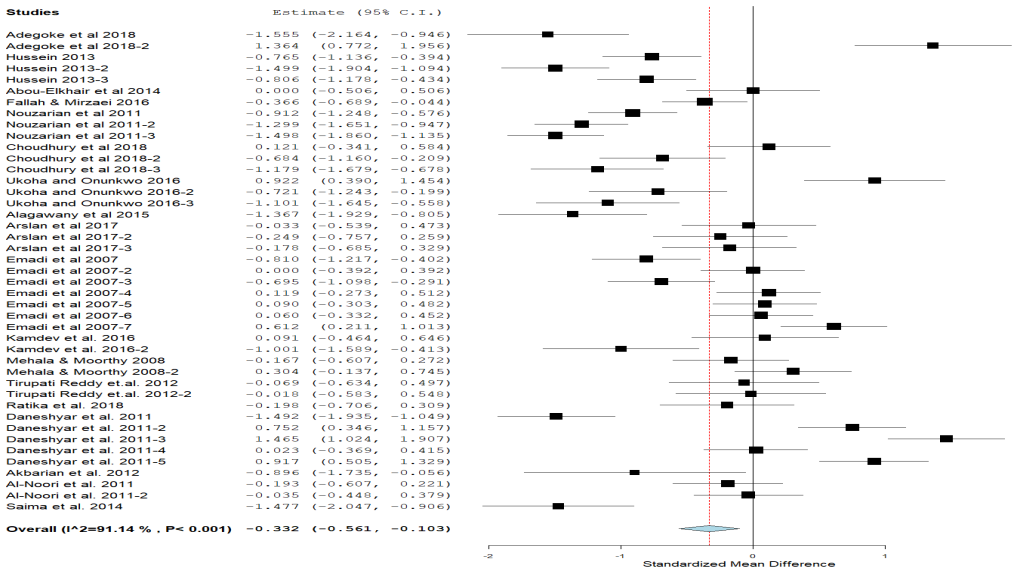

Figure 1. *Forest plots of the effect of dietary turmeric supplementation on TC (mg/dl) in broiler chickens. The solid vertical line represents the line of no effect. Points to the left of the vertical line depict a reduction in the parameter of interest and point to the right depicts an increase. Each square in the plot represents the mean effect size for the study, while the line that joined the individual square depict the lower and upper confidence interval of the effect size. The dotted line with the diamond at the base showing the 95% CI depicts the pooled estimation. I^2 is a measure of variance above chance among studies included in the analysis. Pooled estimation is considered significant when the diamond did not touch the solid vertical line*

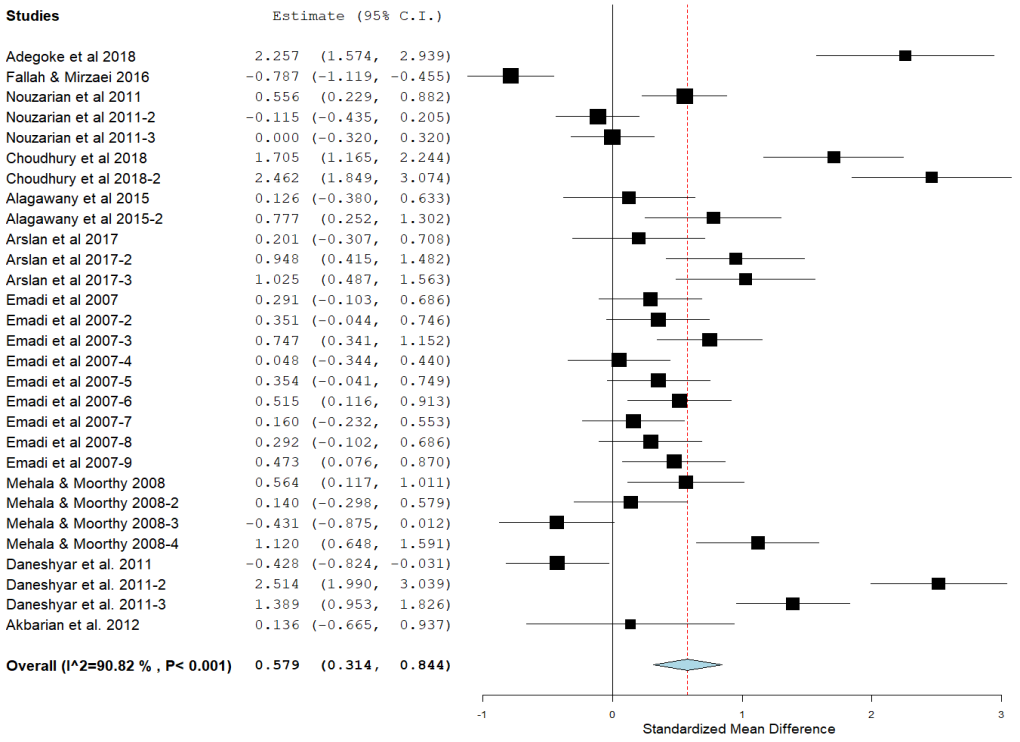

Figure 2. *Forest plots of the effect of dietary turmeric supplementation on HDL-c (mg/dl) in broiler chickens*

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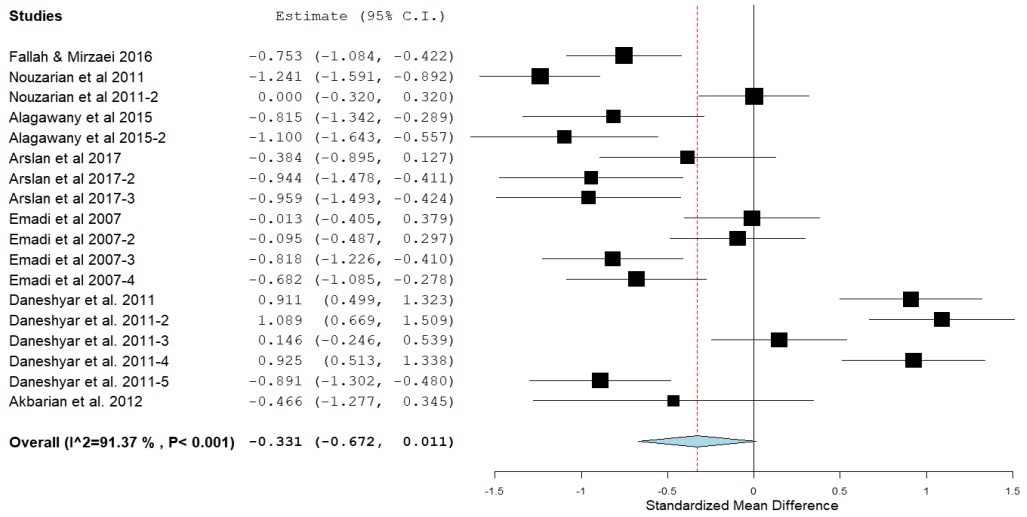

Figure 3. *Forest plots of the effect of dietary turmeric supplementation on LDL-c (mg/dl) in broiler chickens*

Table 2. *Subgroup analysis and meta-regression of effects of the moderators on TC in broiler chickens*

Covariates	Sub group analysis			Meta-regression		
	SMD	95% CI	I^2 (%)	Q_M	df	R^2 (%)
Supplementation level				0.600	1	0.00
< 6 g/kg	-0.259	-0.514 to -0.004	88.27*			
> 6 g/kg	-0.452	-0.890 to -0.013	93.73*			
Mean	-0.332*	-0.561 to -0.103	91.14*			
Duration of supplementation				1.030	3	0.00
21 d	-0.188	-0.800 to 0.423	93.70*			
35 d	-0.168	-0.464 to 0.128	69.39*			
42 d	-0.444*	-0.758 to -0.129	91.85*			
56 d	-0.300	-1.512 to 0.913	93.57*			
Mean	-0.332*	-0.561 to -0.103	91.14*			
Strain of broiler used				0.149	4	0.00
Ross	-0.321*	-0.644 to -0.002	93.32*			
Arbor acres	-0.388	-1.317 to 0.541	93.70*			
Hubbard	-0.447	-1.018 to 0.124	79.25*			
Cobb	-0.279	-0.684 to 0.126	79.15*			
Anak	-0.300	-1.512 to 0.913	93.57*			
Mean	-0.332*	-0.561 to -0.103	91.14*			
Number of bird / treatment				0.004	1	0.00
< 46 birds	-0.337*	-0.605 to -0.068	84.52*			
> 46 birds	-0.324	-0.700 to 0.052	94.45*			
Mean	-0.332*	-0.561 to -0.103	91.14*			

*Significant at p<0.01; SMD = standardized mean difference; CI = confidence interval; I^2 = heterogeneity; Q_M = moderator coefficient; df = degree of freedom; R^2 = amount of heterogeneity accounted for

The sub-analysis of the impact of turmeric on HDL-c as illustrated in *Table 3* revealed that the effect of turmeric on HDL-c was more pronounced in chickens from studies that fed turmeric at < 6 g/kg feed than studies that fed > 6 g turmeric/kg feed. Birds from studies that fed turmeric for 35 and 42 days had statistically increased plasma HDL-c relative to those that fed the same diet for 21 days. However, we noticed

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that effect estimation was higher in chickens from studies that fed turmeric for 42 days. Strain influenced ($p<0.01$) HDL-c with chickens from studies that used Hubbard and Arbor acres recording higher HDL-c as compared to those studies that used Ross and Cobb. Higher ($p<0.01$) HDL-c value was obtained in chickens from trials that included < 46 birds per treatment group than trials that included > 46 birds. The sub-analysis of the influence of covariates on plasma LDL-c as presented in *Table 4* revealed that birds from studies that fed turmeric at > 6 g/kg feed for 35 days ($p<0.01$) had statistically reduced LDL-c. Additionally, broiler chickens from trials that used Hubbard and included less than 46 birds per treatment group had significantly reduced LDL-c.

Table 3. *Subgroup analysis and meta-regression of effects of the moderators on HDL-c in broiler chickens*

Covariates	Subgroup analysis			Meta-regression		
	SMD	95% CI	I^2 (%)	Q_M	df	R^2 (%)
Supplementation level				0.037	1	0.00
< 6 g/kg	0.602*	0.245 to 0.959	92.72*			
> 6 g/kg	0.511*	0.206 to 0.816	76.97*			
Mean	0.579*	0.335 to 0.827	90.82*			
Duration of supplementation				0.129	2	0.00
21 d	0.601	-0.146 to 1.347	93.87*			
35 d	0.476*	0.225 to 0.726	56.48			
42 d	0.622*	0.214 to 1.030	93.20*			
Mean	0.579*	0.314 to 0.844	90.82*			
Strain of broiler used				17.8*	3	37.35
Ross	0.372	0.063 to 0.680	90.44*			
Arbor acres	2.116*	1.644 to 2.587	44.50			
Hubbard	0.608*	0.235 to 0.981	60.81			
Cobb	0.345	-0.290 to 0.980	87.44*			
Mean	0.579*	0.314 to 0.844	90.82*			
No. bird / treatment				2.33	1	5.05
< 46 birds	0.837*	0.391 to 1.282	89.33*			
> 46 birds	0.383	0.065 to 0.702	91.03*			
Mean	0.579*	0.314 to 0.844	90.82*			

Table 4. *Subgroup analysis and meta-regression of effects of the moderators on LDL-c in broiler chickens*

Covariates	Subgroup analysis			Meta-regression		
	SMD	95%CI	I^2 (%)	Q_M	df	R^2 (%)
Supplementation level				5.70	1	23.53
< 6 g/kg	-0.071	-0.484 to 0.342	91.04*			
> 6 g/kg	-0.842*	-1.286 to -0.399	84.29*			
Mean	-0.331	-0.672 to 0.011	91.37*			
Duration of supplementation				9.65*	2	34.62
21 d	0.322	-0.240 to 0.883	87.10*			
35 d	-0.827*	-1.032 to -0.623	0.00			
42 d	-0.359	-0.892 to 0.174	91.16*			
Mean	-0.331	-0.672 to 0.011	91.37*			
Broiler strain used				3.63	1	14.20
Ross	-0.144	-0.558 to 0.270	92.88*			
Hubbard	-0.831*	-1.076 to -0.586	6.53			
Mean	-0.331	-0.672 to -0.011	91.37*			
No. bird / treatment				3.61	1	14.15
< 46 birds	-0.802*	-1.029 to -0.574	0.00			
> 46 birds	-0.122	-0.555 to 0.311	93.46*			
Mean	-0.331*	-0.672 to -0.011	91.37*			

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Analysis of heterogeneity and publication bias

Results of the potential causes of heterogeneity as shown in the results of Model II showed no significant association between covariates and plasma TC (*Table 2*). However, broiler strain (Q_M = 17.80, p<0.01) influenced HDL-c and accounted for 37.35% of the between-study variance (*Table 3*), while duration of supplementation (Q_M = 9.65, p < 0.01) influenced plasma LDL-c and accounted for 34.62% of between-study variance (*Table 4*). The funnel plots of differences in means of plasma TC, HDL-c and LDC-c between broiler chickens fed diet with or without turmeric as displayed in *Figure 4a, b* and *c*, respectively suggest the existence of publication bias. The Rosenberg's Nfs for the database was 565, which is more than 2 folds of the threshold of 280 (5 × n = 54 + 10) needed to find the mean effect size robust. Therefore, the existence of publication bias was not a serious problem in the current meta-analysis as a large number of unpublished studies would be required to alter the significant difference between the experimental and control groups.

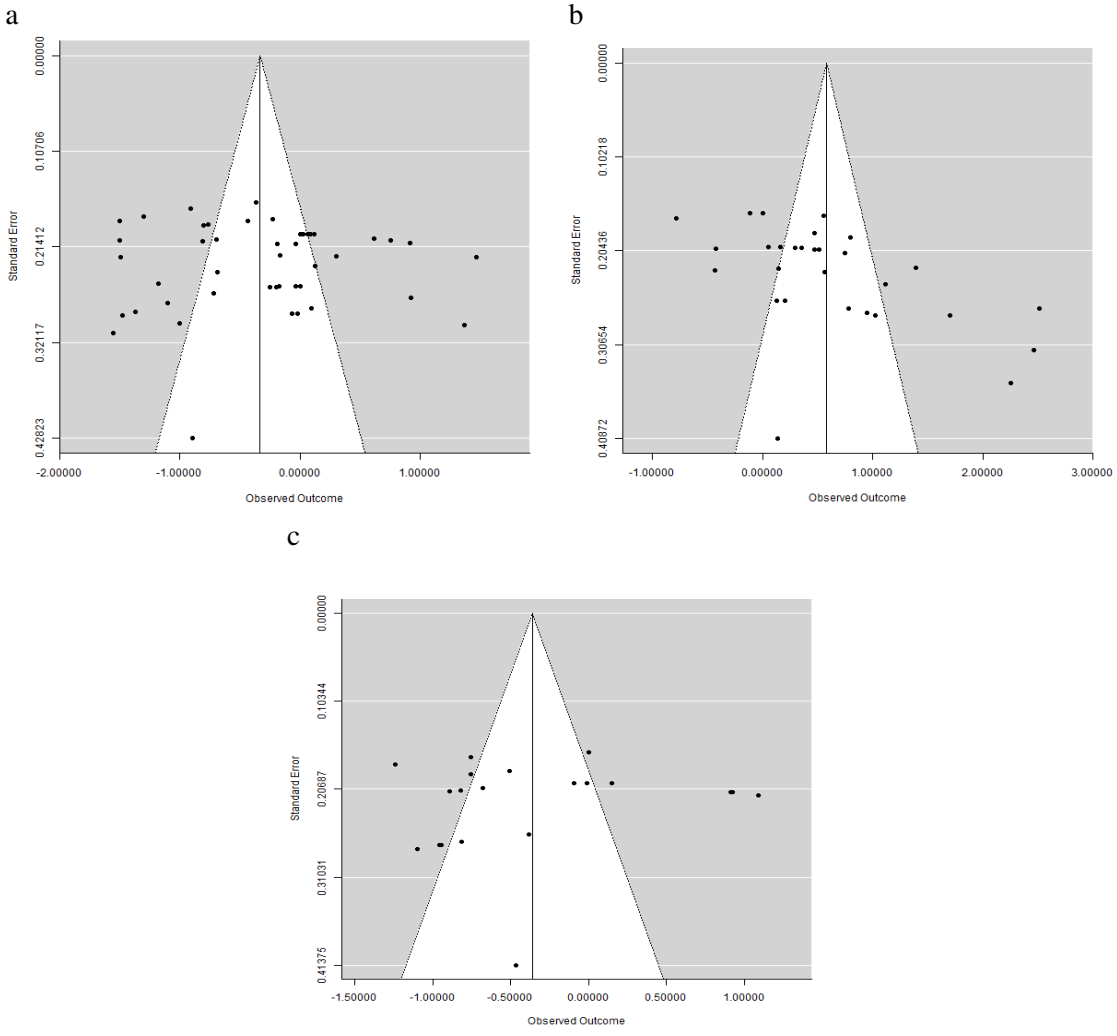

Figure 4. *Funnel plots of the effect of dietary turmeric supplementation on concentrations of TC (a), HDL-c (b) and LDC-c (c) in broiler chickens*

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Discussion

To our knowledge this study is the first meta-analysis that evaluated the effect of turmeric on plasma TC, LDL-c and HDL-c concentrations in broiler chickens. The main thrust of this meta-analysis was to provide evidence that turmeric improves health indices in broiler chickens. Blood biochemical components are indicators of the state of the cells, tissue and organs in farm animals (Akinfola et al., 2007). The association between blood cholesterol and nutrition in animal model has been documented (Okeudo, 2000; Iwuji et al., 2018). The finding from this meta-analysis showed that turmeric influenced plasma cholesterol characteristics in broiler chickens. This is in harmony with Kermanshahi and Riasi (2006) and Hosseini-Vashan et al. (2012) who reported significantly reduced TC and LDL-c value in broiler chickens fed turmeric supplemented diets for 42 days. More so, turmeric supplementation increased plasma HDL-c and reduced plasma TC levels which further confirm the earlier report that turmeric improve plasma HDL-c and reduce the plasma LDL-c in broiler chickens (Emadi et al., 2007). However, the result of present meta-analysis differed from Mehala and Moorthy (2008), who reported lack of significant changes in plasma TC levels of broiler chickens fed turmeric at 2 g/kg feed. The reason for this disparity is not known, however it could be attributed to supplementation dose. The superior effect of dietary turmeric in lowering plasma TC and LDL-c concentrations in broiler chickens as seen in the present meta-analysis may be due to increased bioavailability of turmeric bioactive compounds thus inhibiting the production of hepatic 3-hydroxyl-3-methyglutaryl-CoA reductase and cholesterol 7α-hydroxylase by up-regulation of ATP-binding cassette transporter A1 in foam cells in broiler chickens fed turmeric.

Birds fed turmeric > 6 g/kg feed had reduced TC and LDL-c than birds fed the same diet at < 6 g/kg feed, but birds fed turmeric at < 6 g/kg feed had better HDL-c. This systematic review has shown the effectiveness of turmeric diet at a level more than 6 g/kg feed in lowering plasma TC and LDL-c concentrations in broiler chickens. Importantly, birds from studies that fed less or more than 6 g turmeric/kg feed had better HDL-c value. However, the effect was more pronounced in studies that fed turmeric at > 6 g/kg feed. The reason for the disparity is not known, however, it is likely that blood cholesterol parameters require different dietary levels and nutrient components for its production in the liver. This observation is in line with the earlier results of others (Ogbuewu and Mbajiorgu, 2019; Modisaojang-Mojanaja et al., 2019) who reported that dietary nutrient requirements for optimizing different blood components in animal model are dynamic. However, it is advised that more research be conducted to determine the exact amount of turmeric required to optimize the different blood cholesterol components in broiler chickens using a quadratic optimization model. Birds from studies that fed turmeric for 35 days had significantly reduced LDL-c, whereas birds from studies that fed turmeric for 21 and 42 days had comparable LDL-c concentrations. Conversely, birds from trials that fed turmeric for 35 and 42 days had significantly better HDL-c compared to birds from the experiment that fed the same ration for 21 days. The plasma TC levels were significantly reduced in birds fed turmeric for 35 days relative to those on the other 3 supplementation durations. The superior plasma cholesterol values of broiler chickens on turmeric intervention for 35 and 42 days as compared to those on turmeric intervention for 21 and 56 days is an indication that impact of turmeric in blood cholesterol levels of broiler chickens may be dependent on duration feeding. Thus for improved blood cholesterol production in broiler chickens, turmeric-based diets should be fed between 35 and 42 days. The

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present meta-analysis showed that broilers from Hubbard strains had significantly reduced plasma LDL-c, while those from Hubbard and Arbor acres strains had significantly better plasma HDL-c compared to broiler chickens from the other three strains (Ross, Cobb and Anak). The physiological explanation for this discrepancy is not fully known. However, it may be partially attributed to genetic variations among the different broiler strains included in the meta-analysis (Acar et al., 1991). Results also showed that number of broilers included in each treatment affected plasma cholesterol concentration with those from studies that included less than 46 birds per treatment having significantly better LDL-c and HDL-c than broilers from studies that included more than 46 broiler chickens. The observed poor blood cholesterol levels recorded in broiler chickens from trials that included more than 46 chickens per treatment could be explained in part to overcrowding stress which has been established to negatively affect the biochemical and physiological parameters in broiler chickens (Thaxton et al., 2005; Guardia et al., 2011).

Heterogeneity and publication bias exist in the current meta-analysis. This was envisaged as studies used for the analysis were drawn from different part of the globe. Meta-regression of the moderators that may act as effect modifiers (i.e. chicken strain, supplementation level, duration of supplementation and number of broilers included in each treatment) accounted for most of the heterogeneity. Furthermore, we observed that duration of supplementation and chicken strain are significant predictors of plasma LDL-c and serum HDL-c, respectively in broiler chickens, and explained 72% of the heterogeneity among studies included the analysis. Funnel plots suggest the existence of publication bias and the Rosenberg's Nfs for the database is 565, which is more than 2 folds of the threshold of 280 ($5 \times n = 54 + 10$) needed to find the mean effect size robust. However, Jennions et al. (2013) have reported that meta-analysis results can still be robust in the presence of publication bias provided Nfs is greater than ($5n + 10$). Thus, the existence of publication bias was not an issue in the current meta-analysis as large number of unpublished studies would be required to alter the significant difference found between the mean effect size of birds in experimental and control groups.

Conclusion

The findings of this meta-analysis revealed that dietary turmeric supplementation improved plasma cholesterol values in broiler chickens. However, sub-analysis results indicated that dietary turmeric supplementation at a rate less than 6 g/kg feed or more than 6 g/kg feed reduced plasma TC concentration. Broiler chickens fed diet supplemented with turmeric at a rate more than 6 g/kg feed, on the other hand, reduced the concentrations of LDL-c in broiler chickens. Dietary turmeric supplementation improved plasma HDL-c; however, the effect was higher in the group that received turmeric at a rate more than 6 g/kg feed. Meta-regression analysis revealed significant associations between covariates (duration of supplementation and chicken strain) and outcomes of interest (LDL-c and HDL-c) and explained 72% of the sources of heterogeneity among the studies included in the meta-analysis. It is concluded that turmeric supplementation to the rations of broiler chickens is desirable, mainly due to its beneficial influences on blood TC, LDL-c and HDL-c. However, more studies are needed to ascertain the exact turmeric supplementation dose that optimize blood TC, LDL-c and HDL-c in broiler chickens.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80098021

APPLICATION OF CMSY TO ESTIMATE BIOLOGICAL REFERENCE POINTS OF BOMBAY DUCK (*Harpadon neherus)* FROM THE BAY OF BENGAL, BANGLADESH

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(Received 13th May 2020; accepted 14th Aug 2020)

Abstract. CMSY method was used to calculate Biological Reference Points (BRPs) of *Harpadon nehereus* using seventeen years (2001-2017) of catch data from the Bay of Bengal (BOB), Bangladesh. CMSY requires only catch and resilience data with quantitative stock status information. Also, a Bayesian state-space implementation of the Schaefer production Model (BSM) was used. The lowest Maximum Sustainable Yield (MSY) was provided by BSM (60600 t) that was smaller than last year's catch (82767 t in 2017). The fishing mortality (F) was estimated to be 0.22 and 0.23 for CMSY and BSM, respectively. The intrinsic population growth rate (r) was lower according to BSM (0.37) than the CMSY (0.57). Calculated B/B_{MSY} was 1.13 and F/F_{MSY} was 1.21 from the BSM model for the last data year. Estimated BRPs directed the overfishing status that may lead the *H. nehereus* fishery to be unsustainable. Therefore, proper management strategies are required to ensure the sustainability of *H. nehereus* in the BOB, Bangladesh.
Keywords: *CMSY, BSM, population dynamics, maximum sustainable yield, biomass, exploitation, overfishing, sustainable management*

Introduction

Bangladesh is a Southeast Asian country blessed with the Bay of Bengal (BOB). The BOB is a part of Indian Ocean and the only maritime fishery resource in Bangladesh. The total area of the BOB is 2172000 square km whereas Bangladesh occupies 121110 square km after the maritime border settlement with India and Myanmar (Kumar et al., 2016). This vast area is enriched with 475 fish species but only few of them are commercially harvested as target species. *Harpadon nehereus* (Hamilton, 1822) commonly known as Bombay duck and locally called "*lottia*". Bombay duck is one of the most commercially important species constituted above 10% of the total marine catch over the past decades from the BOB, Bangladesh (DoF, 2018). This is not only well demanded by the national and coastal population of Bangladesh but also has good export value both in fresh and dry condition (Barua et al., 2014; Fernandes et al., 2015; Sarker et al., 2017). However, due to increasing market demand, fishing pressure is also increasing. As a result, BOB fisheries stocks are in crisis including excessive fishing efforts, illegal fishing, and other

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80238034

man made causes (Hossain et al., 2015). Scientific research and studies are prerequisite to ensure the sustainable management of this valuable fisheries in the BOB. Some work has done on growth, mortality and length-weight relationships (Mustafa et al., 1998; Amin, 2001; Sarker et al., 2017) but there is no research on the Biological Reference Points (BRPs) of *H. nehereus* from the BOB, Bangladesh.

The widely used and accepted target BRP for fisheries management is Maximum Sustainable Yield (MSY) while other BRPs are also used for fisheries management (Siddeek et al., 2004; Panhwar et al., 2012; Wang and Liu, 2013; Zhang and Chen, 2015; Mohsin et al., 2017; Karim et al., 2018; Zhang et al., 2018; Ji et al., 2019). Several computer tools and packages such as CEDA (Kirkwood et al., 2003), ASPIC (Prager, 2005), Catch-MSY (Martell and Froese, 2013), CMSY (Froese et al., 2017) are used to calculate the MSY and other BRPs by the researchers and fishery managers. Among them, CMSY is one of the most recent method developed by Froese et al. (2017). CMSY is a Monte Carlo Method based model and the most suited methods to calculate the MSY and BRPs of data limited fisheries (Froese et al., 2017). It was reported that CMSY can provide better performances than other methods to estimate the BRPs and convenient for developing and least-developed countries who have a short time data series (MacCall, 2009; Dick and MacCall, 2011; Martell and Froese, 2013; Rosenberg et al., 2014; Ji et al., 2019). The CMSY model needs only catch and resilience data and when catch and catch per unit effort (CPUE) data are available, a Bayesian state-space implementation of the Schaefer production model (BSM) can also be used (Froese et al., 2017). CMSY and BSM can estimates the intrinsic population growth rate (r), carrying capacity or unexploited stock size (k), maximum sustainable yield (MSY) and other fisheries reference points. Additionally, CMSY can display a sensible evaluation of exploitation rate and relative biomass (Froese et al., 2017). This study aims to calculate the MSY and other BRPs of *H. nehereus* and to provide the baseline stock information to the fishery administrator for the sustainable management of *H. nehereus* in the BOB, Bangladesh.

Materials and Methods

Data acquisition

The study was conducted based on the catch and effort data collected from the Bay of Bengal area of Bangladesh (*Fig. 1*). The BOB is the northeastern part of the Indian Ocean, bordered by India on the west and northwest, by Bangladesh on the north, and Myanmar and India's Andaman and Nicobar Islands on the east. Bangladesh covers an area of 5.59% while the total area of the BOB is 2172000 square km (Kumar et al., 2016). This 5.59% area includes 24077 square km of shallow coastal and estuarine waters, 42007 square km of shallow shelf sea, 44383 square km of deep-sea, and 10644 square km of continental shelf (Chowdhury, 2014). For the *H. Nehereus*, data were collected from the estuarine, shallow coastal, and shelf-sea region where fishing activity is prominent. In this research, total of 17 years (2001-2017) catch and effort data were used from the Yearbook of Fisheries Statistics (YFS), produced by the Department of Fisheries (DoF), Bangladesh (*Table 1*). The total catch data was valued in tons (t) while fishing efforts was total number of fishing gears (trawl net, gill net and set bag net) per year (g/yr) and tons/gear/year (t/g/yr) is the unit of catch per unit effort (CPUE). A single voyage for fishing in the BOB may take 3 to 4 weeks by the mechanize vessels while non-mechanize vessel voyages on a daily or an overnight basis. The voyaging duration may be influence by weather of the BOB (Barua et al., 2014; Karim et al., 2018).

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DOI: http://dx.doi.org/10.15666/aeer/1806_80238034

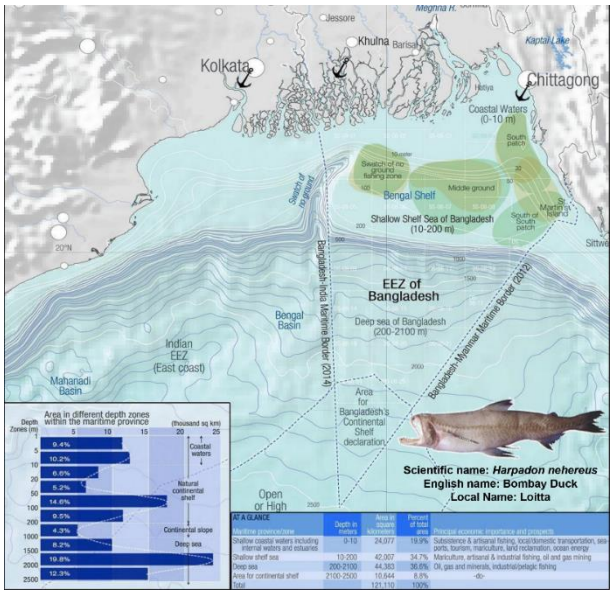

Figure 1. *Map showing the information of the Bay of Bengal, Bangladesh (Chowdhury, 2014) and the figure of Bombay duck (Harpadon nehereus) species*

Table 1. *The seventeen years (2001-2017) time series fishery statistics of Harpadon nehereus from the Bay of Bengal, Bangladesh*

Year	Catch (t)	Effort (g/yr)	CPUE (t/g/yr)
2001	37698	115274	0.33
2002	39537	115274	0.34
2003	41556	115274	0.36
2004	41804	115274	0.36
2005	43355	115354	0.38
2006	39693	115362	0.34
2007	40763	115369	0.35
2008	64224	152595	0.42
2009	64446	156102	0.41
2010	66965	156110	0.43
2011	69244	174708	0.40
2012	79085	174579	0.45
2013	56960	171239	0.33
2014	59470	171914	0.35
2015	64535	171914	0.38
2016	76313	177477	0.43
2017	82767	177492	0.47
Maximum	82767	177492	0.47
Menimum	37698	115274	0.33
Averege	56966	146548	0.38

t= tons, g= gears and yr= year

CMSY and BSM model

CMSY model was used to estimate the biomass, exploitation rate, MSY and related BRPs from catch data and resilience of Bombay duck fishery. Monte Carlo approach was applied to filter probable ranges for the maximum intrinsic growth rate (r) and carrying

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capacity (*k*). Viable *r*-*k* pairs were depicted in a triangular-shape in log-space if there is a positive growth trend. CMSY searched the most probable *r* (maximum increase rate) of *H. nehereus* in the tip of the triangle. A Bayesian state-space implementation of Schaefer Model (BSM) used to calculate *r*, *k* and MSY from CMSY (*Eq. 1*). Another equation used to explain the reduced recruitment at severely depleted stock sizes where biomass falls below ¼*k* (*Eq. 2*).

$$B_{t+1} = B_t + r\left(1 - \frac{B_t}{k}\right)B_t - C_t \quad \text{(Eq.1)}$$

$$B_{t+1} = B_t + 4\frac{B_t}{k}r\left(1 - \frac{B_t}{k}\right)B_t - C_t \left| \frac{B_t}{k} < 0.25 \right. \quad \text{(Eq.2)}$$

where (B_{t+1}) was exploited biomass in the year *t*+1, B_t was current biomass, and C_t was catch in year *t*. The *H. nehereus* species resilience from FishBase were medium and the prior *r*-ranges was 0.2–0.8 (*Table 2*). The prior *k*-range was attaining from three assumptions: (1) unexploited stock size *k* is larger than the largest catch in the time series, (2) maximum sustainable catch (F_{MSY}) depends on productivity, (3) maximum catch constitute larger fraction of *k* in substantially depleted rather than lightly depleted stocks. By default, depending on assumed depletion level, possible biomass ranges (*Table 3*) provide prior estimates of relative biomass at the beginning and end of time series.

Table 2. *Prior ranges for parameter r*

Prior *r*-range	Resilience
0.6–1.5	High
0.2–0.8	Medium
0.05–0.5	Low
0.015–0.1	Very Low

Table 3. *Prior biomass ranges relative to k*

B/k	Prior biomass
0.5–0.9	High
0.2–0.6	Medium
0.01–0.4	Low

In the BSM, the uniform *r*-ranges from *Table 2* were translated into prior densities with a central value. Also, abundance index was related to stock biomass by a catchability coefficient *q* (*Eq. 3*) where $CPUE_t$ was mean CPUE in year *t*, B_t was biomass in year *t* and *q* was catchability coefficient for data-limited fishery.

$$CPUE_t = qB_t \quad \text{(Eq.3)}$$

The CMSY and BSM were implemented using an R-code (CMSY_0_7q.R), downloaded from http://oceanrep.geomar.de/33076/, and proposed by Froese et al. (2017). The JAGS software (Plummer, 2003) also applied for sampling the parameter's probability distributions with the Markov chain Monte Carlo method. To facilitate mixing of Gibbs samples, annual biomass was explicit relative to unexploited biomass with $P_t = B_t/k$. CMSY and BSM estimated parameters of MSY = *rk/4*, fishing mortality

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corresponding to MSY was $F_{MSY} = 0.5r$, biomass corresponding to MSY was $B_{MSY} = 0.5k$ (Schaefer, 1954; Ricker, 1975) and biomass below which recruitment may compromised as half of B_{MSY} (Haddon, 2011; Carruthers et al., 2014; Froese et al., 2015).

CMSY derived input values for prior distribution ranges were tabulated in *Table 4*. The prior initial relative biomass was 0.2-0.6, prior intermediate relative biomass was 0.5-0.9 (in the year 2011), and prior final relative biomass was 0.4-0.8. The prior *r, k, q* ranges were 0.2-0.8/yr, 186000-4472000 t and 7.05e-07-2.82e-06, respectively (*Table 4*).

Table 4. *Input values of prior distributions of biomass, r, k, q ranges*

Input parameters	Value ranges
Prior initial relative biomass	0.2 - 0.6
Prior intermediate relative biomass	0.5 - 0.9 (in year 2011)
Prior final relative biomass	0.4 - 0.8
Prior range for *r*	0.2 - 0.8
Prior range of *q*	7.05e-07 - 2.82e-06
prior range for *k*	186000 - 4472000

Results

A gradual upgrading trend of catch and effort observed from the catch series data of Bombay duck in *Table 1*. Maximum effort (177492 g/yr) observed in the year 2017, where the maximum catch was 82767 t. Similarly, in the year 2001, minimum effort (115274 g/yr) was recorded with a minimum catch (37698 t). The average catch of the 17 years was 56966 t while the average effort recorded as 146548 g/yr and average CPUE was 0.38 t/g/yr. The Gillnet and Set Bag Net (SBN) were the predominantly used gears for fishing in the BOB, Bangladesh. Both the mechanized and non-mechanized fishing vessels voyaged for the fishing of *H. nehereus* fishery in BOB, Bangladesh.

The CMSY and BSM derived biological reference points for the Bombay duck fishery of BOB, Bangladesh are presented in *Table 5*. The maximum carrying capacity (*k*) for *H. nehereus* estimated from BSM (652000 t) was larger than the CMSY estimate of 549000 t. For the BSM, a steno range of *k* was observed at 95% confidence level compare with CMSY. The BSM derived catchability coefficient (*q*) was 1.18e-06, while the value of q_{low} and q_{high} was 8.86e-07 and 1.56e-06, respectively. The larger intrinsic rate of population size increase (*r*) value 0.57/yr depicted from the CMSY model indicates the stock was able to increase its size above 50% of the actual population size per year. At the 95% confidence level, BSM produced a narrow range of *r* compare to the CMSY model. At the 95% confidence level, a wide range of MSY values simulated from CMSY than BSM. The smallest MSY was estimated from BSM (60600 t), while CMSY produced MSY was 77600 t. Both models provided lower MSY values than the last year catch (82767 t in 2017), which indicates an over-exploitation status of Bombay duck fishery from BOB, Bangladesh.

Both CMSY and BSM model showed very closer biomass value, while larger B_{MSY} observed from the BSM model. BSM calculated biomass (B=367076 t) was larger than the B_{MSY} (326000 t) that indicate the stock was not depleted and in safe condition. The CMSY and BSM output also showed underfished biomass condition of *H. Nehereus* as the biomass ratio of B/B_{MSY} for both models depicted >1.0 value (*Table 6*). The BSM computed fishing mortality (F=0.23/yr) was greater than F_{MSY} (0.19) depicted overfishing happening. Higher fishing mortality observed from the BSM model

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(F=0.23/yr) than the CMSY model (F=0.22/yr), but the inverse scenario depicted for the F_{MSY}. The fishing mortality ratio (F/F_{MSY}) value from CMSY and BSM was 0.76 and 1.21, respectively (*Table 6*). Based on the output from the Bayesian state-space Schaefer surplus production model, overfishing status was recognized for the Bombay duck fishery (*Fig. 2*). In summary, BSM derived B/B_{MSY} on F/F_{MSY} with time indicates healthy stock sizes of *H. nehereus* that are about to be depleted by overfishing in the BOB, Bangladesh (*Fig. 2d*).

Table 5. *Estimated biological reference points (BRPs) for the Harpadon nehereus from the Bay of Bengal, Bangladesh, with the 95% confidence intervals*

Model Name	Model Parameters		
	***k* (t)**	***r* (/yr)**	**MSY(t)**
CMSY	549000 (310000 - 974000)	0.57 (0.41 -0.79)	77600 (48000 - 125000)
BSM	652000 (467000 - 911000)	0.37 (0.26 - 0.54)	60600 (50000-73500)

Table 6. *Estimated F, F_{MSY}, F/F_{MSY}, B, B_{MSY}, and B/B_{MSY} of the Harpadon nehereus from the Bay of Bengal, Bangladesh*

	***F* (/yr)**	**F_{MSY} (/yr)**	***B* (t)**	**B_{MSY} (t)**	**F/F_{MSY}**	**B/B_{MSY}**
CMSY	0.22	0.285	387045	274500	0.76	1.41
BSM	0.23	0.19	367076	326000	1.21	1.13

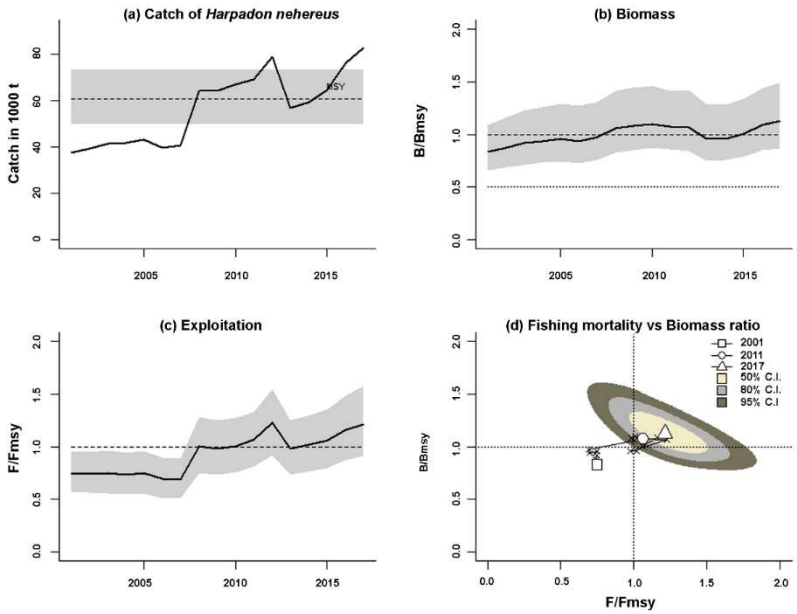

Figure 2. *Exhibition of the information for management of Bombay duck (H. nehereus) fishery in the BOB, Bangladesh from BSM. (a) Catches and MSY (the grey color indicates 95% confidence intervals). (b) The ratio of the relative biomass (B/B_{MSY}) with uncertainty displayed with the grey area. (c) Exploitation ratio (F/F_{MSY}), and F_{MSY} are adjusted for the recruitment below 0.5 B_{MSY}. (d) Stock size (B/B_{MSY}) and exploitation (F/F_{MSY}) relationship at 50%, 80% and 95% confidence intervals (C.I.) with different shaded areas*

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Discussion

Sustainable fisheries management mostly depends on outcomes of stock assessment study that provide valuable information for effective policy formulation for the fisheries resources. Scientists evaluate fishery stock status to propose the biological reference points (BRPs) estimated by different statistical methods. Fisheries scientist and managers consider *k, q, r* and MSY values for the sustainable management of any fisheries (Hilborn and Walters, 1992; Froese et al., 2017; Baset et al., 2017; Mohsin et al., 2018; Ji et al., 2019; Khatun et al., 2019). The recent developed CMSY and BSM models were applied to estimate the BRPs for *H. nehereus* from the BOB, Bangladesh in this study.

For the CMSY and BSM, the *k* and *r* were the required prior information which can be attained from the Fishbase resilience data (Martell and Froese, 2013; Froese and Pauly, 2015). As per ICES (2015) simulation tests, CMSY is not well suited for the very low resilience or very lightly harvested fishery. The resilience value of Bombay duck fishery was medium (prior $r = 0.2$-0.8) from the Bay of Bengal. So far, CMSY may be the best alternative approach for this data poor stock from BOB, Bangladesh (Froese et al., 2017). Catch, effort, and CPUE are major indicators to evaluate the fishery stock status. Catchability coefficient (q) denotes the ratio between fishing mortality and fishing efforts, so-called the efficiency of fishing gears. The q was also important where catch and effort data used as a critical component for the study of the fish population dynamics. The q may be influenced by the variability of the temporal and spatial distribution of the fish stock and fishing fleets (Hoggarth et al., 2006).

FiSAT is a computer software package able to calculate r using the von Bertalanffy growth parameters (Sullivan, 1991; Gayanilo et al., 2005). Sullivan (1991) suggested a prognostic equation was: $r = 0.947 + 1.189k - 0.095 \ln(W_\infty)$. These equations recommended that the intrinsic growth rate was proportionate with the von Bertalanffy growth rate while inverse with body size. Furthermore, the prior value of r was relayed with natural mortality (M) and calculated as $r=2M$ (Froese et al., 2017). Also, Pauly (1980) proposed an empirical formula for M estimation that was further reviewed by the Kenchington (2014) for data-limited fisheries. Compare with other models, the r-value from CMSY ($r=0.57$) indicated the medium growth rate for the Bombay duck fishery in the BOB, Bangladesh, and the fishery was capable enough of adding above 50% more biomass in the existing population per year. It was mentioned that r of 0.1 implies that a population will increase by 10% in a time interval (McAllister et al., 2001; Hoggarth et al., 2006). There is a high correlation between the population growth rate r and the population size k, either large r or large k can produce large catch, this a difficult question in fish population dynamics. Additionally, increasing fishing intensity may increase catch that may decrease the population size.

MSY was the foundation of most BRPs, so-called technical reference point (TRP), that exhibited an explicit assumption about a fishery status (ICES, 2015). If the observed catch was lower than the estimated MSY, it means the flourishing fishery stock, and the higher observed catch than estimated MSY indicates the overexploited status of fishery stock (Gabriel and Mace, 1999; Hoggarth et al., 2006; Rosenberg et al., 2014). The estimated MSY from CMSY and BSM model was 77600 t and 60600 t respectively for the *H. nehereus* fishery from BOB, Bangladesh. Though both models produced smaller MSY value, BSM made the lowest MSY value compare with the last year catch (82767 t in 2017), indicated the Bombay duck fishery overexploited from the BOB, Bangladesh. In the literature several authors studied the length-weight relationship of Bombay duck

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fishery from the BOB, Bangladesh and found the over-exploited status (Mustafa et al., 1994; Islam, 1995; Mustafa et al., 1998; Sarker et al., 2017).

Both CMSY and BSM model produced B/B_{MSY} ratio >1.0 denotes the under-fished condition and safe biomass of the Bombay duck fishery. The demarcation of B/B_{MSY} ratio varies by governing body (such as UN, FAO signifies under-fished when the value under 0.8, while the USA considers it under 0.5) (Hoggarth et al., 2006). On the other hand, F/F_{MSY} ratio <1 was produced by CMSY, while BSM produced F/F_{MSY} ratio was >1.0. From the BSM output results and figure, it is clarified that the Bombay duck fishery in the BOB, Bangladesh was in overfishing condition, but the biomass was in under-fished status, which depicted that the fishery remains sustainable. However, this healthy stock sizes are about to be depleted by overfishing and become unsustainable (Sumaila and Tai, 2020). Overfishing may lose the balanced with millions of fish species and other marine animals in the marine water and overfishing fishing for particular specie may lead it to be extinct. Contineus overfishing practice may distort the food chain and truncating the food web that are threats for the ocean health and will ripple up and down to all the living organisms (Chuenpagdee et al., 2003; Halpern et al., 2015; Gattuso et al., 2018; Sumaila and Tai, 2020). Therefore, management strategies should be strict to control the overfishing status for this fishery to ensure the sustainability of marine fisheries of Bangladesh. This research recommends that the annual catch of *H. nehereus* from the BOB, Bangladesh should not exceed the MSY limit estimated by the BSM model (60600 t) because the smallest value of the MSY should be recommended for the management target of any fishery (Martell and Froese, 2013). Also, illegal fishing and non-license fishing vessel operation should be controlled strictly to ensure the sustainable harvest of this fishery from the Bay of Bengal, Bangladesh.

However, more research on population biology and dynamics are recommended to restrict the overexploitation of this fishery. Because it is much controversial to imply catch data for stock estimation (Pauly et al., 2013), but for the unassessed fishery, most of the method depends on it. The outcomes of this research can be helpful to study growth and other biological parameters to have accurate knowledge of the population dynamics of this fish species.

Conclusion

Harpadon nehereus is one of the essential fisheries in Bangladesh. In the Bay of Bengal, the catch of this fishery was gradually increased up to 82767 t from 2001 to 2017 with the continues lifting of fishing effort. Fishery reference points derived from the CMSY and BSM model indicated that the *H. nehereus* biomass in the BOB was still in sustainable condition, but the overfishing scenario may lead the fishery unsustainable. To ensure the sustainability of this fishery, the results recommend that total allowable catch (TAC) should not exceed the 60600 t from the BOB, Bangladesh. To verifiy this prediction, further studies could be done to assess the stock status accurately along with the length-weight data. More exclusive research on biology and ecology of this fishery is recommended to establish a complete management plan. Also government should take immediate measures to reduce the fishing efforts. Fishery managers and policymakers may use this research information to ensure sustainable management of the Bay of Bengal fishery as well as other data-limited fishery of Bangladesh.

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DOI: http://dx.doi.org/10.15666/aeer/1806_80238034

Acknowledgments. The authors would like to express gratefulness to the Chinese Scholarship Council (CSC) and SOA (State Oceanic Administration) and Central University Basic Research Fund (NO: 201562030) of Ocean University of China for sponsorship of this research. The authors are also grateful to Sylhet Agricultural University authority for their valuable care and supports.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80238034

MICROBIAL METABOLIC ACTIVITY ON A DRIP IRRIGATED COTTON FIELD AFTER TEN YEARS OF SALINE WATER IRRIGATION AND NITROGEN FERTILIZER APPLICATION ON ARID SOIL

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(Received 30th May 2020; accepted 6th Oct 2020)

Abstract. Irrigation with saline water can impact metabolic activity of soil microbial by altering the soil environment. The aim of this study was to reveal the influence of saline water irrigation on soil physicochemical properties and microbial metabolic activities. For this, a ten years saline water irrigation experiment was conducted in Shihezi, Xinjiang Province, China. Results indicated that saline water irrigation reduced soil pH, total N (TN), organic matter (SOM), and basal respiration (SBR), however, soil salinity increased significantly. Nitrogen application increased soil salinity, SOM, TN, and SBR, but reduced soil pH. Saline water irrigation had significant negative effect on soil microbial metabolic activities. Nitrogen application significantly reduced soil microbial metabolic activities in the fresh water irrigation but had no significant effect under saline water irrigation treatment. Water salinity and N amount remarkably affected carbon (C) utilization patterns of the microbial communities, especially the utilization of amino acids, amines, and phenols. The D-Cellobiose, D-Xylose, D-Mannitol, β-Methyl-DGlucoside, i-Erythritol, α-D-Lactose, L-Serine, L-Asparagine, LPhenylalanine, γ-Hydroxybutyric acid, Putrescine and 4-Hydroxy Benzoic Acid were the sole carbon sources. The results of this study increase our understanding of soil biological processes under saline conditions in arid regions.
Keywords: *irrigation water salinity, nitrogen amount, basal respiration, microbial activity, biolog EcoPlate*

Introduction

Salt-affected soils are common in arid areas. In these areas, there is not enough rainfall to leach salts from the root zone. Salt accumulation affects many soil properties. The Xinjiang province in northwest China has an arid continental climate, with low precipitation and high evaporation rates. Most shallow groundwater sources in this region have relatively high salinity, with an electrical conductivity of >2 dS m^{-1} (Chen et al., 2010). Therefore, it is important to identify appropriate irrigation practices to avoid salt accumulation under saline water irrigation (Pereira et al., 2002). Drip irrigation is widely considered as the best method for saline water irrigation (Karlberg et al., 2007). Drip irrigation consists of frequent applications of small amounts of water over extended periods of time. However, even with careful management, irrigation with saline water may cause salt accumulation in soil. Increasing in soil salinity can affect soil water content, pH, SOM, and other soil physicochemical properties (Wong et al., 2010; Mavi et al., 2012), and may affect soil microbial communities. However, understanding about the effect of irrigation with saline water on the function of soil microbial metabolic activities is still incomplete and fragmented.

In the soil ecosystem, soil microbial are indispensable agents, responsible for decomposing organic matter, forming soil aggregates, and driving nutrient cycling

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(Critter et al., 2004; Kaye et al., 2005; Amini et al., 2016). Soil microbial is also sensitive indicators of environmental change. Soil microbial activity has direct impact on the soil ecosystems stability and function (Manzoni et al., 2012). Salt as a major stress effect to soil microbial has been received increasing attention. Previous studies have been done about the influences of salt on soil microbial physiology (Kakumanu and Williams, 2014), respiration (Chowdhury et al., 2011a), biomass (Yan and Marschner, 2012), community structure (Campbell and Kirchman, 2013), and C use efficiencies (Malik and Gleixner, 2013). For example, soil respiration from natural salt gradients is in most cases significantly negatively correlated with salt (Muhammad et al., 2008; Chowdhury et al., 2011b,c; Setia et al., 2011). Soil salinity may change soil microbial community composition (Gros et al., 2003; Gennari et al., 2007) because microbial species differ in their salt tolerance (Mandeel, 2006; Llamas et al., 2008). Sardinha et al. (2003) and Oren (2008) reported that soil salinity negatively affects microbiological processes either by reducing water availability to microorganisms or by influencing their cellular physiology and metabolic processes. However, less work has been done about the effect of water salinity on the soil microbial metabolic activity.

Soil microbial metabolic activity is widely used to evaluate soil processes and soil ecological functions. Increasing attention is being given to the effects of salinity on soil microbial metabolic activity. Studies have found that average well color development (AWCD) values decreased markedly with water salinity increased (Jin et al., 2014), indicating that water salinity may change the C utilization patterns of the soil microbial. Shen et al. (2008) also found that soil salt markedly decreased soil microbial community functional diversity. In addition, fertilization is another important agricultural management measure. Many studies found that fertilization can increase (Conde et al., 2005), reduce (Craine et al., 2007; Ramirez et al., 2010), or have no effect on soil microbial activity (Hobbie and Vitousek, 2000; Pangle and Seiler, 2002). However, little research has been done about the combined effects of irrigation with saline water and nitrogen (N) fertilization on soil microbial metabolic activities under field conditions.

Therefore, it is important to understand the response of soil microbial metabolic activities to the changes in soil salinity that are induced by irrigation with saline water. We hypothesized that ten years saline water irrigation decreases soil nutrients and microbial metabolic activities in an arid area soil in China. The aim of our field located experiment was to use the Biolog EcoPlate method to reveal the effects of different irrigation water salinity and N fertilizer amount on soil microbial metabolic activity. We also compared C utilization patterns by the soil microbial communities in the different treatments. Our study can increase understanding about how water salinity and N amount influence soil microbial process. These results are useful for managing agricultural fields with saline water irrigation.

Materials and methods

Field site and experimental design

The long-term field located experiment that began in 2009 at the Shihezi University Agricultural Experiment Station, Shihezi City, Xinjiang Province, China (44°18′ N, 86°02′ E). The area has a continental climate. Annual precipitation and evaporation at the experiment station were 210 mm and 1660 mm, respectively. The soil at the site is an alluvial, gray desert soil. Some of the soil physicochemical properties (0–20 cm

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depth) at the start of the field located experiment in 2009 were as follows: bulk density, 1.33 g/cm^3; electrical conductivity ($EC_{1:5}$), 0.13 dS m^{-1}; pH, 7.48; SOM, 16.8 g kg^{-1}; TN, 1.08 g kg^{-1}; available phosphorus(P), 25.9 mg kg^{-1}; and available potassium (K), 253 mg kg^{-1}.

The experimental design was a 2 × 2 factorial with two irrigation water salinity levels [ECw of 0.35 and 8.04 dS m^{-1}, representing as fresh water (FW) and saline water (SW), respectively] and two N application rates (0 and 360 kg N ha^{-1}, representing as N0 and N360, respectively). These treatments were arranged in a randomized block design with 3 replications. The cropping system was continuous cotton (*Gossypium hirsutum Xinluzao 52*).

Figure 1 shows a diagram of the plot layout. Each plot (16 m long × 1.2 m wide) had four rows of cotton. The plots were mulched with one sheet of plastic film. Two drip irrigation lines were installed under the plastic film. The emitters were 0.4 m apart. Water was applied by drip irrigation at a rate of 2.7 L h^{-1}. The same amount (450 mm) of water was applied to each plot annually, the irrigation water amount was controlled by a water meter. The timing of the irrigation events (irrigation intervals of 7 to 10 d) was based on cotton growth requirements and soil moisture condition.

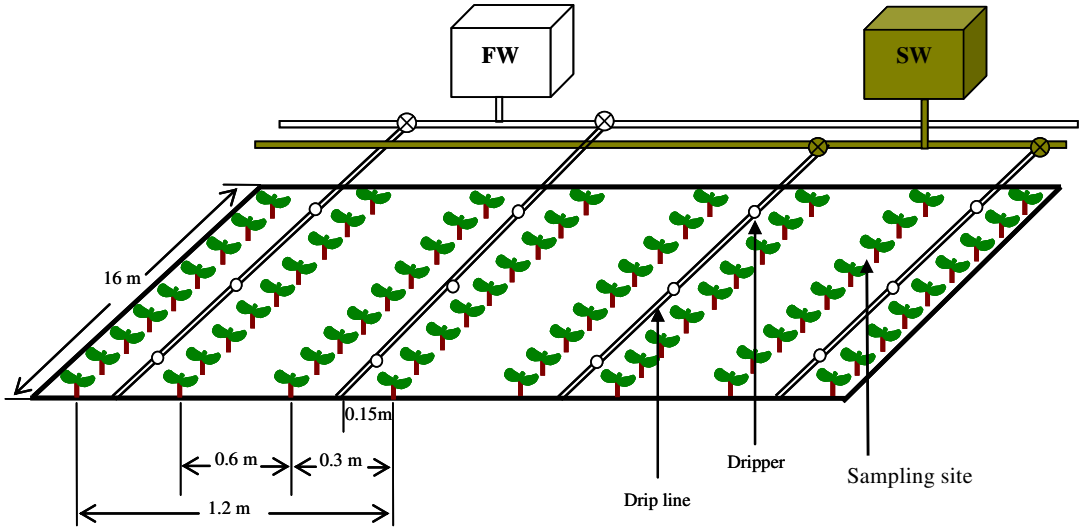

Figure 1. *Layout of an experimental plot*

The fresh irrigation water was obtained from a local well. The saline irrigation water was provided by adding NaCl and $CaCl_2$ (a weight ratio of 1:1) to the well water. The ion concentrations of the irrigation fresh and saline water are presented in *Table 1*.

Table 1. *Selected chemical characteristics of the irrigation water. EC, electrical conductivity; SAR, sodium adsorption ratio. The symbols FW and SW represent irrigation water salinities (EC) of 0.35 and 8.04 dS/m, respectively. SAR, sodium adsorption ratio*

Water	EC (dS m^{-1})	pH	SAR	HCO_3^- (meq L^{-1})	Cl^- (meq L^{-1})	SO_4^{2-} (meq L^{-1})	Ca^{2+} (meq L^{-1})	Mg^{2+} (meq L^{-1})	K^+ (meq L^{-1})	Na^+ (meq L^{-1})
FW	0.35	7.52	0.16	0.98	2.46	0.73	2.44	1.18	0.33	0.22
SW	8.04	7.09	8.91	1.15	88	0.83	45.5	1.18	0.33	43.04

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Phosphorous and potassium fertilizers (105 kg ha^{-1} P_2O_5 and 60 kg ha^{-1} K_2O) were applied at planting. The N fertilizer (urea) was applied in six equal amounts on June 20, June 27, July 4, July 12, July 19, and July 26 through the drip irrigation system. The urea solution was stored in a plastic 15 L container and pumped into the irrigation system. The agronomic operations were the same in all of the plots. The 360 kg N ha^{-1} amount is commonly used by local farmers.

Soil sampling

The soil samples in this study were collected on July 28, 2018. The cotton was at the boll opening stages. Three soil samples (0–20 cm) were collected from each plot. The soil samples were then mixed to form one composite sample per experimental plot. After sieving to remove stones, plant roots, then, the samples were put into sterile plastic bags and transported to the laboratory in a cooler and were refrigerated at 4 °C for microbial analysis.

Soil chemical and microbial analysis

TN and SOM were measured using the semimicro-Kjeldahl and the $K_2Cr_2O_7$-H_2SO_4 oxidation-reduction titration method, respectively. Soil pH was measured using an MP521 Lab pH meter (Soil to water ratios: 1:2.5). Soil salinity was measured using an MP521 Lab conductivity meter (Soil to water ratios: 1:5). Soil basal respiration in laboratory conditions was measured according to the method of Alef and Nannipieri (1995).

Soil microbial metabolic activity was measured by Biolog Ecoplates™ (Biolog, Hayward, CA, USA). The plates had 96 wells (i.e., one water blank and 31 sole C sources, each replicated three times). Soil suspensions were prepared by mixing 5 g soil into 45 mL of phosphate-buffered saline solution in sterile 100 mL centrifuge tubes, and then shaken 30 min on an orbital shaker. The suspension was diluted 1:1000 and then 150 μL aliquots of each suspension were inoculated into the wells of the biolog microtiter plates. The biolog microtiter plates were incubated at 25 °C in constant temperature incubator. The color development in each plate well was recorded at 12 h intervals for 168 h at an optical density of 590 nm. The microbial activity was expressed as AWCD value was determined according to the methods of Garland and Mills (1991). To calculate the utilization of C sources, the 96 h optical density values were normalized by dividing them by their AWCD.

Data analyses

All variance (ANOVA) analyses were conducted using SPSS19.0 statistical software. The difference between the groups used Tukey's test method ($P<0.05$). Principal components analysis (PCA) was conducted using Canoco 4.5 software. The heatmap and correlation analyses were generated using R package.

Results

Soil properties

Irrigation with saline water increased markedly soil salinity but reduced soil pH, total N, and SOM (*Figure 2*). Nitrogen application significantly increased soil salinity, SOM, and total N, but reduced soil pH. Soil salinity was 474% greater in SWN0 than that in

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FWN0, and 346% greater in SWN360 than that in FWN360. Soil pH, total N, and SOM were 3.7, 6.2, and 8.2% less in SWN0 than that in FWN0. In comparison, soil pH, total N, and SOM were 2.5, 14.2, and 5.1% less in SWN360 than that in FWN360.

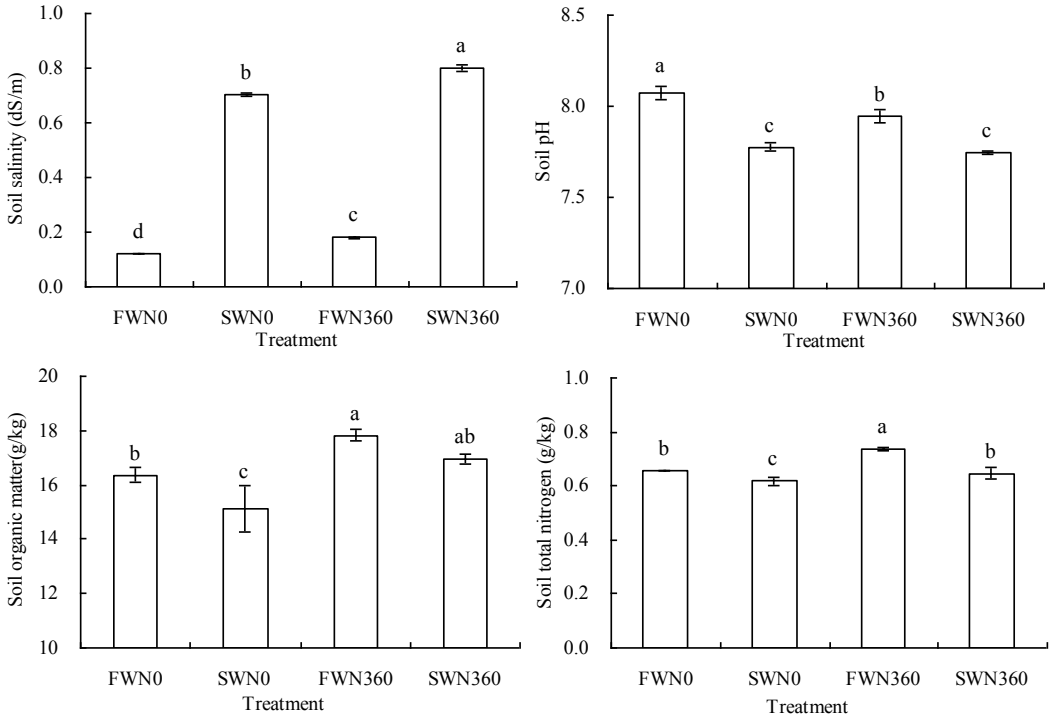

Figure 2. *Soil salinity, pH, organic matter, and total N as affected by irrigation water salinity and N amount. Note: Values are the mean of three replicates. Error bars represent standard deviations. Different letters within the same panel indicate significant differences at P < 0.05. Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}*

Soil basal respiration (SBR)

Saline water irrigation significantly decreased SBR (*Figure 3*). Nitrogen application significantly increased SBR. SBR was 51.6% greater in FWN0 than that in SWN0, and 31.6% greater in FWN360 than that in SWN360. Averaged across two N amounts, irrigation with saline water reduced SBR by 28.4%. Averaged across two water salinities, N application increased SBR by 35.1%.

Soil microbial metabolic activity

AWCD is widely used to quantify soil microbial metabolic activity. There was little change in AWCD during the first 48 h of the incubation (*Figure 4*). After this lag phase, however, the AWCD values increased markedly. The increase rates varied depending on the treatment. For example, AWCD after 60 h increased more slowly in SWN0, and SWN360 than that in FWN0 and FWN360. There was no significant difference in AWCD between SWN0 and SWN360 after 96 h. The accumulative AWCD values decreased in the order FWN0 > FWN360 > SWN0 > SWN360.

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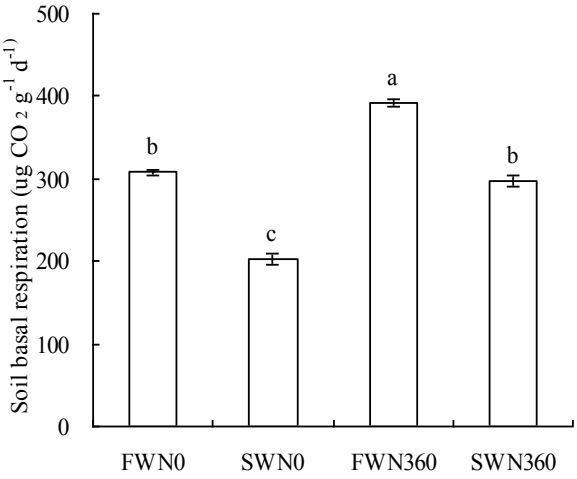

Figure 3. *Soil basal respiration as affected by irrigation water salinity and N amount. Note: Values are the mean of three replicates. Error bars represent standard deviations. Different letters within the same panel indicate significant differences at $P < 0.05$. Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}*

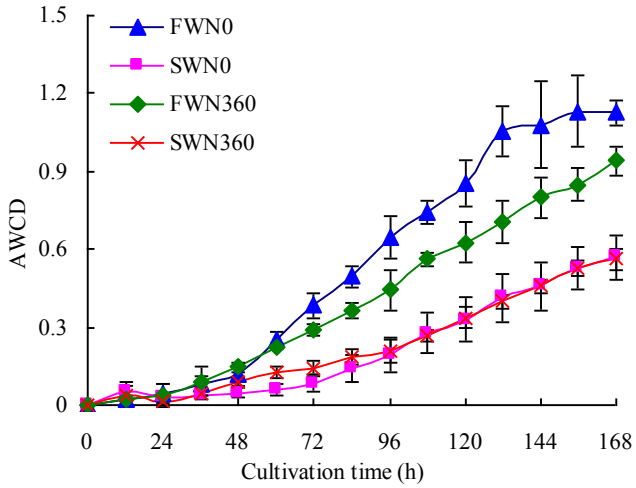

Figure 4. *Average well color development during a 168-h incubation as affected by irrigation water salinity and N amount. Note: Values are the mean of three replicates. Error bars represent standard deviations. Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}*

The ANOVA analyses results also showed that saline water irrigation and N fertilizer amount significantly altered soil microbial metabolic activity (*Table 2*). N fertilizer application significantly reduced AWCD in the FW treatments. However, N fertilizer application had no significant impact on AWCD in the SW treatments. The main effect means were 175% higher in FW than that in SW, and 23% higher in N0 than that in N360.

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Table 2. *Average well color development (AWCD) and six biochemical categories of substrates as affect under different treatments*

Treatment	AWCD	Carbohydrates	Amino acids	Carboxylic acids	Amines	Phenols	Polymers
FWN0	0.648 a	1.006 a	0.587 a	0.522 a	0.471 a	0.386 a	0.281 a
SWN0	0.191 c	0.325 c	0.149 c	0.153 b	0.052 b	0.022 b	0.139 a
FWN360	0.443 b	0.778 b	0.332 b	0.386 a	0.046 b	0.036 b	0.271 a
SWN360	0.206 c	0.423 c	0.097 c	0.162 b	0.039 b	0.015 b	0.080 a

Note: Different letters within a column and within a factor indicate significant differences among individual treatments ($P < 0.05$). Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}

Different biochemical categories of substrates

The average absorbance of each carbon source category at 96 h were shown in *Figure 5*. There were significant differences in substrate use patterns under water salinity and N amount treatments. For example, amines and phenols were the least utilized by soil microbial communities in FWN360, whereas phenols were least utilized in SWN0 and SWN360. These results indicated distinct differences in microbial community structures among the treatments. Nitrogen application increased the utilization of carboxylic acids and carbohydrates both in the FW and SW treatments. Saline water irrigation increased carbohydrate utilization both in N0 and in N360 treatment.

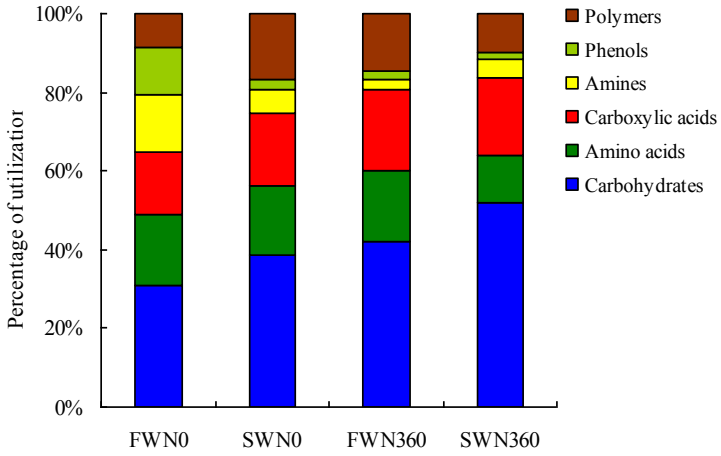

Figure 5. *Utilization percentages of six categories of carbon substrates as affected by irrigation water salinity and N amount. Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}*

The ANOVA analyses results indicated that saline water irrigation and N fertilizer amount significantly affected the utilization of amino acids, amines, and phenols (*Table 2*). The three substrates utilization rates were greatest in FWN0. Irrigation water salinity also significantly affected the utilization of carbohydrates, carboxylic acids, and polymers. In addition, the highest substrates utilization rates of those compounds were

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in FWN0 and FWN360. Nitrogen fertilizer had no significant effect on carbohydrate, carboxylic acid, and polymer utilization.

Figure 6 shows a heatmap of C substrate utilization as influenced by water salinity and N amount. The sole C source uptake varied among the treatments. Saline irrigation water significantly decreased the utilization of D-cellobiose, D-xylose, β-methyl-d-glucoside, i-erythritol, and D-mannitol ($P < 0.05$) in both the N0 and N360 treatments. Nitrogen fertilizer significantly decreased i-erythritol utilization but had no significant influence on the utilization of D-cellobiose, β-methyl-D-glucoside, and D-xylose in FW or in SW. In addition, α-D-lactose utilization was greatest in FWN0. Irrigation water salinity and N amount had no significant influence on the utilization of N-acetyl-D-glucosamine, glucose-1-phosphate, D, L-α-glycerol phosphate, and D-galactonic acid γ-lactone ($P > 0.05$).

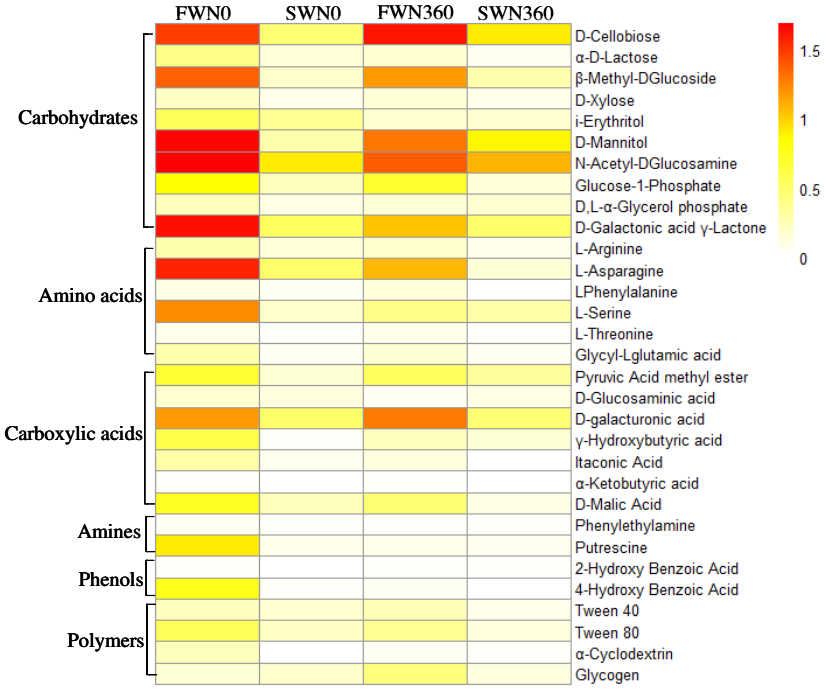

Figure 6. *Heatmap showing the utilization of 31 carbon substrates as influenced by irrigation water salinity and N amount. Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}*

Regarding amino acids, L-serine utilization was greatest in FWN0. L-asparagine utilization was significantly higher (i) in FW than that in SW, and (ii) in N0 than that in N360 ($P < 0.05$). Compared with FW treatment, SW treatment significantly reduced L-phenylalanine utilization, whereas there was no significant difference in L-phenylalanine utilization between N0 and N360. Neither irrigation water salinity nor N amount had significant impact on the utilization of L-arginine, L-threonine, and glycyl-L-glutamic acid. The SW treatment significantly reduced carboxylic acid utilization, especially the utilization of γ-hydroxybutyric acid ($P < 0.05$). The SW treatment significantly reduced the utilization of amines and phenols, especially the utilization of putrescine and 4-hydroxy benzoic acid ($P < 0.05$).

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A PCA analysis was used to confirm the impact of water salinity and N amount on sole carbon source utilization. The first axes and the second axes together explained 88.8 % of the total variation (*Figure 7*). The PCA clearly separated the soil samples according to the water salinity or N amount. This indicated significantly different microbial communities and patterns of potential carbon utilization among the treatments. There were three clusters: (1) FWN0; (2) FWN360; (3) SWN0 and SWN360.

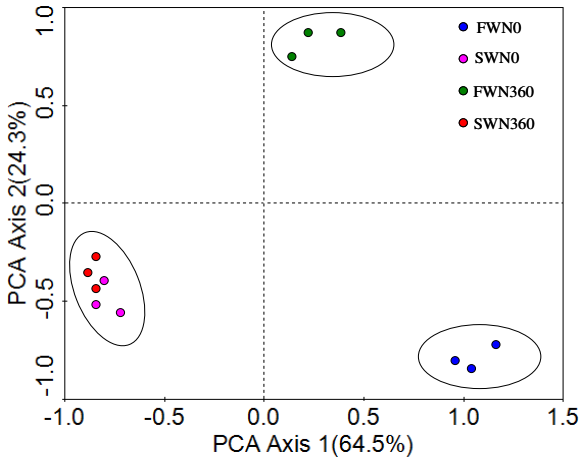

Figure 7. *Principal coordinate analysis (PCA) of carbon source utilization profiles as affected by irrigation water salinity and N fertilization. The resulting plots of 12 samples. Abbreviations: FWN0, fresh water with no N fertilizer; FWN360, fresh water with 360 kg N ha^{-1}; SWN0, fresh water with no N fertilizer; SWN360, fresh water with 360 kg N ha^{-1}*

Correlation analyses

Soil salinity (EC1:5) was negatively correlated with soil basal respiration, AWCD and the six categories of carbon source utilization (*Figure 8*). Soil pH was positively correlated with AWCD and the six categories of carbon source utilization. SOM and total N was positively correlated with soil basal respiration However, neither SOM nor total N were significantly correlated with AWCD or carbon source utilization.

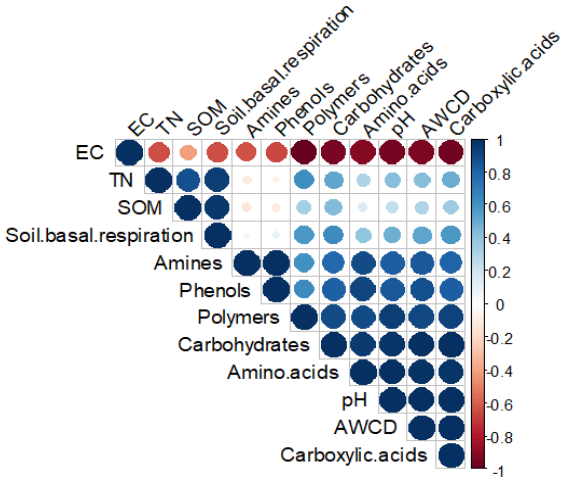

Figure 8. *Correlations between selected soil chemical properties and carbon source utilization*

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Discussion

The use of saline irrigation water is likely to increase in the future, especially in developing countries where there is extreme shortage of freshwater. However, long term saline water irrigation may alter soil properties, for instance, soil physicochemical properties and microbial metabolic activity. In our study, irrigation with saline water increased soil salinity. It was previously also found that saline water irrigation increased soil salinity (Huang et al., 2011; Ahmed et al., 2012). Our results agree with Villa-Castorena et al. (2003) who found that N fertilizer application increased soil salinity. Soil pH declined when the soil was fertilized with N, this agrees with the report of Enwall et al. (2005). In this study, soil pH decreased with irrigation water salinity increased, one explanation is that the accumulation of strong acidic ions (e.g. NO_3^-, SO_4^{2-} and Cl^-) in salinized soil (Fan et al., 2009). Salinity was identified as one of the most potent environmental factors affecting soil microorganisms. Some researchers have reported on interaction between soil salinity and microbial activity. For instance, Rietz and Haynes (2003) and Yuan et al. (2007) found that salt conditions obviously inhibit soil microbial communities and their biochemical activities. Saline water irrigation will increase salt accumulation in the root zone and have adverse effects not only on soil physicochemical properties but also on microbial metabolic activities. Our results agree with observations that saline water irrigation for ten years decreased soil basal respiration, mainly due to osmotic stress in salinized soil (Mavi and Marschner, 2013). The carbon source utilization efficiency was an important factor influencing soil microbial communities activities in salt affected soils (Chen et al., 2017a). In this study, AWCD was less in saline water irrigation than in fresh water irrigation. This result indicated that irrigation with saline water could restrain soil microbial metabolic activities and affect substrate utilization patterns. In addition, Rietz and Haynes (2003) also reported that a negative relationship between soil microbial activity and soil salt. One explanation is that increased soil salinity lead to more stressed, a smaller soil microbial community which was lower carbon source utilization efficiency.

Fertilization application may indirectly or directly influence soil chemical, physical, and biological properties (Marschner et al., 2003; Hai et al., 2010). Nitrogen fertilization may influence soil microbial metabolic activities through a variety of mechanisms, for example by altering microbial biomass, diversity, and labile carbon inputs by soil microbial (Phillips et al., 2015; Miura et al., 2016). However, the effects of N fertilization vary depending on soil environment, original N content, plant species, and other factors (Rath and Rousk, 2015). In this study, N fertilization for ten years reduced soil microbial metabolic activities in fresh water irrigation, but had no significant effect in saline water irrigation. In addition, N fertilization application increased soil basal respiration in this study. Sarathchandra et al. (2001) also reported that 200 and 400 kg N/ha markedly reduced soil microbial metabolic activities compared with an unfertilized treatment. He et al. (2007) reported that the primary reason why soil microbial metabolic activities declined in N fertilized soil was that the N fertilizer reduced soil pH.

The Biolog Ecoplates can be used as a method to make preliminary comparisons of the soil microbial metabolic activities on all the substrates; however, this method does not provide specific metabolic property on each categories of carbon source utilization (Zhang et al., 2014). Some soil microbial may prefer to utilize amino acids, and others may tend to utilize carbohydrates. Chen et al. (2017b) found that different utilization rate of carbohydrates, amino acids, carboxylic acids, and polymers by soil microbial

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communities under different saline irrigation and fertilization regimes. Our study showed that the utilization of six substrate categories was markedly different in saline water irrigation than that in fresh water irrigation. In addition, irrigation water salinity and N amount markedly influenced the utilization rates of amines, amino acids, and phenols, which demonstrated that these three categories are sensitive indicators for distinguishing carbon source utilization under different irrigation water salinity and N amount treatments by soil microbial communities. Overall, the results showed that microbial in saline water irrigated soils may decrease their use of one carbon source, but increase their use of another. The result is that the total metabolic activity is sustained. Meanwhile, the significant correlations between above carbon categories and soil chemical properties were highly consistent with the AWCD. For example, soil salinity ($EC_{1:5}$) was negatively correlated with AWCD and the six categories of carbon source utilization. This agrees with the report of Min et al. (2016). Based on experimental results, we concluded that D-cellobiose, β-methyl-d-glucoside, D-xylose, i-erythritol, D-mannitol, α-D-lactose, L-serine, L-asparagine, L-phenylalanine, γ-hydroxybutyric acid, putrescine and 4-hydroxy benzoic acid were the sole carbon substrate categories for distinguishing the carbon source utilization rate of each category by the soil microorganisms. However, the Biolog Ecoplates technique only analyzes the cultivable soil microbial community activities. For more accurate assessment, future work needs to be done based on functional groups and molecular methods.

Conclusions

In this study, we quantified the effects of ten years of saline water irrigation on soil properties and microbial metabolic activities under drip-irrigated cotton field in arid area. Ten years saline irrigation water and N application may alter soil properties and significantly influence microbial metabolic activities. The soil basal respiration and microbial metabolic activities were higher in fresh water irrigation than in saline water irrigation. Nitrogen application significantly reduced soil microbial metabolic activities in the fresh water treatments but had no significant effect in the saline water treatments. The amino acids, amines, and phenols utilization rate decisively impact the carbon source utilization under different irrigation water salinity and N fertilizer rate. This study results increase understanding about soil biological processes under saline conditions. In the future, we should focus on the improvement of saline soil, to explore the improvement effect of straw returning and biochar application on saline soil, and provide a theoretical basis for the utilization of saline water and improvement of saline soil in arid areas.

Acknowledgments. This work was jointly funded by The National Natural Science Foundation of China [41661055], the Youth Innovation Talent Cultivation Program of Shihezi University [CXRC201706].

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80358048

EFFECTS OF ELEVATION ON THE ABOVEGROUND BIOMASS AND CARBON STOCK IN THE ORIENTAL BEECH (*Fagus orientalis* Lipsky) FORESTS OF THE SINOP REGION, TURKEY

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(Received 2nd Jun 2020; accepted 6th Oct 2020)

Abstract. In this study it was our aim to assess the amount of aboveground biomass and carbon stock of beech stands based on elevation. To achieve this, in the research area, 55 trial sites were chosen by selective sampling method depending on elevation climatic zones (400–600 m, 600–800 m, 800–1000 m). In these trial areas, 55 trees were cut down and the weights of their bark, leaves, stem and branches were determined. The carbon content of each component was calculated. The results of the study showed that, in all elevation zones, the stem wood had the highest average biomass and carbon stock values of all the tree components (for the 400–600 m, biomass was 199.19 kg and carbon stock was 95.82 kg; for the 600–800 m, biomass was 378.75 kg and carbon stock was 182.49 kg; for the 800–1000 m, biomass was 372.56 kg and carbon stock was 183.39 kg). This was followed by the branches, bark and leaves in descending order. The aboveground biomass in the single tree components of leaves, branches, stem and total biomass, all except the biomass of their bark varied with elevation in oriental beech stands, which are economically significant species in Turkey.
Keywords: *biomass, carbon stock, elevation zones, stem, branches, leaves*

Introduction

The most important geomorphological features affecting the spread of beech forests are elevation, aspect and slope. The effects of climatic factors such as light, temperature, precipitation and evaporation change according to these factors and habitat type (Atalay, 1983). Beech forests are more widespread in shady environments on the northern slopes of mountains that are foggy during the vegetation season. There are also beech forests on the southern slopes where fog is observed and on the upper parts of foggy southern slopes (Atalay, 1992).

The optimal conditions for beech tree growth are low temperature and high precipitation. These conditions are related to elevation from the sea (Atalay, 1992). As the elevation increases, the temperature and the relative humidity to water vapor ratio decrease while precipitation, evaporation and radiation intensity increase (Atalay, 1983). Beech forests can grow on areas at 150–200 m elevation, as well as at 1200 m and mostly at 1800 m. Generally, above 1600 m, the proportion of beech decreases and instead coniferous trees (fir and spruce) are observed (Atalay, 1983, 1992).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80498063

Various factors affect the aboveground biomass. The most important ones include the age and density of the stand, precipitation, temperature, latitude, physiographic factors (elevation, slope, aspect, relief) and soil factors.

In a study performed in the Himalayas, Sharma et al. (2018) investigated the differences in the growth behavior of tree species at different elevations in terms of diversity, regeneration dynamics, biomass and carbon stock. Depending on the elevation, the total biomass density was found to be the greatest between 1600–1900 m and 3100-3400 m. Accordingly, the total carbon density varied at different elevation ranges. Thus, it was concluded that *A. pindrow*, *A. spectabilis*, *A. acuminatum*, *B. utilis*, *Cedrus deodora*, *Q. semecarpifolia* and *R. arboreum* species found in the high regions of the Himalayas had more carbon stock potential and thus could be recommended for carbon management in afforestation works.

Fehse et al. (2002) performed a study in tropical forests and surprisingly found that the total aboveground biomass and annual biomass production were higher at high elevations, considering the assumption of biomass decrease with increasing elevation. These results were attributed to different forest systems, tree species, soils, parent materials and climatic conditions. This study concluded that the positive characteristics of tree species, rapid growth, productive nitrogen and phosphorus compatibility, the suitability of climatic conditions at high elevations, might be the reasons for the increased levels of biomass at higher elevations.

In a study by Zhu et al. (2010) in Northeast China, vegetation cover, eroded material and the amount of carbon stored in the soil were determined in 22 different sites, depending on elevation (700–2000 m). The authors concluded that the carbon density in the vegetation decreased with increasing elevation.

Nagar et al. (2017) reported that biomass and carbon amounts at different elevations and aspects in the humid, temperate Western Himalayan ecosystems were the highest on the northern aspect and in the 2101–2400 m elevation belt. In a study of 444 sample areas performed in the Agarta region of India, it was revealed that the biomass and carbon content of a tree are related to tree diameter and are not dependent only on the number of trees (Majumdar and Selvan, 2018). In a study conducted in Poland, it was found that there was a significant relationship between tree species diversity and aboveground biomass (Gazda et al., 2015).

Kobler et al. (2019) found that net ecosystem production, aboveground biomass and soil CO_2 emissions were higher on the south-west facing slope rather than the north-east facing slope. They concluded according to these results that there may be changes due to elevation and aspect over small distances in mountainous areas and the forest carbon dynamics in similar complex topographies will not give reliable results on large scales. Similarly, Güner (2019) examined the aboveground biomass and carbon concentrations found in the tree components in different habitats in *Abies nordmanniana* subsp. *equi-trojani* (Bursa-Uludağ and Kastamonu-Ilgaz Mountain). Root, stem, branch, bark and seed samples were taken from 25 sampling points with different elevation, slope and aspect characteristics. As a result of carbon measurements and statistical analyses, significant differences were found in the carbon concentrations. While the lowest carbon concentration was in the roots, the highest was found in the bark. For *Abies nordmanniana* subsp. *equi-trojani* forests, the weighted carbon concentration was found to be 52.31% for aboveground biomass and 52.15% for total tree biomass. They concluded that the carbon concentrations found in this study could be used to calculate the carbon stocks

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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stored in the existing tree components in the *Abies nordmanniana* subsp. *equi-trojani* forests at different locations.

In light of the research discussed above, the purpose of this study is to investigate elevation-based changes in the amounts of aboveground biomass and carbon stocks of the components of Oriental beech trees (*Fagus orientalis* Lipsky.) growing in the Sinop region.

Materials and methods

General overview of the research area

This study was conducted in Turkey in 2013. The research was carried out in the pure beech forests on the border between Sinop province and Türkeli district in the Western Black Sea Region (*Fig. 1*). The whole area of its present distribution has a North to South extensions from 41°57'54'' N to 41°44'42'' N and East to West extensions from 34°23'32'' E (Greenwich) to 34°16'25'' E.

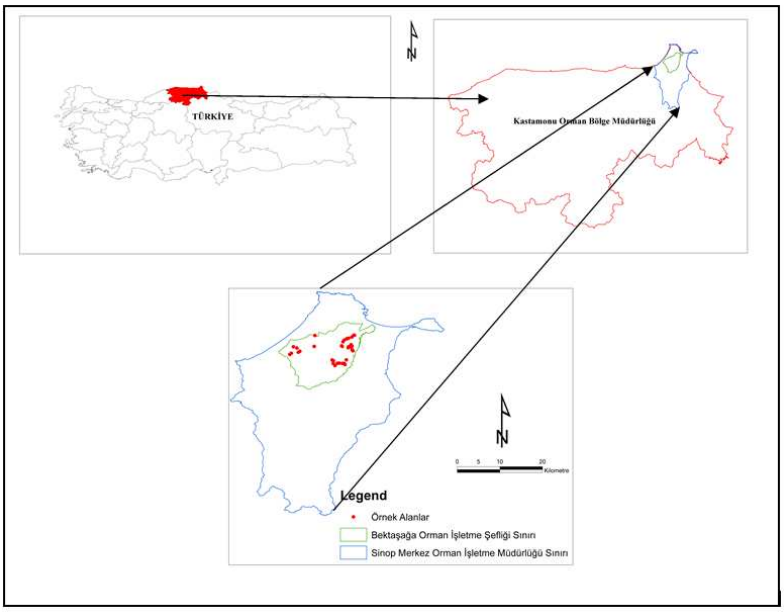

Figure 1. *Overview of the research area*

Climate properties of the research area

In the research area; according to the Thornthwaite method, all of the sample areas the climate is humid, moderately warm, with little or no water deficiency, similar to the oceanic climate.

Soil characteristics of the research area

The soil texture in the sample areas between different elevations (400–600 m, 600-800 m and 800–1000 m) is heavy clay, loamy clay, clay loam, sandy loam, sandy clay and sandy clay loam. The soils of the research area were generally in the clay soil class. Loamy clay soil was dominant in all elevation zones. The soils of the study area have high acidity, low lime content and no salinity (*Table 1*) (Güvendi, 2013).

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Table 1. *Average soil characteristics of the research area*

Elevation Zones	Sand (%)	Silt (%)	Clay (%)	Soil Texture	SWHC (%)	pH (H_2O)	EC (dS/m)	Organic Substance (%)	Lime (%)
400–600 m (1)	40	19	41	Loamy clay	11.7	5.2	0.07	1.6	0.65
600–800 m (2)	47	19	34	Loamy clay	13.4	5.9	0.14	2.7	0.97
800–1000 m (3)	37	23	40	Loamy clay	11.3	5.7	0.13	2.9	0.85

SWHC: soil water holding capacity; EC: electrical conductivity

Trial areas and determination of sample trees

The research area was divided into three elevation zones (400–600 m, 600–800 m and 800–1000 m). A total of 55 trial areas of 400 m^2 were selected from each elevation zone, in almost equal numbers. In each of these trial areas, one beech tree with representative characteristics of the trial area was cut (Kahyaoğlu, 2017). *Figure 2* shows the cutting of sample trees representing the trial areas, separation into sections and taking cross-section samples from sample trees.

Figure 2. *Taking samples from the research area*

Sample trees in the trial area were selected from trees of different diameters to represent each diameter class. The sample trees were all alive, single stem, healthy, with an intact top and natural branch pruning. Some information about the sample areas is given in *Table 2*.

After measuring the breast height diameters of the determined sample trees, they were cut to the nearest surface and their lengths were measured. The branches of the trees were then separated from their stems and the stem diameters were measured at 0.30 m and 1.30 m above soil level and also at heights above 1.30 m at 2 m intervals; 5 cm thick cross-sections were taken and their fresh weights were determined by weighing with an electronic scale. Following that, the bottom diameters and lengths of all branches of the stem were measured. Then a sample branch was taken to represent the growth and top structure of the trial tree and weighed with its leaves. Subsequently, the branch and fresh leaf weights of the sample branch were determined separately. Following that, a 5 cm cross section of the sample branch wood and all leaves of the sample branch were put into polyethylene bags, then numbered and transferred to the laboratory.

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Table 2. *Summarized data from the sample plot areas*

Plots No.	Coordinates		Elevation Zones (m)	Altitude (m)	Aspect	$d_{1.30}$ (cm)	Height (m)
	x	y					
1	623951	4641842		375	N	27	21.3
2	624028	4641571		460	N	32.5	28.3
3	624307	4641696		390	N	21	18.6
4	624508	4641845		410	S	28	25.5
5	624623	4641864		435	N	30	26.3
6	624610	4641566		500	S	21.5	21.1
7	623592	4641586		450	S	28	26
8	623599	4641880		360	S	19.9	17.3
9	623278	4641956		350	N	26.3	25.9
10	623176	4641656	400-600 (1)	440	N	20	20.1
11	622990	4641943		335	N	21.5	23.6
12	622995	4641651		450	N	21	21.3
13	622711	4641904		330	N	17	18
14	622223	4642053		310	S	29	25.8
15	622423	4641661		400	S	23	16.1
16	621882	4642035		300	S	25	22.8
17	621952	4641706		410	S	19	19.8
18	623356	4641737		375	S	18	18.1
19	623589	4641496		490	S	21	18.8
20	614311	4627415		770	S	26	23.5
21	619753	4629219		670	S	30	27.6
22	614075	4627096		790	S	33	28.6
23	619837	4629470		660	S	29	26.2
24	620591	4629528		760	N	25.5	25.1
25	620767	4629236		750	N	31	26.8
26	611248	4629423		780	N	21	16.5
27	611352	4630025		750	N	22	19.6
28	610727	4629577	600-800 (2)	780	S	20.8	18.7
29	610606	4630079		770	S	16.3	16
30	610237	4630315		790	S	19	19.6
31	612330	4633007		650	N	15.5	16.1
32	612137	4633312		750	N	16.8	14.8
33	612247	4632715		610	N	26	21.3
34	614338	4633365		712	N	23	25.9
35	615000	4632674		770	S	24	25.1
36	611703	4632630		650	N	30	23.1
37	612564	4629611		990	N	29	25.6
38	612970	4628370		1100	N	31	27.9
39	612774	4629192		1012	S	25.5	23.0
40	615713	4634136		975	S	36.5	40.7
41	615332	4634157		990	N	25	29.2
42	615028	4633365		1040	S	21.1	17.6
43	616590	4632263		930	S	20	24.9
44	616059	4631675		872	N	21.8	24.9
45	616562	4631720		915	N	25.5	20.3
46	616719	4631556	800-1000 (3)	940	N	25.5	25.6
47	615616	4631610		860	N	23	29.2
48	611866	4626218		1140	S	26	27.2
49	612007	4626459		1165	S	19	23.2
50	612146	4626745		1182	S	30	25.9
51	612095	4627094		1170	S	29	30
52	611807	4627513		1140	N	24	24.1
53	611499	4627561		1075	N	24	26.4
54	611401	4627732		1005	N	28	25.1
55	611886	4627031		1090	S	18.5	17.4

$d_{1.30}$ (cm): diameter at breast height of tree; Height (m): height of tree

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80498063

Determination of tree biomass

In order to determine the fresh and oven dry trunk weights, first the trunk volume of each sample tree was calculated. In the stem volume calculation, the stem was divided into three parts. The bottom stump is between 0 m and 0.30 m and its volume was calculated using the formula for the volume of a cylinder with the measured bottom stump diameter ($d_{0.30}$ m) and a bottom stump height of 0.30 m. The volumes of 2 m long sections from the bottom stump to the top, such as 0.30–2.30 m and 2.30–4.30 m, were calculated using the Huber (median surface) formula by using mid-section diameters such as $d_{1.30}$ and $d_{3.30}$. The volume of the top was calculated by assuming it to be a cone and lastly the volumes of the bottom stump section and the top were added to obtain the stem volume.

The base diameters and lengths of all living branches of each sample tree were measured and the volumes of all branches were calculated by assuming them to be cone shaped. Then, the volumes of all branches were summed to obtain the total branch volume of the sample tree. The total weight was then calculated by selecting a sample branch to represent all branches and the development of the sample tree. Then, the sample branch was stripped of leaves and the leaf and branch weights were determined separately. The fresh branch weight of a tree was calculated by first calculating the ratio of the volume of the sample branch to the total branch volume, which was then multiplied by the fresh weight of the sample branch. Similarly, the total dry weight of a tree was calculated by using the ratio between the dry and fresh weights of a cross section taken from the sample branch.

In order to determine the total fresh leaf weights of each sample tree, the leaf weight in the sample branch selected to represent the branching of the tree was determined and then multiplied by the total branch volume of the sample tree; the result obtained was then divided by the volume of the sample branch to give the total fresh leaf weight of the sample tree. After that, the total fresh leaf weight of the sample tree was multiplied by the dry leaf weight of the sample branch and then divided by the fresh leaf weight of the sample branch to obtain the total dry leaf weight of the sample tree.

Because the beech tree bark was thin, it was difficult to separate the bark from the trees when they were fresh. The dry bark weight of each tree was calculated from the difference between the total stem weight with dry bark and total stem weight without dry bark. The volumization of the branches taken from the sample trees and the determination of the fresh leaf and branch weight of the sample branch are shown in *Figure 3*.

Figure 3. *Biomass measurements in the research area*

The stem, branch and leaf samples, whose fresh weights were determined in the field, were brought to the laboratory. The stem and branch samples were then dried in an oven

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at 65°C for 7 days while the leaf samples were kept at 65°C for 48 hours (Peichl and Arain, 2007). All samples were completely dried and their weights were determined.

Determination of carbon stock amounts

After completing the required biomass measurements for all components of each sample tree, the stem, branch, bark and leaf samples were crushed and ground into a powder. The amounts of carbon in the tree components forming the ecosystem biomass were then determined. A LECO Truspec 2000 device was used to determine carbon content (C%) by dry combustion method. The amount of carbon stored for all wood components in each sample area was calculated by multiplying the measured biomass values of the wood components by the C% values.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine whether there was a difference in the amounts of biomass and carbon of aboveground single tree components depending on elevation at the 5% significance level.

Duncan's post hoc test was performed to identify homogeneous subgroups from the differences obtained from the one-way ANOVA results.

Results

Distribution of the diameter at breast height ($d_{1.30}$) to elevation zones

When the distribution of $d_{1.30}$ diameters of trees representing the study area to elevation zones was examined, it was observed that the trees in the II. elevation zone had a thicker diameter than the trees in the I. and III. elevation zone (*Fig. 4*).

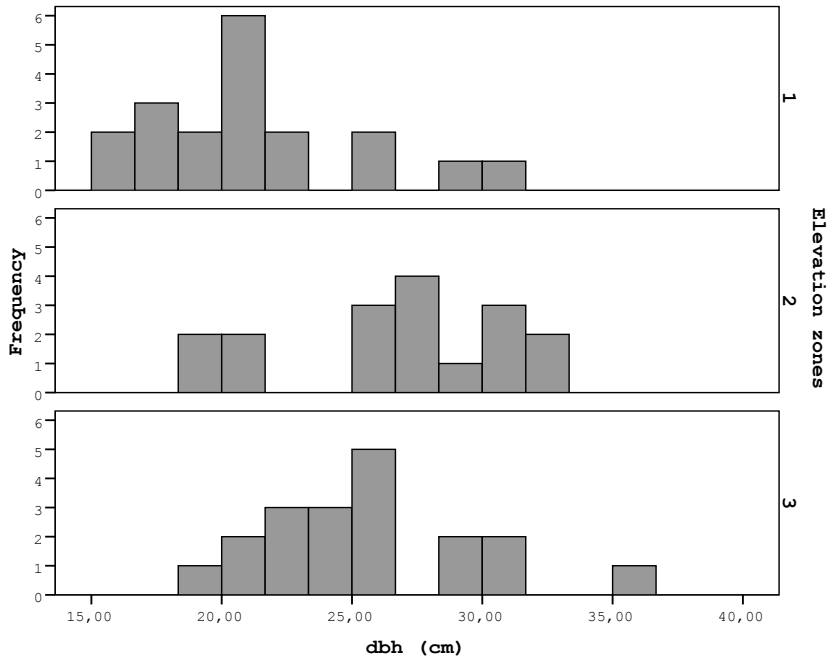

Figure 4. *The diameter at breast height (cm) distributions against elevation zones (1: 0-400, 2: 401-800, 3: 801-1200 m)*

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Biomass amounts in single tree components with respect to elevation zones

As shown in *Fig. 5*, for 400–600 m, the highest average biomass value of Oriental beech tree components was found for the stem wood in a single tree with 199.19 kg. This was followed by branches with 49.27 kg, bark with 22.43 kg and leaves with 7.03 kg. For 600–800 m, the highest average biomass value of Oriental beech tree components was found for the stem wood in a single tree with 378.75 kg. This was followed by branches with 76.48 kg, bark with 38.49 kg and leaves with 10.95 kg. For 800–1000 m, the highest average biomass value of Oriental beech tree components was found for the stem wood in a single tree with 372.56 kg. This was followed by branches with 40.13 kg, bark with 30.84 kg and leaves with 4.94 kg.

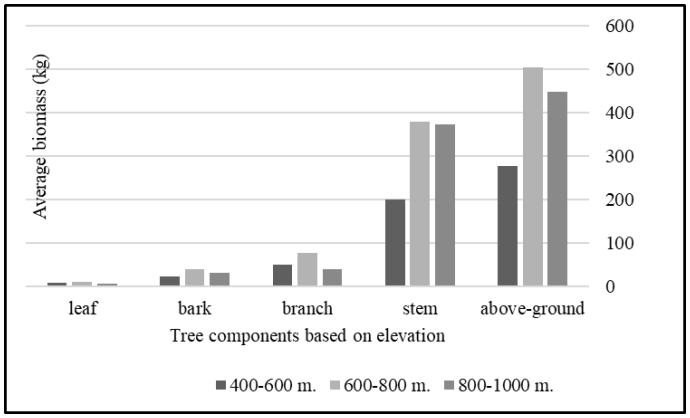

Figure 5. *Measured biomass amounts of sample tree components for elevation zones*

The relationship between diameter at breast height ($d_{1.30}$) and biomass amounts according to elevation zones

When we look at the biomass amounts according to the elevation zones of the trees representing the study area; It is seen that the trees representing the I. elevation zone have lower diameter at breast height and therefore biomass amounts. It is seen that of the trees in the II. and III. elevation zone has higher diameter at breast height and therefore biomass amounts (*Fig. 6*).

Simple variance analysis results showing the change in single tree component biomass with respect to elevation

One-way ANOVA was performed to determine whether the biomass amounts for the aboveground single tree components differed with elevation at the 5% significance level (*Table 3*). According to the one-way ANOVA results, significant differences were found with respect to elevation at the 95% confidence level for the leaf, branch, stem and total biomass amounts, but not for the bark.

Also, Duncan's post hoc test was performed to identify homogeneous subgroups from the differences shown by the one-way ANOVA results. According to Duncan's post hoc test results for the leaf biomass, the 800–1000 m (average=4.96 kg) and 400–600 m (average=6.99 kg) elevation zones were in the same group, whereas 600–800 m elevation zone (average=10.77 kg) was different from the other zones with a higher biomass average. For the branch biomass, the 800–1000 m (average=40.25 kg) and 400–600 m (average=48.51 kg) elevation zones were in the same group while the medium elevation

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zone (600–800 m) was found to have a higher amount of branch biomass than the other elevation zones (average=82.05 kg). For the stem component, the 800–1000 m (average=372.64 kg) and 600–800 m (average=378.76 kg) elevation zones were in the same group while the 400–600 m elevation zone (average=198.43 kg) was in another group with significantly lower biomass content. Regarding the aboveground total tree biomass, the 800–1000 m (average=448.90 kg) and 600–800 m (average=503.88 kg) elevation zones were in the same group while the 400–600 m elevation zone (average=276.47 kg) was in another group with significantly lower total biomass (*Table 4*).

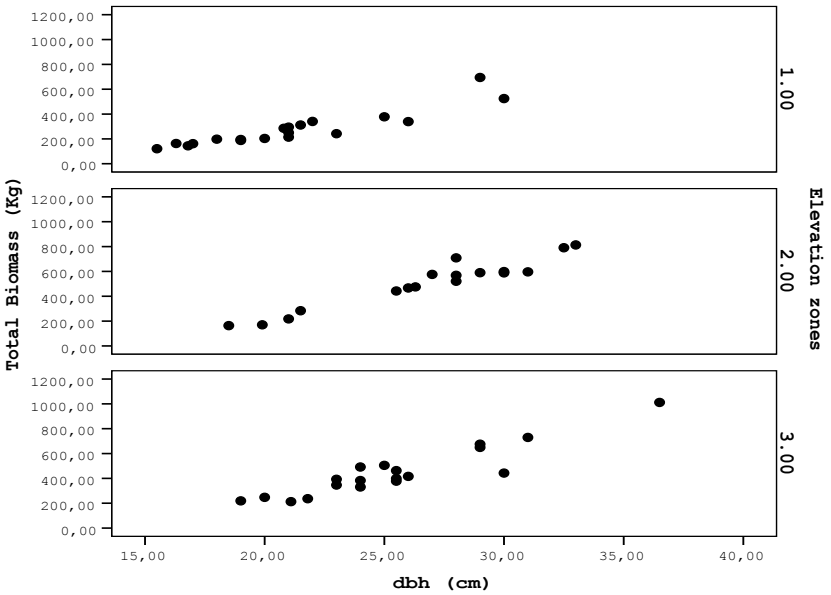

Figure 6. *The relationships between total biomass (kg) and diameter at breast height (cm) distributions against elevation zones (1: 0-400, 2: 401-800, 3: 801-1200 m)*

Table 3. *Simple variance analysis results showing the difference in biomass (kg) amounts of single tree components with respect to elevation*

Tree Components	Sources of Variation	Total of Squares	Degree of Freedom	Average of Squares	F Value	Significance Level (P)
Leaves	Between-groups	310.095	2	155.048	7.106	0.002*
	Within-groups	1134.613	52	21.819		
	Total	1444.708	54			
Bark	Between-groups	2281.292	2	1140.646	2.651	0.080ns
	Within-groups	22377.656	52	430.340		
	Total	24658.948	54			
Branches	Between-groups	17317.995	2	8658.97	6.251	0.004*
	Within-groups	72030.872	52	1385.209		
	Total	89348.867	54			
Stem	Between-groups	390400.52	2	195200.259	9.170	0.000*
	Within-groups	1106946.6	52	21287.434		
	Total	1497347.1	54			
Total	Between-groups	516601.24	2	258300.618	7.843	0.001*
	Within-groups	1712497.8	52	32932.649		
	Total	2229099.0	54			

P: limit probabilities in ANOVA with one factors; *: P<0.05; **: P<0.01; ***: P<0.001; ns: P>0.05

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Table 4. *Duncan's post hoc test results for single tree components and total biomass amounts*

Elevation	N	Tree Components			Total Biomass
		Leaves	Branches	Stem	
400–600 m	19	6.99a	48.50a	198.43a	276.47a
600–800 m	17	10.77b	82.05b	378.76b	503.88b
800–1000 m	19	4.96a	40.25a	372.64b	448.90b

Different letters in each column indicate significant differences (Duncan, P<0.05)

Carbon stock amounts of single tree components with respect to elevation zones

For the 400–600 m elevation zone, the highest average carbon stock value of Oriental beech tree components was found in the stem wood with 95.82 kg, followed by branches with 23.43 kg, bark with 10.48 kg and leaves with 3.22 kg. For the 600–800 m elevation zone, the highest average carbon stock value was found in the stem wood with 182.49 kg, followed by branches with 33.93 kg, bark with 17.86 kg and leaves with 5.06 kg. For the 800–1000 m elevation zone, the highest average carbon stock value was found in the stem wood in a single tree with 183.39 kg, followed by branches with 21.88 kg, bark with 16.58 kg and leaves with 2.32 kg (*Fig. 7*).

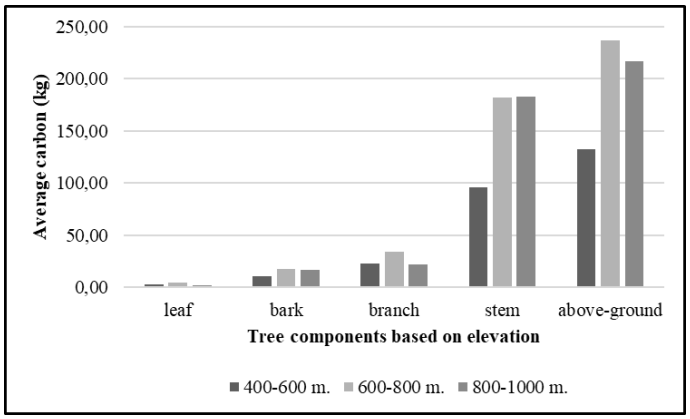

Figure 7. *Measured carbon stock amounts of single tree components by elevation zone*

The relationship between diameter at breast height ($d_{1.30}$) and carbon stock amounts according to elevation zones

When we look at the carbon stock amounts according to the elevation zones of the trees representing the study area; It is seen that the trees representing the I. elevation zone have lower diameter at breast height and therefore carbon stock amounts. It is seen that the trees in the II. and III. elevation zone have higher diameter at breast height and therefore carbon stock amounts (*Fig. 8*).

Simple variance analysis results showing change of carbon stock amounts of single tree components with respect to elevation

One-way ANOVA was performed to determine whether there was a difference between carbon stock amounts of aboveground single tree components and elevation factor according to the 95% significance level (*Table 5*).

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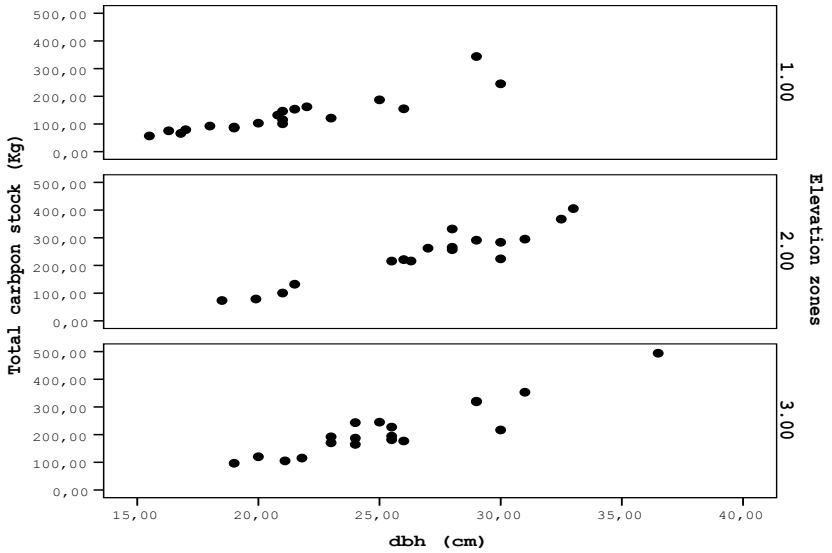

Figure 8. *The relationships between total carbon (kg) and diameter at breast height (cm) distributions against elevation zones (1: 0-400, 2: 401-800, 3: 801-1200 m)*

Table 5. *Simple variance analysis results showing change of carbon (kg) stock amounts of single tree components with respect to elevation*

Tree Components	Sources of Variation	Total of Squares	Degree of Freedom	Average of Squares	F Value	Significance Level (P)
Leaves	Between-groups	66.450	2	33.225	7.119	0.002*
	Within-groups	242.700	52	4.667		
	Total	309.150	54			
Bark	Between-groups	487.963	2	243.982	2.484	0.093ns
	Within-groups	5107.808	52	98.227		
	Total	5595.771	54			
Branches	Between-groups	1984.421	2	992.210	2.839	0.068ns
	Within-groups	1817.699	52	349.533		
	Total	20160.120	54			
Stem	Between-groups	89548.865	2	44774.433	8.724	0.001*
	Within-groups	266872.09	52	5132.156		
	Total	356420.96	54			
Total	Between-groups	113808.10	2	56904.052	7.242	0.002*
	Within-groups	408563.42	52	7856.989		
	Total	522371.52	54			

P: limit probabilities in ANOVA with one factors; *: P<0.05; **: P<0.01; ***: P<0.001; ns: P>0.05

The results of the one-way ANOVA showed that there were significant differences with respect to elevation at the 95% confidence level for the stem, branch and total carbon stock amounts, but not for the bark and branch carbon stocks.

Duncan's post hoc test was performed to identify any homogeneous subgroups from the differences obtained according to the one-way ANOVA results.

According to the results of Duncan's post hoc test for the amount of carbon stock in the leaves, the 800–1000 m (average=2.29 kg) and 400–600 m (average=3.20 kg)

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DOI: http://dx.doi.org/10.15666/aeer/1806_80498063

elevation zones were in the same group while the 600–800 m elevation zone was in another group with a significantly higher carbon stock value (average=4.98 kg). For the stem component, the 400–600 m (average=95.41 kg) elevation zone had a significantly lower amount of carbon in the stem than the other two elevation zones. The 600–800 m (average=179.98 kg) and 800–1000 m (average=180.52 kg) elevation zones were in the same group and had significantly higher amounts of carbon. For the total aboveground total tree components, the 400–600 m (average=132.05 kg) elevation zone had a significantly lower amount of carbon than the other two elevation zones. The 600–800 m (average=236.58 kg) and 800–1000 m (average=217.02 kg) elevation zones were in the same group and had significantly higher carbon averages (*Table 6*).

Table 6. *Duncan's post hoc test results for single tree components and total carbon amounts*

Elevation	N	Tree Components		Total Carbon
		Leaves	Stem	
400–600 m	19	3.20a	95.41a	132.05a
600–800 m	17	4.98b	179.98b	236.58b
800–1000 m	19	2.29a	180.52b	217.02b

Different letters in each column indicate significant differences (Duncan, P<0.05)

Discussion

According to the one-way ANOVA results, significant differences were found with respect to elevation at the 95% confidence level in the leaf, branch, stem and total biomass values, but not for the bark, as well as in the changes of leaf, stem and total carbon amounts, but not for the bark and branch. The biomass amounts of the tree components were generally found to be higher in the 600–800 m and 800–1000 m elevation zones than in the 400–600 m elevation zone. This may be due to drought at low elevations (400–600 m) because beech trees have high moisture demands. On the other hand, it has been shown in previous studies that as elevation increases (>1000 m), the shortening of the growth period and low temperatures adversely affect the development of beech stands (Wardle, 1984). In terms of biomass production, our results show that the 600–800 m zone has the most suitable habitat conditions for beech stands. Fehse et al. (2002) assumed that the productivity of forests and hence the carbon sequestration, falls as elevation increases and their research results support this assumption. Simard et al. (2006) in their study performed in the mangrove forests in the Everglades National Park (Florida, USA), revealed that most of the biomass were available in medium height forests when they matched the elevation and biomass data. Zhu et al. (2010) found that carbon storage and partitioning of different components in Mt Changbai temperate forests in Northeast China varied substantially with forest type and elevation. Aiba et al. (1999) in their study performed at 700, 1700, 2700 and 3100 m, determined that as elevation increased, average leaf areas and the diversity of tree species decreased. Rajput et al. (2017) revealed that total biomass production increased with increasing elevation from 1100–1400 m to 2000–2300 m. Likewise, the increase in biomass with increasing elevation was supported by studies by Zhu et al. (2010) and Gairola et al. (2011). This can be explained by the fact that large conifers in areas with higher elevations have dominance over those in lower elevations. Also, it was found that the highest biomass and carbon storage were in forest land as compared with other land covers. Chen et al. (2019) in a study conducted to determine the amount of carbon storage in forest ecosystems in Hunan state in southern

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China, found that carbon storage increased over more than 20 years. They pointed out that efforts should be made to prevent negative human effects in young and middle-aged forests and appropriate tree species should be selected in order to maximize the carbon storage potential in forest ecosystems. Numerous studies have reported that stand development changes with elevation. In some studies, stand development decreased with elevation, while in others it increased up to a certain elevation, and then decreased (Kalay et al., 1993). Mankou et al. (2017) concluded that the changes in forest structure were mainly caused by elevation. Lucas-Borja et al. (2012) reported that soil moisture and temperature varied significantly with elevation. Soil respiration, microbial carbon and enzyme activity tended to be lower at low elevations, but no differences were found between pure and mixed pine forests. This study suggested that the soils of the Cuenca Mountains may be more sensitive to changes in tree composition under certain physical and chemical conditions, such as soil temperature and humidity. Bagroo et al. (2017) found that soil organic carbon and nitrogen stocks in mountainous forests were affected by forest diversity, topographic characteristics and climate change, concluding that elevation had a positive effect on soil organic carbon. Ali et al. (2017) reported that soil organic carbon stock values increased with increasing elevation in all land uses (agriculture, forest, pasture) but decreased with soil depth. These results show that in areas of high elevation, the recovery of degraded agricultural land into forests and the decrease in land use intensity may increase soil organic carbon stocks in the study area and in similar mountainous areas.

Conclusion

This study found that for Oriental beech stands, which are economically important for Turkey, the amount of leaf, branch, stem and total biomass, but not bark among aboveground single tree components varied with respect to elevation. When examining the carbon contents of beech specimens, the leaf, stem and total carbon contents significantly varied with elevation. In the study area, the higher biomass and carbon values obtained in the 600–800 m elevation zone are related to the favorable characteristics of this habitat for beech. Particularly, the fact that temperature and humidity conditions are optimal for beech at this elevation zone may cause this difference. The lower zone (400–600 m) is dry and the upper zone (800–1000 m) is cold, which may adversely affect the biomass and carbon content of the beech trees that grow in those zones. In the mountainous habitat where the study was carried out, the mutually complex relations between the location, microclimate and soil properties make generalization difficult. For this reason, in future studies, detailed climate and soil investigations should be done to reveal the factors affecting the biomass and carbon contents of oriental beech with respect to elevation.

Acknowledgements. This work was supported by the Karadeniz Technical University Project Department (KTU-BAP), Project No: 5913.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80498063

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80498063

MORPHO-GENETIC INSIGHTS IN BREAD WHEAT (*TRITICUM AESTIVUM* L.) TO COMBAT PECULIAR WATER STRESS CONDITIONS

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(Received 24th Jun 2020; accepted 17th Sep 2020)

Abstract. The present research was undertaken to estimate the yield traits genetics of wheat in normal and water stress conditions. Six lines and three testers were crossed according to line × tester mating fashion to develop eighteen crosses. Results depicted that variances due to genotypes and crosses were significant for all studied traits in both conditions. Significant differences between lines were witnessed for Plant height (cm), grains spike^{-1} and biological yield plant^{-1} under normal but significant for grains spike^{-1} under water stress condition. Chakwal 86 possessed the highest general combining ability for number of tillers plant^{-1}, biological yield plant^{-1} and grain yield plant^{-1} in both conditions. Similarly, in both conditions, the best specific cross combinations were B 215 B 1103 × Galaxy 2013 and GA 2002 × 2511. Gene action was studied to evaluate the nature for inheritance of evaluated traits. All the observed traits reflected non-additive type of gene action except grains spike^{-1} under both conditions. High heritability (H^2) under both conditions was witnessed for plant height, flag leaf area, tillers plant^{-1}, grains spike^{-1} and biological yield plant^{-1}. The genotypes Chakwal 86, Galaxy 2013 and crosses B 215 B 1103 × Galaxy 2013 and GA 2002 × 2511 can be used in future for developing high yielding wheat varieties under water stress conditions.
Keywords: *line × tester, combining ability, gene action, heritability*

Introduction

Wheat is grown almost all over the globe with a total production of 732.40 million tonnes. Major wheat producing countries include the European Union (136.86 million tonnes), China (131.43 million tonnes), India (99.87 million tonnes), Russia (71.69 million tonnes), United States (51.29 million tonnes), Canada (32.20 million tonnes) and Pakistan having production of 25.20 million tonnes (FAO, 2019). Wheat occupies a major place in the economic system of Pakistan and shares 8.9% value addition in agriculture and adds 1.6% to GDP of Pakistan. Production of wheat reached 25.195 million tonnes during 2018-19 with an increase of 119 million tonnes over 25.076 million tonnes during 2017-18 (Ministry of Food, Agriculture and Livestock, 2019).

Currently, the population of Pakistan is more than 210 million and is growing at a fast pace causing an increase in food demand. Therefore, we need to develop wheat varieties that can give better production even with less water requirement (Noorka et al., 2013). Scarcity of water is a global issue due to shrinkage of fresh water resources. It is a major concern for plant breeders to encounter various kinds of drought (HongBo et al., 2006). Different yield associated traits like plant height, spikelets spike, spike length, 1000-grain weight and grains per spikes are considerably reduced by water stress (Mirbahar et al., 2009; Noorka and Teixeira da Silva, 2014). We can enhance our overall production of wheat by improving our existing varieties by better utilization of available germplasm resources and breeding techniques (Ullah

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

et al., 2019). For checking the yield barrier levels and make wheat cultivation more attractive, it has now become necessary to explore alternative approaches. Among the potential alternatives, an important access for increasing wheat production has been the search for a desirable cross combination which can withstand vagaries of climate change (Noorka et al., 2013). The study of combining ability and heritability are important pre-requisites for a sound crop improvement program following conventional hybridization. Several mating designs have been used in different crop plants for estimating combining ability effects of the parents and identification of desirable genotypes for the production of hybrid. These designs provide information on various genetic parameters of variance. The line × tester mating technique designed by Kempthorne (1957; Noorka and Taufiqullah, 2015) appears to be an effective method to evaluate diverse genotypes for assessing their combining ability effects. The design has an added advantage of its application in a situation of complete set of crosses. Different types of gene actions are estimated from combining ability variances which direct the manifestation of yield and yield relating parameters (Dreisigacker et al., 2005). Additionally, comprehensive information about the association among parents and hybrids is necessary to improve wheat hybrid breeding program (Longin et al., 2012; Noorka et al., 2020).

Keeping in view, the current research was commenced to identify the potential parents, crosses and their segregates for viable breeding program and to estimate the combining ability effects of parents and crosses both under normal and water stress conditions.

Materials and methods

Research areas

The present investigation was undertaken at experimental area of the Department of Plant Breeding and Genetics, University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Pakistan (the latitude of Bahawalpur, Pakistan is 29.39, and the longitude is 71.68; Bahawalpur is located with the GPS coordinates of 29° 24' N and 71° 40' E; *Table 1*) during the year 2016-17 by using six wheat genotypes as lines (Chakwal 86, BARS 2009, Chakwal 50, GA 2002, ESWYT P-117 and B215 B1103) and three genotypes as testers (Galaxy 2013, 2511 and Lasani 2008). These six lines (females) and three testers (males) were crossed according to line × tester mating design to develop eighteen crosses.

Experimental details

During the year 2017-18, the F_0 seeds of eighteen crosses along with their parents were planted to develop F_1 under both environmental conditions in randomized complete block design (RCBD) with three replications. Plant to plant and row to row distance was maintained as 15 cm and 30 cm, respectively. All recommended cultural practices were applied for both environmental conditions except non application of irrigation at tillering stage for water stress environment. When the plants reached maturity, data of ten guarded plants for each genotype of normal and water stress conditions were taken for morphological traits like plant height (cm), flag leaf area (cm^2), tillers $plant^{-1}$, spike length (cm), grains $spike^{-1}$, 1000-grain weight, biological yield $plant^{-1}$ and grain yield $plant^{-1}$.

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Table 1. *Monthly minimum, maximum, average temperature and total rainfall*

Month	Temperature (Minimum °C)	Temperature (Maximum °C)	Temperature (Average °C)	Rainfall (Average mm)
December 2017	15	25	19	6.88
January 2018	14	24	18	0.00
February 2018	16	27	21	0.17
March 2018	21	34	27	0.02
April 2018	26	38	32	2.77
May 2018	32	43	38	3.65

Statistical analysis

Analyses of variances (ANOVA) were performed according to Steel et al. (1997). The general combining ability (GCA) of lines and testers and specific combining ability (SCA) of crosses were worked out by using line × tester mating technique as suggested by Kempthorne (1957). The ANOVA for Line × Tester used in the experiment is given in *Table 2*. Falconer (1989) formula was used to calculate broad sense heritability as under:

$$H^2 = V_G/V_P$$

where: H^2 = broad sense heritability, V_G = genotypic variance, V_P = phenotypic variance.

Table 2. *Analysis of variance (ANOVA) for line × tester*

Source of variation	Degree of freedom	Mean square
Replication (r)	$(r-1)$	
Genotypes (g)	$(g-1)$	MS_2
Parents (p)	$(p-1)$	
Parents versus crosses	1	
Crosses (c)	$(c-1)$	
Lines (l)	$(l-1)$	M_l
Testers (t)	$(t-1)$	M_t
Lines × testers	$(l-1)(t-1)$	$M_{l\times t}$
Error	$(r-1)(t-1)$	MS_1

MS_2 = genotypic mean square, M_l = line mean square, M_t = tester mean square, $M_{l\times t}$ = line × tester mean square, MS_1 = error mean square

Results and discussion

Line × tester - analysis of variance (ANOVA)

The analysis of variance was worked out for Plant height (cm), flag leaf area (cm^2), tillers $plant^{-1}$, spike length (cm), grains $spike^{-1}$, 1000-grain weight (g), biological yield $plant^{-1}$ (g) and grain yield $plant^{-1}$ (g) for testing the differences among treatments. The mean sums of squares (MSS) for all the studied traits are reflected in *Table 3a-d*. The results depicted that the variances due to genotypes were significant for all the traits under both conditions. The variability present among hybrids is very useful in improvement of wheat varieties for water stress condition. Significant variation among

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

yield related traits has also been reported by Nawaz et al. (2013), Singh et al. (2014), Yao et al. (2014), Salman et al. (2014) and Saeed et al. (2016).

Table 3a. *Analysis of variance through L × T for studied traits under normal and water stress conditions*

Source of variance	Degree of freedom	Plant height		Flag leaf area	
		Normal	Water stress	Normal	Water stress
Replications	2	3.10	0.26	3.30	2.12
Genotypes	26	133.05**	71.89**	28.75**	29.44**
Parents	8	43.49**	41.23**	40.54**	37.09**
Crosses	17	152.97**	81.08**	24.88**	27.50**
Parents vs crosses	1	510.86**	160.98**	0.27	1.17
Lines	5	347.98**	132.76	26.50	22.65
Testers	2	78.59**	54.74**	30.46**	25.18**
Line × tester	10	70.34**	60.51**	22.95**	30.39**
Error	52	1.60	2.91	0.75	0.71

Table 3b. *Analysis of variance through L × T for studied traits under normal and water stress conditions*

Source of variance	Degree of freedom	Tillers plant^{-1}		Spike length	
		Normal	Water stress	Normal	Water stress
Replications	2	1.53	0.62	0.94	0.21
Genotypes	26	8.19**	6.51**	1.32**	1.05**
Parents	8	7.49**	6.24**	1.58**	1.40**
Crosses	17	8.90**	6.97**	1.12**	0.94**
Parents vs crosses	1	1.64	0.74	2.62**	0.01
Lines	5	9.73	7.67	1.35	1.11
Testers	2	18.76**	11.92**	0.05	0.14
Line × tester	10	6.51**	5.63**	1.21**	1.02**
Error	52	0.33	0.26	0.25	0.07

Table 3c. *Analysis of variance through L × T for studied traits under normal and water stress conditions*

Source of variance	Degree of freedom	Grains spikes^{-1}		1000-grain weight (g)	
		Normal	Water stress	Normal	Water stress
Replications	2	0.56	0.54	14.20	1.93
Genotypes	26	128.44**	77.79**	10.44**	16.56**
Parents	8	78.10**	46.61**	8.65	16.78**
Crosses	17	159.64**	96.66**	11.89**	17.02**
Parents vs crosses	1	0.58	6.52**	0.03	6.92**
Lines	5	527.82**	319.85**	16.15	18.07
Testers	2	13.71**	9.42**	17.98	20.65**
Line × tester	10	4.74**	2.51**	8.55	15.76**
Error	52	1.78	0.90	4.78	0.40

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DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

Table 3d. *Analysis of variance through L × T for studied traits under normal and water stress conditions*

Source of variance	Degree of freedom	Biological yield plant^{-1}(g)		Grain yield plant^{-1} (g)	
		Normal	Water stress	Normal	Water stress
Replications	2	6.19	1.72	3.47	1.05
Genotypes	26	107.54**	68.91**	26.99**	12.77**
Parents	8	139.58**	70.93**	35.24**	17.63**
Crosses	17	88.93**	70.43**	24.60**	10.94**
Parents vs crosses	1	167.44**	26.89**	1.85	4.91**
Lines	5	203.30**	115.48	19.00	7.40
Testers	2	124.06**	86.69**	13.48**	8.52**
Line × tester	10	24.73**	44.65**	29.62**	13.20**
Error	52	1.72	1.08	2.07	0.58

Mean variability, general combining ability and specific combining ability

Mean variability

Among parents for plant height, the variation was between 102.27 cm (Chakwal 86) to 114.67 cm (GA 2002) under normal condition while mean values ranged from 68.23 cm (Galaxy 2013) to 80.36 cm (Chakwal 86) under water stress condition. Among crosses, this range varied from 90.61 cm (BARS 2009 × 2511) to 114.33 cm (GA 2002 × Galaxy 2013) under normal whereas under water stress condition, the mean performance ranged from 63.92 cm (BARS 2009 × Galaxy 2013) to 78.39 cm (B215 B 1103 × 2511). Among parents for flag leaf area, the mean performance was witnessed between 29.56 cm^2 (ESYWT P 117) to 40.90 cm^2 (B 215 B 1103) for this parameter under normal condition while mean performance ranged from 23.12 cm^2 (ESYWT P 117) to 33.46 cm^2 (B 215 B 1103) under water stress condition. However, among crosses, the mean value varied from 31.81 cm^2 (ESYWT P 117 × Lasani 2008) to 41.56 cm^2 (B 215 B 1103 × Galaxy 2013) under normal condition whereas the mean performance ranged from 22.24 cm^2 (B 215 B 1103 × Lasani 2008) to 34.32 cm^2 (B 215 B 1103 × Galaxy 2013) under water stress condition. Among parents, the variation was between 9.54 (Galaxy 2013) to 14.22 (B 215 B 1103) for tillers plant^{-1} under normal condition while mean values ranged from 6.10 (B 215 B 1103) to 10.71 (ESYWT P 117) for this character under water stress condition. Similarly, under normal condition among crosses, this range varied from 8.14 (ESYWT P 117 × Lasani 2008) to 15.43 (B 215 B 1103 × Galaxy 2013) whereas under water stress condition, the mean performance for crosses varied from 5.14 (ESYWT P 117 × 2511) to 12.02 (B 215 B 1103 × Galaxy 2013). For spike length among parents under normal condition, the mean values were between 8.10 cm (2511) to 10.17 cm (B 215 B 1103) while mean performance ranged from 6.75 cm (2511) to 8.95 cm (B 215 B 1103) under water stress condition. Among crosses, the mean values ranged from 7.73 cm (GA 2002 ×x Galaxy 2013) to 10.28 cm (B 215 B 1103 × Galaxy 2013) under normal condition whereas this mean value range in crosses under water stress condition was found between 7.04 cm (ESYWT P 117 × Galaxy 2013) to 9.36 cm (B 215 B 1103 × Galaxy 2013). For grains spike^{-1} among parents under normal condition, the mean performance was observed between 49.70 (Galaxy 2013) to 65.27 (B 215 B 1103) while mean performance values ranged from 33.60 (Galaxy 2013) to 46.57 (B 215 B

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DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

1103) for this trait under water stress condition. Likewise, among crosses under normal condition, the mean value varied from 48.59 (BARS 2009 × 2511) to 68.13 (B 215 B 1103 × Galaxy 2013). The mean performance range in crosses under water stress condition for this character was found from 33.05 (BARS 2009 × 2511) to 47.40 (B 215 B 1103 × Galaxy 2013). Among parents, the mean values were between 49.00 (Galaxy 2013) to 54.48 (B 215 B 1103) under normal condition while mean performance ranged from 33.51 (Galaxy 2013) to 41.07 (B 215 B 1103) under water stress condition for 1000-grain weight. Among crosses, the mean values ranged from 49.70 (Chakwal 86 × 2511) to 56.53 (B 215 B 1103 × Galaxy 2013) for this trait under normal condition while under water stress condition the mean values range in crosses was found between 33.99 (Chakwal 86 × 2511) to 42.90 (B 215 B 1103 × Galaxy 2013). The mean performance range among parents varied between 50.74 (Galaxy 2013) to 68.39 (B 215 B 1103) for biological yield plant^{-1} under normal conditions while mean values ranged from 40.10 (2511) to 52.69 (Chakwal 86) under water stress condition. Among crosses, the range varied from 53.03 (Chakwal 50 × Lasani 2008) to 70.84 (B 215 B 1103 × Galaxy 20131) under normal condition whereas this mean performance under water stress condition ranged from 38.31 (Chakwal 50 × Lasani 2008) to 53.40 (B 215 B 1103 × Galaxy 2013). Under normal condition, for grain yield plant^{-1} among parents, the mean performance was found between 22.73 (Galaxy 2013) to 32.50 (B 215 B 1103) whereas this mean performance values ranged from 17.05 (Galaxy 2013) to 23.23 (B 215 B 1103) for this character under water stress condition. Similarly, among crosses, the mean values varied from 22.20 (B 215 B 1103 × Lasani 2008) to 35.55 (B 215 B 1103 × Galaxy 2013) under normal condition. Likewise, this mean performance range in crosses under water stress conditions was found from 16.73 (B 215 B 1103 × Lasani 2008) to 25.71 (B 215 B 1103 × Galaxy 2013).

Estimation of general combining ability (GCA)

Combining ability is an assessment of the value of parents on the basis of performance of their progenies by using a specific breeding approach. When crosses are more productive, they are supposed to have good combining ability. GCA effects of lines and testers for evaluated traits are reflected in *Table 4a-d*. The plant height with negative values of GCA effects is considered favorable as short stature plants are considered good due to their ability to withstand lodging (Muneer et al., 2016). Among lines under normal condition, Chakwal 86 and BARS 2009 were observed as good combiners having GCA values of (- 4.87) and (- 8.89), respectively whereas under water stress condition, lines BARS 2009 (-6.67), Chakwal 50 (-0.61) and ESWYT P 117 (-1.40) were good general combiners. Similarly, among testers under normal condition, Galaxy 2013 and 2511 were observed good combiner having GCA values of (-1.31) and (-1.10), respectively whereas under water stress condition, testers Galaxy 2013 (-0.97) and Lasani 2008 (-0.94) were found good general combiners. These results are in accordance with the findings of Muneer et al. (2016). Negative GCA effects for flag leaf area are favorable due to less transpirational loss. Lines BARS 2009, GA 2002 and ESWYT P 117, under both conditions, reflected negative and significant results while testers Lasani 2008 under normal condition and 2511 and Lasani 2008 under water stress condition were good general combiners. Tillers plant-1 is a yield associated character and plants with higher number of fertile tillers result in more grain yield. Under both condition, lines BARS 2009, Chakwal 86

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

and Chakwal 50 exhibited positive and significant GCA effects with values 1.02, 0.58 and 0.83 for normal and 0.94, 0.35 and 0.53 under water stress condition, respectively. For testers, Galaxy 2013 was a good general combiner under both conditions. The findings of Majeed et al., (2011), Muneer et al. (2016), Saeed et al. (2016) and Iqbal et al. (2007) are also supportive to these findings. Spikes with greater length are desirable because of more spikelets per spike resulting in greater grain yield. Under normal condition, line B 215 B 1103 and under water stress condition, lines BARS 2009 and B 215 B 1103 were positive and significant general combiners. Grains spike-1 is a significant attribute contributing to overall grain yield and hence positive and significant GCA effects are desirable owing to yield per spike. Under both conditions among lines, GA 2002, ESWYT P 117 and B 215 B 1103 reflected positive and significant combining ability results. Among testers, Lasani 2008 was a good general combiner under both conditions. Muneer et al. (2016) and Iqbal et al. (2007) also witnessed the same results. Thousand-grain weight is a significant yield related trait and could be practiced as a selection criterion for high production in wheat. Among lines, B 215 B 1103 under both conditions showed maximum desirable GCA effects and thus proved to be good general combiners for this particular trait. However, testers Galaxy 2013 and Lasani 2008 under water stress condition reflected significant and positive GCA effects. For lines, biological yield plant-1, was positive and significant for Chakwal 86, ESWYT P 117 and B 215 B 1103 under both conditions whereas for testers, it proved to be positive and significant for Galaxy 2013 and 2511 under both conditions. Grain yield being a complex character, the plant breeders are particularly interested in developing high yielding varieties to cope with food demand of the masses by improving directly or indirectly this trait. Line Chakwal 86 demonstrated as a best general combiner under both conditions whereas the tester Galaxy 2013 reflected significant and positive GCA effects under both environmental conditions. These findings are in line with the results of Muneer et al. (2016), Sattar et al. (2018) and Jatav et al. (2014). It is suggested that line Chakwal 86 having high GCA for tillers plant-1, biological yield plant-1 and grain yield plant-1 under both conditions and tester Galaxy 2013 having good GCA for plant height, tillers plant-1, biological yield plant-1 and grain yield plant-1 under both environmental conditions can be easily employed for exploring high yielding wheat varieties in future.

Table 4a. *GCA effects of lines and testers under normal and water stress conditions*

Genotypes	Plant height		Flag leaf area	
	Normal	**Water stress**	**Normal**	**Water stress**
Chakwal 86	-4.87**	3.03**	-0.01	-0.16
BARS 2009	-8.89**	-6.67**	-0.97**	-0.73**
Chakwal 50	0.95**	-0.61**	2.11**	2.26**
GA 2002	9.07**	3.74	-0.95**	-0.72**
ESWYT P 117	1.45**	-1.40**	-2.14**	-2.08**
B 215 B 1103	2.30**	1.92**	1.96**	1.43**
Galaxy 2013	-1.31**	-1.07**	1.41**	1.36**
2511	-1.10**	2.01**	-0.26	-0.58**
Lasani 2008	2.41**	-0.94**	-1.15**	-0.78**

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

Table 4b. *GCA effects of lines and testers under normal and water stress conditions*

Genotypes	Tillers plant^{-1}		Spike length	
	Normal	Water stress	Normal	Water stress
Chakwal 86	1.02**	0.94**	0.24	0.07
BARS 2009	0.58**	0.35**	0.20	0.26**
Chakwal 50	0.83**	0.53**	-0.21	-0.29**
GA 2002	-0.32	-0.35**	-0.64**	-0.43**
ESWYT P 117	-1.77**	-1.678**	-0.03	-0.11
B 215 B 1103	-0.34	0.20	0.44**	0.51**
Galaxy 2013	1.12**	0.83**	0.05	0.09
2511	-0.25	-0.80**	-0.05	-0.08
Lasani 2008	-0.87**	-0.30	-0.01	-0.01

Table 4c. *GCA effects of lines and testers under normal and water stress conditions*

Genotypes	Grains spike^{-1}		1000-grain weight	
	Normal	Water stress	Normal	Water stress
Chakwal 86	-5.36**	-4.06**	-1.04	-0.55**
BARS 2009	-7.79**	-6.15**	-1.01	-0.94**
Chakwal 50	-7.57**	-5.92**	-0.78	-0.27
GA 2002	5.62**	4.64**	-0.35	-0.56**
ESWYT P 117	6.88**	5.15**	0.86	-0.54**
B 215 B 1103	8.22**	6.35**	2.32**	2.86**
Galaxy 2013	0.16	-0.17	0.56	0.69**
2511	-0.94**	-0.62**	-1.15**	-1.23**
Lasani 2008	0.78**	0.79**	0.60	0.54**

Table 4d. *GCA effects of lines and testers under normal and water stress conditions*

Genotypes	Biological yield plant^{-1}		Grain yield plant^{-1}	
	Normal	Water stress	Normal	Water stress
Chakwal 86	2.45**	2.44**	1.97**	1.57**
BARS 2009	-3.26**	-1.19**	-2.36**	-1.09**
Chakwal 50	-7.85**	-6.02**	-0.66	-0.53**
GA 2002	0.55	-0.67	0.14	-0.24
ESWYT P 117	4.94**	4.34**	0.80	-0.06
B 215 B 1103	3.17**	1.11**	0.11	0.35
Galaxy 2013	2.16**	1.54**	0.98**	0.72**
2511	0.76**	0.98**	-0.33	-0.07
Lasani 2008	-2.92**	-2.51**	-0.65	-0.65**

Estimation of specific combining ability (SCA)

SCA results of crosses are reflected in *Table 5a-d*. Crosses Chakwal 86 × Lasani 2008, Chakwal 50 × Galaxy 2013, GA 2002 × Lasani 2008, ESWYT P 117 × Lasani

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

2008 and B 215 B 1103 × Galaxy 2013 exhibited positive and significant SCA effects for plant height under both conditions. Crosses Chakwal 86 × Galaxy 2013, BARS 2009 × 2511, GA 2002 × Galaxy 2013, ESWYT P 117 × 2511, ESWYT P 117 × Lasani 2008 and B 215 B 1103 × Lasani 2008 were best specific combiners under normal condition environment whereas for water stress condition, best combiners were Chakwal 86 × Galaxy 2013, GA 2002 × Galaxy 2013, ESWYT P 117 × 2511 and B 215 B 1103 × Lasani 2008 for flag leaf area. The same findings were reported by Srivastava et al. (2012), Singh and Kumar (2014) and Sattar et al. (2018). Under normal condition, crosses like Chakwal 86 × Lasani 2008, Chakwal 50 × Lasani 2008, GA 2002 × 2511, ESWYT P 117 × 2511 and B 215 B 1103 × Galaxy 2013 displayed significant and favorable SCA effects and proved to be good combiners for tillers plant^{-1}. Likewise, under water stress condition, for tillers plant^{-1}, the crosses Chakwal 86 × 2511, BARS 2009 × Lasani 2008, Chakwal 50 × 2511 and B 215 B 1103 × Galaxy 2013 proved to be good specific combiners. Jain and Sastry (2012), Hammad et al. (2013), Singh and Kumar (2014), Muneer et al. (2016), and Kumar et al. (2018) also reported similar results in their experiments for tillers plant^{-1}. Two crosses ESWYT P 117 × Lasani 2008 and B 215 B 1103 × Galaxy 2013 under normal condition and three crosses GA 2002 × 2511, ESWYT P 117 × Lasani 2008 and B 215 B 1103 × Galaxy 2013 under water stress condition had significantly positive SCA effects for spike length. For grains spike^{-1}, all cross combinations under both conditions showed non-significant results except GA 2002 × 2511. Similar results were shown by Shabbir et al. (2012) and Bibi et al. (2013) for this trait. Positive and favorable SCA effects for 1000 grain weight were witnessed for six crosses like Chakwal 86 × Lasani 2008, Chakwal 50 × Lasani 2008, GA 2002 × Lasani 2008, ESWYT P 117 × Galaxy 2013, B 215 B 1103 × Galaxy 2013 and B 215 B 1103 × 2511 under water stress condition while no cross had significantly positive SCA effects under normal condition. Out of these six crosses under water stress condition, the best three in order of merit were Chakwal 86 × Lasani 2008, Chakwal 50 × Lasani 2008 and B 215 B 1103 × 2511. Similar findings were given by Ahmad et al. (2011), Majeed et al. (2011), Hammad et al. (2013) and Muneer et al. (2016). Under both conditions, six cross combinations Chakwal 86 × Galaxy 2013, BARS 2009 × Galaxy 2013, Chakwal 50 × Lasani 2008, GA 2002 × Lasani 2008, ESWYT P 117 × Lasani 2008 and B 215 B 1103 × Galaxy 2013 reflected positive and significant SCA effects for biological yield plant^{-1}. However, B 215 B 1103 × Galaxy 2013 and Chakwal 86 × Galaxy 2013 were the best combiners. Crosses with desired and significant SCA effects under normal condition for grain yield plant^{-1} were GA 2002 × 2511 and B 215 B 1103 × Galaxy whereas under water stress condition, the positive and significant results were obtained for Chakwal 86 × Lasani 2008, GA 2002 × 2511, ESWYT P 117 × 2511 and B 215 B 1103 × Galaxy 2013. Similarly, under both conditions, the best combiners were GA 2002 × 2511 and B 215 B 1103 × Galaxy 2013 for grain yield plant^{-1}. These results are in confirmation with Majeed et al. (2011), Bibi et al. (2013), Fellahi et al. (2013), Muneer et al. (2016) and Sattar et al. (2018).

Heritability

Genotypic variance (Vg), Phenotypic variance (Vp), Environmental variance (Ve) and Heritability (H^2) estimates for studied traits evaluated under both environmental conditions are presented in *Table 8*. The characters like plant height, flag leaf area, tillers plant^{-1}, grains spike^{-1} and biological yield plant^{-1} under both environments showed high heritability. However, under water stress environments, all traits reflected

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

moderate to high heritability. Similarly, spike length, 1000-grain weight and grain yield plant^{-1} reflected medium to high heritability.

Table 5a. *SCA effects of crosses under normal and water stress conditions*

Crosses	Plant height		Flag leaf area	
	Normal	Water stress	Normal	Water stress
Chakwal 86 x Galaxy 2013	2.31**	3.70**	-3.77**	-3.00**
Chakwal 86 x 2511	-0.38	0.76	-0.35	-0.21
Chakwal 86 x Lasani 2008	-1.93**	-4.46**	4.12**	3.21**
BARS 2009 x Galaxy 2013	0.75	-0.06	0.10	-0.07
BARS 2009 x 2511	-2.45**	-1.36	-1.13**	-0.49
BARS 2009 x Lasani 2008	1.70**	1.43	1.03**	0.56
Chakwal 50 x Galaxy 2013	-6.38**	-5.69**	0.08	-0.51
Chakwal 50 x 2511	-0.26	-2.45**	0.86	0.58
Chakwal 50 x Lasani 2008	6.64**	8.14**	-0.94	-0.07
GA 2002 x Galaxy 2013	3.52**	3.86**	-1.13**	-2.66**
GA 2002 x 2511	0.39	-0.05	-0.09	-0.32
GA 2002 x Lasani 2008	-3.91**	-3.81**	1.22**	2.97**
ESWYT P 117 x Galaxy 2013	5.54**	1.82	3.08**	2.32**
ESWYT P 117 x 2511	-0.97	0.36	-1.64**	-1.69**
ESWYT P 117 x Lasani 2008	-4.57**	-2.18**	-1.44**	-0.64
B 215 B 1103 x Galaxy 2013	-5.74**	-3.63**	1.65**	3.91**
B 215 B 1103 x 2511	3.66**	2.74**	2.35**	2.12**
B 215 B 1103 x Lasani 2008	2.08**	0.89	-4.00**	-6.03**

Table 5b. *SCA effects of crosses under normal and water stress conditions*

Crosses	Tillers plant^{-1}		Spike length	
	Normal	Water stress	Normal	Water stress
Chakwal 86 x Galaxy 2013	-0.61	-1.30**	0.14	0.01
Chakwal 86 x 2511	-0.99**	1.24**	-0.04	0.13
Chakwal 86 x Lasani 2008	1.60**	0.06	-0.09	-0.14
BARS 2009 x Galaxy 2013	-0.65	0.50	-0.33	-0.22
BARS 2009 x 2511	0.37	-0.16	0.36	0.24
BARS 2009 x Lasani 2008	0.27	0.66**	-0.03	-0.03
Chakwal 50 x Galaxy 2013	-0.88**	-1.27**	0.06	0.04
Chakwal 50 x 2511	-0.52	1.44**	0.19	0.14
Chakwal 50 x Lasani 2008	1.40**	-0.17	-0.25	-0.18
GA 2002 x Galaxy 2013	-0.39	0.12	-0.32	-0.29
GA 2002 x 2511	0.88**	-0.15	0.46	0.34**
GA 2002 x Lasani 2008	-0.49	0.02	-0.14	-0.05
ESWYT P 117 x Galaxy 2013	0.06	0.37	-0.71**	-0.62**
ESWYT P 117 x 2511	1.34**	-0.82**	-0.11	-0.27
ESWYT P 117 x Lasani 2008	-1.40**	0.44	0.82**	0.89**
B 215 B 1103 x Galaxy 2013	2.47**	2.57**	1.16**	1.08**
B 215 B 1103 x 2511	-1.09**	-1.56**	-0.85**	-0.58**
B 215 B 1103 x Lasani 2008	-1.38**	-1.01**	-0.31	-0.49**

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

Table 5c. *SCA effects of crosses under normal and water stress conditions*

Crosses	Grains spike^{-1}		1000-grain weight	
	Normal	Water stress	Normal	Water stress
Chakwal 86 x Galaxy 2013	0.68	0.61	-0.76	-0.92**
Chakwal 86 x 2511	-1.31	-0.86	-1.05	-1.73**
Chakwal 86 x Lasani 2008	0.62	0.25	1.81	2.65**
BARS 2009 x Galaxy 2013	0.90	-0.45	-0.12	-0.09
BARS 2009 x 2511	-1.32	-0.32	-0.41	0.01
BARS 2009 x Lasani 2008	0.42	0.77	0.53	0.09
Chakwal 50 x Galaxy 2013	0.12	0.50	-0.68	-1.00**
Chakwal 50 x 2511	0.07	-0.09	-0.64	-1.32**
Chakwal 50 x Lasani 2008	-0.19	-0.41	1.32	2.32**
GA 2002 x Galaxy 2013	-1.79**	-1.09	-0.78	-1.49**
GA 2002 x 2511	1.66**	1.22**	-0.41	0.57
GA 2002 x Lasani 2008	0.14	-0.13	1.18	0.93**
ESWYT P 117 x Galaxy 2013	-1.02	-0.65	1.63	1.67**
ESWYT P 117 x 2511	0.76	0.04	1.06	0.41
ESWYT P 117 x Lasani 2008	0.25	0.62	-2.69**	-2.07**
B 215 B 1103 x Galaxy 2013	1.10	1.08	0.71	1.84**
B 215 B 1103 x 2511	0.14	0.01	1.45	2.07**
B 215 B 1103 x Lasani 2008	-1.24	-1.09	-2.16	-3.91**

Table 5d. *SCA effects of crosses under normal and water stress conditions*

Crosses	Biological yield plant^{-1}		Grain yield plant^{-1}	
	Normal	Water stress	Normal	Water stress
Chakwal 86 x Galaxy 2013	2.73**	1.67**	-1.74**	-1.59**
Chakwal 86 x 2511	-1.61**	-1.17	0.13	0.33
Chakwal 86 x Lasani 2008	-1.11	-0.50	1.61	1.25**
BARS 2009 x Galaxy 2013	2.06**	2.80**	0.05	0.37
BARS 2009 x 2511	0.56	-1.06	-0.87	-1.07**
BARS 2009 x Lasani 2008	-2.62**	-1.74**	0.82	0.69
Chakwal 50 x Galaxy 2013	-1.75**	-0.54	-1.43	-0.21
Chakwal 50 x 2511	0.21	0.38	-0.15	-0.66
Chakwal 50 x Lasani 2008	1.53**	0.16	1.58	0.87
GA 2002 x Galaxy 2013	-3.72**	-5.87**	-2.04**	-1.08**
GA 2002 x 2511	1.02	0.80	1.72**	1.19**
GA 2002 x Lasani 2008	2.70**	5.06**	0.32	-0.10
ESWYT P 117 x Galaxy 2013	-2.56**	-2.12**	-1.73**	-1.69**
ESWYT P 117 x 2511	0.09	-0.92	1.24	0.99**
ESWYT P 117 x Lasani 2008	2.47**	3.05**	0.49	0.71
B 215 B 1103 x Galaxy 2013	3.24**	4.06**	6.90**	4.20**
B 215 B 1103 x 2511	-0.26	1.97**	-2.08**	-0.78
B 215 B 1103 x Lasani 2008	-2.98**	-6.03**	-4.82**	-3.42**

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

Component of variance and proportional contribution of parents and crosses

Genetic component of variance i.e. variance due to GCA ($б^2g$) and variance due to SCA ($б^2s$) were worked out from analysis of variance for combining ability under both conditions and are presented in *Table 6*. The ratio between $б^2g/б^2s$ was calculated to determine the relative role of gene action. The ratio between $б^2g/б^2s$ greater than 1 reflected additive type of gene action and the ratio less than one indicated non-additive (dominance) type of gene action. Variance due to GCA greater than SCA variance showed additive types of gene action but variance due to SCA greater than GCA variance indicated non-additive type of gene action. Variance due to GCA equal to the SCA variance reflected the epistasis (non-allelic interaction).

In the present studies, the estimated values of genetic components of variance due to $б^2g$ and $б^2s$ revealed the predominance of SCA indicating the role of non-additive (dominance) type of gene action in the expression of characters in both conditions except grains spike^{-1} which shows the additive type of gene action under both conditions. Dominance and additive type of gene actions were studied for various yield related traits under normal and water stress conditions. Non-additive gene action for plant height, flag leaf area, tillers per plant^{-1}, spike length, 1000-grain weight, biological yield plant^{-1} and grain yield plant^{-1} was observed. These results are in accordance with Srivastava et al. (2012) and Sattar et al. (2018) who observed non-additive type of gene action for plant height, flag leaf area, tillers plant^{-1}, 1000-grain weight and grain yield plant^{-1}. Additive type of gene action for spike length and grain weight per spike has been reported earlier by Ahmad et al. (2011) and Hammad et al. (2013).

Proportional contribution of lines, testers and line × tester interaction to the total variance for different plant characters is presented in *Table 7*. While observing the proportional contribution of lines, testers and their hybrids to the total variance of eight studied traits, lines were more prominent for the characters like plant height, grains spike^{-1} and biological yield plant^{-1} under both conditions. The contribution of testers was very low in proportion in most of the studied traits under both conditions. Line × tester interaction contributed predominantly to flag leaf area, tillers plant^{-1}, spike length, 1000-grain weight and grain yield plant^{-1} under both conditions. Similar findings were reported by Shabbir et al. (2012) and Fellahi et al. (2013) and Sattar et al. (2018).

Table 6. *Variances due to GCA, SCA under normal and water stress conditions*

Traits	$б^2$ GCA		$б^2$ SCA		$б^2$ GCA/$б^2$ SCA	
	N	WS	N	WS	N	WS
Plant height	2.477	0.617	22.914	19.202	0.108	0.032
Flag leaf area	0.058	-0.087	7.399	9.891	0.008	-0.009
Tillers plant^{-1}	0.072	0.040	2.061	1.788	0.035	0.023
Spike length	-0.003	-0.002	0.322	0.316	-0.009	-0.007
Grains spike^{-1}	4.644	2.823	0.986	0.534	4.712	5.283
1000-grain weight	0.100	0.038	1.256	5.122	0.080	0.007
Biological yield plant^{-1}	1.925	0.773	7.669	14.523	0.251	0.053
Grain yield plant^{-1}	-0.151	-0.068	9.185	4.206	-0.016	-0.016

N = normal, WS = water stress, L×T = line × tester

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DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

Table 7. *Gene action, contribution of parents and their crosses under normal and water stress conditions*

Traits	Gene action				Contribution of parents and their crosses					
	Additive		Dominance		Line		Tester		L×T	
	N	WS	N	WS	N	WS	N	WS	N	WS
Plant height	4.95	1.23	22.91	19.20	66.91	48.16	6.04	7.94	27.05	43.90
Flag leaf area	0.12	-0.17	7.40	9.89	31.33	24.23	14.40	10.77	54.26	65.00
Tillers plant^{-1}	0.14	0.08	2.06	1.79	32.16	32.37	24.80	20.13	43.04	47.50
Spike length	-0.01	-0.005	0.32	0.32	35.54	34.59	0.50	1.72	63.96	63.69
Grains spike^{-1}	9.29	5.65	0.99	0.53	97.25	97.33	1.01	1.15	1.74	1.53
1000-grain wt.	0.20	0.08	1.26	5.12	39.95	31.24	17.78	14.28	42.27	54.49
Biological yield plant^{-1}	3.85	1.55	7.67	14.52	67.23	48.23	16.41	14.48	16.35	37.29
Grain yield plant^{-1}	-0.30	-0.14	9.18	4.21	22.72	19.88	6.45	9.16	70.83	70.96

Table 8. *Genotypic variance (Vg), phenotypic variance (Vp), environmental variance (Ve) and heritability (H^2) estimates for studied traits evaluated under normal and water stress conditions*

Traits	Environmental variance		Genotypic variance		Phenotypic variance		Heritability (b.s)	
	N	WS	N	WS	N	WS	N	WS
Plant height	1.60	2.91	43.82	22.99	45.42	25.90	96.48	88.78
Flag leaf area	0.75	0.71	9.33	9.57	10.08	10.28	92.52	93.05
Tillers plant^{-1}	0.33	0.26	2.62	2.08	2.95	2.34	88.91	88.78
Spike length	0.12	0.19	0.33	0.36	0.45	0.55	73.33	65.45
Grains spike^{-1}	1.78	0.90	42.22	25.63	44.00	26.53	95.95	96.59
1000-grain weight	0.68	1.11	3.38	1.88	4.06	2.99	83.25	62.87
Biological yield plant^{-1}	1.72	1.08	35.27	22.61	36.99	23.69	95.35	95.43
Grain yield plant^{-1}	2.08	2.06	8.31	8.32	10.39	10.38	80.07	80.15

Conclusion

The parent Chakwal 86 and Galaxy 2013 possessed highest general combining ability for number of tillers plant^{-1}, biological yield plant^{-1} and grain yield plant^{-1} under both environments. Crosses B 215 B 1103 × Galaxy 2013, GA 2002 × 2511 and ESWYT P 117 × Lasani 2008 were the ideal specific combiners for flag leaf area, tillers plant^{-1}, spike length, and biological yield plant^{-1} under both environments. Similarly, High heritability (H^2) under normal and water stress environments was observed for plant height, flag leaf area, tillers plant^{-1}, grains spike^{-1} and biological yield plant^{-1}.

Keeping in view the overall results of the study, it is recommended that parents Chakwal 86 and Galaxy 2013 having highest general combining ability for various traits under normal and water stress conditions. Similarly crosses B 215 B 1103 × Galaxy 2013, GA 2002 × 2511 and ESWYT P 117 × Lasani 2008 being best specific combiners under both conditions can be safely employed for future breeding programs to explore high yielding wheat genotypes under water stress conditions.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80658079

THE PHYSIOLOGICAL AND QUALITY RESPONSE OF *PELARGONIUM GRAVEOLENS* (L.) GROWN ON NITRATE AND AMMONIUM NUTRIENT SOLUTIONS

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(Received 29th Jun 2020; accepted 17th Sep 2020)

Abstract. The objective of this study was to evaluate the effects of nitrate (NO_3^-) and ammonium (NH_4^+) on the yield, mineral content and oil quality of rose geranium. Nitrate, at concentrations between eight and 14 meq L^{-1}, and, ammonium at concentrations between 0.0 and 1.5 meq L^{-1}, was applied to rose geranium. Nitrate (10 meq L^{-1}) produced the most significant increase in plant height, foliar fresh mass, foliar dry mass, oil yield and oil content. Ammonium applied at 1.0 meq L^{-1} had no significant effects on agronomic-related attributes (e.g. plant height, foliar fresh mass, and foliar dry mass, etc.), only folia N (5%), P (10%) and S (22%) levels were increased. Principal component analysis was used to reduce the redundancy of the NO_3^- and NH_4^+ data, and the eigenvalue of the correlation matrix. For the NO_3^- study, PC1 and PC2 accounted for most of the variability, showing a cumulative variability> 55.5%: PC1 accounted for 42.20%, while PC2 accounted for 13.31% of the total variance. For NH_4^+, PC1 and PC2 accounted for most of the variability, with a cumulative variability> 47.1%: PC1 accounted for 26.54%, while PC2 accounted for 20.56% of the total variance. For successful cultivation of rose geraniums, nutrient solutions should contain optimal concentrations of NO_3^- and NH_4^+.
Keywords: *chlorophyll, geranium oil, hydroponically, ion, mineral*

Introduction

Production of rose geranium (*Pelargonium graveolens* L.) oil occurs in South Africa (Sedibe and Allemann, 2012); local producers export geranium oil to the USA, Japan, and Europe, with an annual income estimated between 34 and 57 million USD (DAFF, 2017). In South Africa, geranium oil is a key component in the production of many food and beverage products (Rao et al., 1996; Sedibe, 2012), and is an active ingredient in skin care products (Sedibe, 2012).

In order to obtain a profitable income, high quality standards of rose geranium oil have to be met. This is an on-going challenge for South African producers, since weather parameters such as temperature and rainfall affect oil quality and these effects are profound on geraniol, citronellol and citronellylformate compounds of rose geranium oil (Prakasa Rao et al., 1995). Growers mitigate the effects of weather by management of the plants' nutrient regime (Sedibe and Allemann, 2012; Nyakane et al., 2019).

Rose geraniums need between 100 kg to 200 kg N ha^{-1}, depending on the soil nutrient status (Singh et al., 1996, 1999; Ram et al., 2003; Araya et al., 2006). Nitrogen is the only nutrient element that can be formulated in ionic solution either as the cation (NH_4^+) or the anion (NO_3^-). For greenhouse crops, most of the N is supplied in the form of NO_3^-. However, small quantities of NH_4^+, are used by soil-less growers to acidify their nutrient solution, consequently preventing precipitating Fe and Zn (Combrink, 2019). The objective of this study was to evaluate the effects of NO_3^- and NH_4^+ on the yield, the mineral content and the oil quality of rose geranium oil, using a multivariate principle component analysis (PCA).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

Material and methods

Research site

This trial was conducted in a greenhouse at the west campus facility at the University of the Free State in Bloemfontein, situated in the Free State province of South Africa. The site is at an altitude of 1351 m above sea level (29°07'16.78" S, 26°12'45.95" E). A wet wall pad and two fan systems were used to control temperature to ±26° in the greenhouse.

Experimental design

Rose geranium cuttings were rooted for eight weeks by a commercial grower (Pico-gro, RSA) prior to planting. Plants were then planted in 5 L pots filled with 2 mm sterile silica sand. Plants were grown for five months during 2016 growing season. Each treatment was replicated five times in a randomised complete block design. Each experimental unit had eight pots containing one plant (Sedibe, 2012). *Table 1* shows concentrations of each ion in the nutrient solutions, in order to fulfil the experimental concentrations, NO_3^- levels were applied at 8, 10, 12 and 14 meq L^{-1} concentrations and NH_4^+ were applied using 0.0, 0.5, 1.0 and 1.5 meq L^{-1} concentrations.

Table 1. *Ion ratio used in the nutrient solutions to test the effect of NO_3^- and NH_4^+ concentrations on rose geranium growth and oil quality parameters*

Nitrate ion (meq L^{-1})						
NH_4^+	K^+	Ca^{2+}	Mg^{2+}	NO_3^-	$H_2PO_4^-$	$SO_4^=$
1.00	5.50	6.50	2.50	8.00	2.11	4.83
1.00	5.50	6.50	2.50	10.00	1.50	3.44
1.00	5.50	6.50	2.50	12.00	0.89	2.05
1.00	5.50	6.50	2.50	14.00	0.28	0.66
Ammonium ion (meq L^{-1})						
NH_4^+	K^+	Ca^{2+}	Mg^{2+}	NO_3^-	$H_2PO_4^-$	$SO_4^=$
0.00	5.88	6.95	2.67	10.00	1.50	3.44
0.50	5.69	6.72	2.59	10.00	1.50	3.44
1.00	5.50	6.50	2.50	10.00	1.50	3.44
1.50	5.31	6.28	2.41	10.00	1.50	3.44

A "drain-to-waste" drip system was used to fertigate all the pots, using four irrigation cycles per day, scheduled at 08:00, 11:30, 14:00 and 18:00 for 30 min (Sedibe et al., 2005). The emitter flow rate was set to 4 L $hour^{-1}$. As plants developed and the demand for water and nutrients increased, volumes were increased to ensure that 10% to 25% solution drained-to-waste, to prevent salt accumulation in the potting bags (Combrink, 2019). A fresh solution was mixed every two weeks.

During the experimentation period no diseases occurred. Aphids were controlled with organophosphate, using levels prescribed for ornamentals in South Africa (0.5 ml L^{-1}). A full cover spray was applied and then this application was repeated after three and six days, following the method of Nyakane et al. (2019) and Sedibe and Allemann (2012).

The nutrient solutions were maintained at pH 6.98 (±0.02), with electrical conductivity (EC) of 1.60 (± 0.1) mS cm^{-1}. The EC level of the solution was maintained by proportionally lowering the concentrations of phosphate and sulphate with increased

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

nitrate concentrations (*Table 1*). Micronutrients (mg L^{-1}) used in all the experiments were 6.0 Fe; 0.21 B; 0.03 Cu; 0.2 Zn; 0.55 Mn; and 0.05 Mo (Sedibe and Allemann, 2012; Combrink, 2019). The nutrient solutions were made up in 1000 L tanks, which served as reservoirs for the treatments. The feeding water had an EC of 0.2 mS cm^{-1} and a pH of 6.92. Expressed as meq L^{-1}, it contained 0.48 Na; 0.05 K; 1.0 Ca; 0.45 Mg; 0.01 N; 0.15 SO_4; 0.21 Cl; and a total alkalinity of 1.18 (HCO_3). The alkalinity was lowered to 0.04 meq L^{-1} by applying 60% nitric acid at 0.78 meq L^{-1}.

Plant height, foliar fresh mass (FM), and foliar dry mass (DM) were measured at harvesting. DM was determined by drying the harvested material at 68ºC for 72 hours. The dried leaves were milled to 0.25 mm diameter using a micro hammer mill (Culatti, Zurich) (Jones et al., 1991). These leaves were subsequently used for the analyses of Ca, Mg, P, K, S, and N. Content of Ca, Mg, P, and K in the plant tissue was determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 4300 DV, PerkinElmer Inc. USA) (Van Maarschalkerweerde and Husted, 2015). Sulphur content was also determined by ICP-OES using an extract solution (ICP-OES; JY Horiba Ultima, USA) and set at a wavelength of 181.978 nm (Van Maarschalkerweerde and Husted, 2015). The chlorophyll content was determined from the upper six mature leaves on the crop, using a chlorophyll meter (Optisciences CCM 200, USA), following the procedure described by Chen and Black (1992).

Nitrogen content was determined following the Dumas combustion method in a Leco FP-528 combustion N analyser (LecoCorp. St. Joseph, MI, USA) (Matejovic, 1995; Etheridge et al., 1998). Fresh biomass (2 kg - 5 kg) was collected for the extraction of essential oil. The oil was extracted from the stem and leaves using water and steam distillation for one hour (Motsa et al., 2006) in a customised 5 kg test distiller (PA Pretorius). Oil quality was determined by gas chromatography (GC); an Aligent GC (FID; model 6890N) fitted with a 30 mm x 0.25 mm DB-5 fused silica capillary column, and film thickness of 0.25 μm (Novák et al., 2001; Adams, 2004).

Statistical analysis

Analyses of variance were conducted using the general linear model (GLM) procedure of statistical analysis system (SAS) version 9.3 (SAS, 2017). A regression analysis was also run using the SAS program. Significant results were analysed using Tukey's while Fischer's test was used to calculate the least significant difference test (LSD), described by Steel and Torrie (1980). Statistically significant differences between treatment means was determined at the 5% level of significance.

Basic formulae were used in performing PCA, by adjusting the data matrix, *X*, which consists of *n* observations (rows) on *p* variables (columns) as described by NCSS (2019).

The basic equation of PCA is, in matrix notation, given by:

$$Y=W\,X' \qquad \text{(Eq.1)}$$

where W is a matrix of coefficients that is determined by PCA.

These equations are also written as:

$$Y_{ij} = w_{1i}\,x_{1j}+w_{2i}x_{2j}+\ldots+w_{pi}x_{pj} \qquad \text{(Eq.2)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

As seen, the components are a weighted average of the original variables. The weights, *W*, are constructed so that the variance of y_1, $Var(y_1)$, is maximized. Also, so that $Var(y_2)$ is maximized and that the correlation between y_1 and y_2 is zero. The remaining yi's are calculated so that their variances are maximized, subject to the constraint that the covariance between y_i and y_j, for all *i* and *j* (*i* not equal to *j*), is zero.

Results

Yield, mineral and oil quality attributes

The yield attributes, mineral content and oil quality parameters of rose geranium are shown in *Table 2*. Plant height, FM, DM, oil yield and oil content were all significantly affected by nitrate content ($P<0.05$). The optimal NO_3^- level for all of these parameters was 10.0–12.0 meq L^{-1}. Increases of 24% (plant height,) 40% (FM), 50% (DM), 60% (oil yield) and 17% (oil content) were observed at 10.0 NO_3^- meq L^{-1}. Only chlorophyll content had an optimal NO_3^- concentration that was significantly different (12.0 meq L^{-1}; $P<0.001$). Geraniol and geranyl formate were significantly affected by NO_3^-, though the optimum concentration could not be established as concentrations varied in an inconsistent manner. The optimal NO_3^- concentration for citronellyl formate was also 10.0–12.0 meq L^{-1}, an increase of 8% compared to other treatments ($P<0.05$).

Table 2. *Yield, mineral content and oil quality of rose geraniums cultivated using a nutrient solution with variable nitrate content*

Nitrate (meq L^{-1})	Plant height (cm)	Foliar fresh mass (g $plant^{-1}$)	Oil yield (g $plant^{-1}$)	Oil content (%)	Foliar dry mass (g $plant^{-1}$)	Chlorophyll
8.00	46.60 ± 2.88^{c}	585.00 ± 112.64^{b}	0.68 ± 0.68^{b}	0.12 ± 0.04^{b}	124.93 ± 15.82^{b}	18.33 ± 0.74^{b}
10.00	57.00 ± 6.16^{b}	820.20 ± 159.63^{a}	1.09 ± 1.09^{a}	0.14 ± 0.02^{ab}	187.12 ± 29.79^{a}	20.14 ± 1.13^{b}
12.00	55.80 ± 7.40^{ab}	822.80 ± 59.44^{a}	1.16 ± 1.16^{a}	0.14 ± 0.04^{ab}	192.04 ± 22.38^{a}	24.24 ± 3.33^{a}
14.00	49.40 ± 1.67^{bc}	455.20 ± 101.12^{b}	0.75 ± 0.75^{b}	0.17 ± 0.02^{a}	120.79 ± 13.07^{b}	23.13 ± 1.49^{a}
$LSD_{T0.05}$	7.10*	147.18^{***}	0.26^{*}	0.03^{*}	29.80^{***}	2.87^{***}
Nitrate	**N**	**P**	**K**	**Ca**	**Mg**	**S**
8.00	3.36 ± 0.22^{a}	0.51 ± 0.06^{a}	2.91 ± 0.31^{ab}	2.02 ± 0.15^{a}	0.35 ± 0.03^{a}	0.49 ± 0.05^{a}
10.00	3.39 ± 012^{a}	0.43 ± 0.02^{b}	2.61 ± 0.27^{b}	1.77 ± 0.89^{b}	0.35 ± 0.02^{a}	0.43 ± 0.01^{a}
12.00	3.35 ± 0.08^{a}	0.38 ± 0.03^{b}	2.97 ± 0.28^{a}	2.00 ± 0.18^{a}	0.36 ± 0.04^{a}	0.34 ± 0.07^{b}
14.00	3.39 ± 0.37^{a}	0.27 ± 0.03^{c}	2.78 ± 0.26^{ab}	1.73 ± 0.08^{b}	0.21 ± 0.01^{a}	0.26 ± 0.03^{c}
$LSD_{T0.05}$	0.35^{NS}	0.06^{***}	0.32^{**}	0.19^{*}	0.34^{NS}	0.08^{***}
Nitrate	**Linalool**	**Citronellol**	**Geraniol**	**Citronellyl-formate**	**Geranyl-formate**	**Guaia6,9diene**
8.00	1.96 ± 1.09^{a}	31.91 ± 1.22^{a}	13.94 ± 0.88^{a}	19.66 ± 2.07^{b}	8.61 ± 1.03^{a}	9.69 ± 0.71^{a}
10.00	1.52 ± 0.41^{a}	33.46 ± 1.39^{a}	10.39 ± 1.05^{b}	21.39 ± 0.96^{ab}	7.98 ± 0.45^{ab}	9.49 ± 0.24^{a}
12.00	1.89 ± 0.61^{a}	32.31 ± 1.87^{a}	10.25 ± 0.63^{b}	22.31 ± 0.48^{a}	7.47 ± 0.44^{b}	10.39 ± 0.54^{a}
14.00	1.25 ± 1.18^{a}	31.50 ± 1.50^{a}	12.87 ± 1.32^{a}	20.20 ± 1.36^{b}	8.44 ± 0.28^{a}	10.17 ± 0.59^{a}
$LSD_{T0.05}$	0.72^{NS}	2.17^{NS}	1.40^{***}	1.87^{*}	0.85*	0.74^{NS}

NS not significant at $P<0.05$; *significant at $P<0.05$; ** significant at $P<0.01$; *** significant at $P<0.001$. Means (±standard deviation). The same superscript letter within a column denotes non significance ($P>0.05$)

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DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

As shown in *Table 3*, ammonium had no significant effect on agronomic-related attributes (e.g. plant height, FM, DM, oil yield, oil and chlorophyll content). Significant changes were mostly observed on the foliar mineral contents. NH_4^+ at 1.0 meq L^{-1} increased folia levels of N (5%), P (10%) and S (22%). Foliar Mg content and linalool were also significantly affected by ammonium concentrations ($P<0.05$), but the optimal nutrient level was unclear. Citronellol, geraniol and citronellyl formate were not significantly affected by ammonium content ($P>0.05$). The optimal NH_4^+ concentration for geranyl formate and guai6,9diene was 1.00 meq L^{-1} ($P<0.01$ and $P<0.05$, respectively).

Table 3. *Yield, mineral content and oil quality of rose geraniums cultivated using a nutrient solution with variable ammonium content*

Ammonium (meq L^{-1})	**Plant height (cm)**	**Foliar fresh mass (g $plant^{-1}$)**	**Oil yield (g $plant^{-1}$)**	**Oil content (%)**	**Foliar dry mass (g $plant^{-1}$)**	**Chlorophyll**
0.00	67.00±6.51^a	760.20±119.88^a	0.85±0.18^a	0.112±0.02^a	170.34±8.92^a	21.90±1.51$_a$
0.50	63.20±5.35^a	803.20±118.20^a	0.99±0.20^a	0.124±0.02^a	170.00±14.42^a	24.266±3.20^a
1.00	60.20±5.80^a	790.40±90.50^a	1.00±0.11^a	0.127±0.12^a	158.28±14.02^a	26.17±4.155^a
1.50	60.60±10.18^a	777.80±123.33^a	1.00±0.09^a	0.130±0.01^a	161.05±42.57^a	23.70±2.41^a
$LSD_{T0.05}$	9.39^{NS}	168.53^{NS}	0.20^{NS}	0.01^{NS}	11.12^{NS}	4.41^{NS}
Ammonium	**N**	**P**	**K**	**Ca**	**Mg**	**S**
0.00	3.12±0.14^b	0.40±0.01^b	2.29±0.15^a	1.77±0.10^a	0.37±0.01^b	0.32±0.01^c
0.50	3.11±0.16^b	0.41±0.03^a	2.53±0.24^a	1.74±0.11^a	0.40±0.02^a	0.33±0.05^c
1.00	3.29±0.12^a	0.44±0.01^a	2.38±0.10^a	1.85±0.07^a	0.37±0.01^b	0.39±0.01^b
1.50	3.31±0.17^a	0.40±0.03^b	2.40±0.14^a	1.76±0.12^a	0.30±0.01^c	0.43±0.02^a
$LSD_{T0.05}$	0.12^{***}	0.03^{***}	0.23^{NS}	0.13^{NS}	0.02^{***}	0.04^{***}
Ammonium	**Linalool**	**Citronellol**	**Geraniol**	**Citronellyl-formate**	**Geranyl-formate**	**Guaia6,9diene**
0.00	1.77±0.28^a	33.46±2.32^a	12.68±1.22^a	20.48±0.34a	9.18±0.58^{ab}	9.17±0.61^b
0.50	1.80±0.36^a	33.38±1.09^a	11.78±1.03^a	20.70±0.58a	9.14±0.34^{ab}	9.16±0.42^b
1.00	1.06±0.32^b	31.86±1.14^a	10.82±2.31^a	20.65±2.39a	9.97±0.68^a	10.27±0.69^a
1.50	1.71±0.26^a	33.79±2.47^a	11.99±1.10^a	20.36±1.90a	8.58±0.75^b	9.43±0.27^b
$LSD_{T0.05}$	4.28^{*}	2.75^{NS}	2.16^{NS}	2.41^{NS}	0.88^{**}	0.69^{**}

NS not significant at $P<0.05$; *significant at $P<0.05$; ** significant at $P<0.01$; *** significant at $P<0.001$. Means (±standard deviation). The same superscript letter within a column denotes non significance ($P>0.05$)

Nitrate and ammonium principal component analysis

Table 4 shows the PCA used to reduce the redundancy of the NO_3^- and NH_4^+ data, and the eigenvalue of correlation matrix. *Table 4* and *Fig. 1B* show that for the nitrate treatment, out of nineteen principal components (PC) used, the first two (PC1 and PC2) accounted for most of the variability, with a cumulative variability >55.5%. PC1 accounted for 42.2%, while PC2 accounted for 13.31% of the total variance. *Table 4* and *Fig. 1B* show that plant height, FM, oil yield, DM, total biomass, citronellol, geraniol and Mg content were loaded positively on PC1, however linalool, Ca, P and S were loaded on PC2. This confirms that the majority of the tested variables were significantly affected by NO_3^- levels between 10 and 12 meq L^{-1}. Geraniol was the only compound was positively affected by the application of NO_3^- at 8 meq L^{-1}.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

Table 4. *Eigenvalues and principal component factor loading of parameters affected by nitrate and ammonium*

Nitrate				**Ammonium**			
Principal component	PC1	PC2	PC3	Principal component	PC1	PC2	PC3
Eigenvalue	13.083	4.125	2.652	Eigenvalue	7.433	5.757	3.475
% of variance	42.204	13.307	8.555	% of variance	26.545	20.561	12.409
Cumulative % of total variance	42.204	55.511	64.066	Cumulative % of total variance	26.545	47.106	59.515
Factor loadings				Factor loadings			
Height	**0.398**	0.099	0.130	Height	**0.289**	0.214	0.018
Foliar fresh mass	**0.704**	0.164	0.000	Foliar fresh mass	**0.583**	0.028	0.092
Oil yield	**0.665**	0.000	0.257	Oil yield	0.187	**0.694**	0.026
Oil content	0.002	0.338	0.434	Oil content	0.050	**0.652**	0.009
Foliar dry mass	**0.862**	0.000	0.013	Foliar dry mass	**0.726**	0.081	0.043
Total biomass	**0.903**	0.005	0.007	Total biomass	**0.736**	0.144	0.089
Chlorophyll	0.102	0.297	0.069	Chlorophyll	0.008	**0.329**	0.065
Linalool	0.000	**0.293**	0.219	Linalool	0.070	**0.408**	0.226
isoMenthone	0.046	0.181	0.011	isoMenthone	0.001	0.146	0.002
Citronellol (C)	**0.272**	0.143	0.015	Citronellol (C)	**0.316**	0.018	0.175
Geraniol (G)	**0.572**	0.089	0.114	Geraniol (G)	0.080	0.236	0.107
Citronellyl-formate	0.269	0.188	0.025	Citronellyl-formate	0.001	0.053	0.012
Geranyl-formate	**0.347**	0.038	0.127	Geranyl-formate	0.204	0.066	**0.356**
Guaia6,9diene	0.000	0.024	0.220	Guaia6,9diene	0.011	0.178	0.210
C:G ratio	**0.668**	0,029	0.071	C:G ratio	0.200	0.203	0.033
N	0,038	0,018	0.000	N	0.006	0.011	0.304
Mg	**0.314**	0.198	0.015	Mg	0.009	0.013	0.012
Ca	0.054	**0.236**	0.002	Ca	0.097	0.012	**0.613**
P	0.001	**0.731**	0.017	P	0.015	0.017	**0.566**
K	0.004	0.084	0.013	K	0.086	0.044	0.003
S	0.005	**0.587**	0.018	S	0.083	0.220	0.006

Values in bold correspond to the factor with the greatest squared cosine

In the ammonium treatment, the first two principal components (PC1 and PC2) accounted for most of the variability, with a cumulative variability > 47.1%. PC1 accounted for 26.54%, while PC2 accounted for 20.56% of the total variance. *Table 4* and *Fig. 1B* show that plant height, FM, DM, total biomass, and citronellol were loaded at PC1, while, in contrast to the NO_3^- results, oil yield, oil content, chlorophyll and linalool were loaded on PC2.

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DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

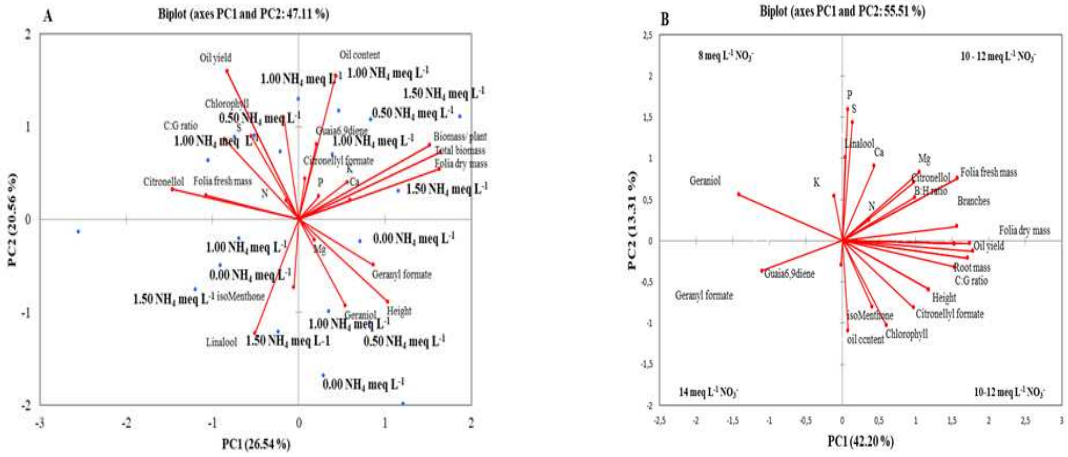

Figure 1. *Rotated principal component analysis of measured yield, mineral and oil quality parameters of rose geraniums grown under A) variable ammonium (NH_4^+) concentrations; and B) variable nitrate (NO_3^-) concentrations*

Discussion

In South Africa, growers use both nitrate and ammonium in nutrient solutions as a source of N, however, most of N comes from NO_3^-. Owing to its small size, and single charge ion, NH_4^+ is easily absorbed by roots, resulting in the secretion of H^+. However, it is often applied in smaller quantities, used mainly to reduce pH at the roots. In other countries, greenhouse crops are grown using nitrate as the sole N source, and the pH of these nutrient solutions tend to increase towards the root zone. Where organic substrates are used, bicarbonate is released during decomposition, thus increasing the alkalinity and raising the substrate pH (Benoit, 2003). Increasing the NH_4^+ level can counteract this, especially on crops that are sensitive to Ca-deficiency (Combrink, 2019), however, Kafkaffi (2003) reported that damage by NH_4^+ increases at high root zone temperatures. Ammonium is taken up by plant cells by means of NH_4^+ transporters in the plasma membrane and distributed to intracellular compartments, such as chloroplasts, mitochondria and vacuoles (Howitt and Udvardi, 2000). The recommended optimum ratio of NH_4^+ to NO_3^- for most summer crops is 1:10. For acid-loving crops, the recommendation is 1:1 (Pienaar, 2005; Combrink, 2019), and for blueberries it is 3:1 (Sonneveld, 2002; Combrink, 2019). Plant growth is impaired by using NH_4^+ as the sole source of N (Claussen and Lenz, 1999).

The optimum NO_3^- concentration for most parameters measured in this study was between 10 and 12 meq L^{-1}. Steiner's universal nutrient solution contains 12, 1 and 7 meq L^{-1} of NO_3^-, H_2PO_4 and SO_4, respectively (Steiner, 1984). Thus, nitrate represents 60% of Steiner' total anion application. In this trial the optimum anion ratio can be estimated at about 11 meq L^{-1} (74%), with 1.2 meq H_2PO_4 L^{-1} and 2.75 meq SO_4 L^{-1}. The level of NO_3^- in rose geranium cultivation is crucial for optimising yield, in terms of height, FM, DM, oil and oil content, and for foliar content of P, K, S, geraniol, citronellyl-formate and geranyl-formate. Nitrogen promotes optimum plant growth by increasing cytokinin production. The synthesized cytokinin encourages cell wall elasticity, by increasing the number of meristematic cells, and cell growth (Razaq et al., 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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There is a positive relationship between chlorophyll and N-content of the leaves (Abasi et al., 2016; Gholizadeha et al., 2017) and chlorophyll content can be used as an alternative measure for N status of most plant species (Fontes and de Araujo, 2006). This relationship between chlorophyll and foliar N content was not found in this study, possibly due to the increase in biomass production that occurred at increased nitrate levels. It is interesting to note that higher ammonium increased foliar N content and leaf chlorophyll was not affected.

It is known that high concentrations of NH_4^+ in nutrient solutions may suppress the uptake of calcium (Abasi et al., 2016). In this study foliage Ca content was not affected by NH_4^+ at levels of up to 1.5 meq L^{-1}. Environmental conditions varied in the greenhouse that could have stimulated or suppressed transpiration, so causing differences in foliar Ca concentrations. As a result, as well as the fact that foliage Ca concentrations were well within limits for healthy plant growth (1.72%-2.06%), inconsistent effects of NO_3^- concentrations on foliage Ca occurred. Calcium accumulation in plant tissues is affected by Ca uptake by the roots as well as the transpiration rate of the involved plant species. (Adams and Holder, 1992; Combrink, 2019). High humidity, high temperature, low temperature and low transpiration rates may induce Ca deficiency (Olle and Bender, 2009).

To ensure high herbage and oil yields, rose geraniums need to be grown at a relatively high nitrate concentration, of between 10 to 12 meq L^{-1}. At these nitrate concentrations, plant height, FM, oil yield, DM, total biomass, citronellol, geraniol, C:G ratio and Mg content were loaded positively on PC1, however linalool, Ca, P and S were loaded on PC2. The ammonium concentrations evaluated in this study had no effect on herbage and oil yield, but affected foliar N and S content. Given that some oil quality parameters were also affected by ammonium; this provides useful information to growers that 1.00 meq L^{-1} ammonium might be sufficient for hydroponically grown rose geraniums. The principal component analysis also revealed that plant height, FM, DM, total biomass, and citronellol were loaded at PC1, while oil yield, oil content, chlorophyll and linalool were loaded on PC2.

Conclusion

Unlike NH_4^+, a relatively high concentration of NO_3^- of 10 to 12 meq L^{-1} ensures high herbage and oil yields. Further investigation of the ratio of NH_4^+ to NO_3^- is required for rose geranium grown using nutrient solution.

Acknowledgements. This work was supported by the Central University of Technology, Free State and the National Research Foundation- Thuthuka funding.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80818090

INVESTIGATING THE ALLELIC VARIATION OF LOCI CONTROLLING RUST RESISTANCE GENES IN WHEAT (*Triticum aestivum* L.) LAND RACES BY SSR MARKER

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(Received 6th Jul 2020; accepted 17th Sep 2020)

Abstract. A study was conducted to determine the status of gene combination for rust (*Puccinia spp.*) resistance in 100 indigenous wheat land races also yield related traits were evaluated at two different locations during the 2016-17 periods with Randomized Complete Block Design. Variations for the studied traits were conferred by Principal Component Analysis (PCA), PC1 with 80.1% variation for the Peshawar, and 93.8% for the Islamabad location. WMC419, XGWM120, GWM174, XGWM140, XGWM410, XGWM111, WMC773, XWMC170, XWMC405, XWMC348 and XWMC407 has no allelic variation because of monomorphic nature while XGWM35 (200&225 bp), GWM 148 (190&200 bp), Barc 86 (200&210 bp), BARC 114 (105&200 bp), CSLV34 (150&190 bp), PSP3000 (300&350 bp), XGWM493 (150&300 bp), XGWM153 (100&300 bp), XGWM44 with 120,285,500 & 700 bp, XBARC4 (90&200 bp), XGWM125 (150&190 bp) show variation in alleles. Analysis of Yellow rust (Yr5,7,10,15,18,19,26,29,34, P138 and Sr13,) and leaf rust (Lr19,34 and 49) revealed A and B resistance groups. Group A comprised of 36% of total accessions, while rest of the accessions (64%) were present in group B. The study revealed high level of diversity assessed through PCA and cluster analysis. Based on marker results, 23 wheat lines ranging 17-21 alleles in combination were identified. These lines have great potential to be used in rust resistance breeding programs.
Keywords: *indigenous germplasm, yield related traits, principal component analysis, cluster analysis*

Introduction

Wheat (*Triticum aestivum* L.) is an essential cereal crop with a variety of uses and is considered the major component of bread. It belongs to the family *Poaceae* and genus *Triticum* and ranks first in cereals. It is a staple diet and provides more nourishment for the people of the world than any crop. There are two kinds of wheat species on which the world wheat production depends. The common/ bread wheat, which is mostly used today, has 42 (42=2n =6x) chromosomes containing AA, BB and DD genomes (Goutam et al., 2013). Wheat has high nutritional value and it gives nearly 21% of calories along with 20% of the protein (Waqar et al., 2018). During

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

2018 it was grown on an 8.7 mha area with 250 million tones production (FAO, 2020). This yield is still not enough to meet global requirements and climatologists also predicted that if existing/available cultivars and agronomic practices are used there is high chance that nearly 30% yield reduction will occur in South Asia's zone (Cgiar, 2017). One of the main factor in decline of yield are wheat diseases particularly Rust which is a common name for *Puccinia* spps i.e. fungal pathogens affecting lots of cereal crops especially wheat (Brown and Hovmøller, 2002; Waqar et al., 2018). Rust disease slows the growth of plant and decrease forage quality, also it results in poor seedling germination, foliar damage and reduced grain size (Chen et al., 2012). Seriousness of this disease can be depicted by its ability to grow everywhere provided that suitable substrate that may be wheat or other species in favorable environmental conditions. It can also pose a high risk to a lot of sustainable wheat production zones (Singh et al., 2004). Pandemic of rusts disease caused for example the 19th century famine that covered large area of the world and affected human. This famine nearly cost 5 billion US dollars losses to cereals especially wheat for many years (Waqar et al., 2018). The severity of the disease affects the yield attributing traits. Therefore, appropriate selection of rust resistance parents is important in crossing nurseries/programs to enhance the genetic recombination for capability of yield enhancement by reducing rust losses (Ajmal et al., 2013).

In Pakistan not much studies have been conducted in order to characterize rust resistant genes using DNA markers particularly SSR for developing rust resistant varieties through gene pyramiding. Developed varieties must be resistant and high yielding. This can be achieved by using the best available technique/designs like ANOVA, PCA, cluster analysis and other statistical tools. PCA is the best tool and is used worldwide for exploring similarities; dissimilarities along with hidden patterns between genotypes especially in land races or if the association on data and grouping is not very clear (Granato et al., 2018). Similarly for markers there are lots of categories but two main groups are broadly used i.e. PCR-based SSRs and AFLPS (Granato et al., 2020). Long term fertility building requires a combined methodology instead of short range approach and targeted way out instead of conventional agriculture approaches, therefore combined efforts of agriculture scientists (of diverse discipline) specially plant breeders and geneticist is required to overcome loss inflicted by lethal disease i.e. rust by incorporation of resistance alleles in various high yielding crops through breeding programs (Waqar et al., 2018). Considering the situation of our country this study was done to evaluate the rust resistance alleles/genes at various loci in indigenous Pakistani wheat germplasm and also to evaluate their performance across different yield related traits for their future incorporation in different breeding programs.

Materials and Methods

The breeding material was collected from BCI, National Agriculture Research Center (NARC) Islamabad, Pakistan (official germplasm of Pakistan). For experimental study and to screen huge breeding material, germination test (15 seeds accession^{-1}) was performed at lab for the selection of 100 elite performer land races (out of 1000) using Filter paper Whatman No. 1 sheet (Punjabi and Basu, 1982). Accessions having higher germination rate i.e. above 70% were registered for further experimental analysis (*Table 1*).

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

Table 1. *Performance of 100 selected accessions on the basis of germination test*

Accessions name	Accessions coding	Germinated seed	Germination %age	Accessions name	Accessions coding	Germinated seed	Germination %age
11123	1	12	80	11222	51	14	93
11126	2	11	73	11223	52	11	73
11144	3	14	93	11224	53	13	87
11145	4	13	87	11225	54	11	73
11152	5	14	93	11226	55	12	80
11154	6	12	80	11227	56	14	93
11155	7	11	73	11228	57	14	93
11160	8	11	73	11229	58	15	100
11161	9	15	100	11231	59	15	100
11162	10	14	93	11233	60	11	73
11163	11	13	87	11236	61	14	93
11164	12	15	100	11237	62	12	80
11166	13	14	93	11239	63	12	80
11168	14	12	80	11240	64	13	87
11170	15	13	87	11242	65	14	93
11171	16	11	73	11243	66	14	93
11173	17	11	73	11244	67	15	100
11174	18	13	87	11246	68	14	93
11177	19	14	93	11248	69	12	80
11178	20	15	100	11249	70	13	87
11179	21	13	87	11250	71	14	93
11181	22	12	80	11252	72	12	80
11183	23	14	93	11253	73	11	73
11184	24	11	73	11255	74	13	87
11185	25	12	80	11256	75	15	100
11186	26	11	73	11259	76	14	93
11187	27	12	80	11261	77	12	80
11188	28	15	100	11265	78	12	80
11189	29	15	100	11272	79	11	73
11190	30	12	80	11274	80	14	93
11192	31	15	100	11275	81	14	93
11193	32	14	93	11278	82	13	87
11194	33	12	80	11288	83	14	93
11195	34	11	73	11290	84	13	87
11197	35	14	93	11292	85	13	87
11198	36	12	80	11293	86	13	87
11200	37	13	87	11294	87	12	80
11202	38	15	100	11295	88	14	93
11205	39	12	80	11296	89	15	100
11207	40	13	87	11297	90	15	100
11208	41	12	80	11299	91	14	93
11209	42	15	100	11304	92	11	73
11210	43	14	93	11317	93	14	93
11211	44	12	80	11553	94	12	80
11214	45	12	80	11558	95	13	87
11215	46	11	73	12087	96	14	93
11216	47	13	87	12100	97	11	73
11217	48	11	73	12231	98	12	80
11218	49	13	87	18668	99	13	87
11221	50	12	80	24740	100	11	73

* Germination test was done prior to field evaluation

** Accessions with less than 70% germination were not selected in present study

For the evaluation morphological parameters RCB-Design was used with 3 replications across two locations i.e. in Islamabad (located at 33.6844°N and 73.0479°E) and in Peshawar region (located at 34.01^0N and 71.35^0E). The climate of the Islamabad region is a humid subtropical climate (Köppen climate classification. The temperature

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ranges from a minimum of -3.9 °C (25.0 °F) in January to a maximum of 46.1 °C (115.0 °F) in June. The *rainy* period of the year lasts for *12 months*, from *November 19* to *November 6*, with a sliding 31-day rainfall of at least *0.5 inches*. *Most rain* falls during the 31 days centered on *July 29*, with an average total accumulation of *7.4 inches*.

Peshawar region lies 331 m above sea level and climate it's referred as a local steppe climate because there is not much rainfall all year long. The Köppen-Geiger climate classification is BSh. The temperature here averages 22.7 °C | 72.8 °F. Precipitation here is about 384 mm |15.1 inch per year. In Peshawar sowing was done on the 20th November while in Islamabad it was on the 1st December. Furthermore, proper agronomic practices (one ploughing with soil turing plough followed by 2-3 harrowing) were followed in order to have data accuracy. Crop was planted when soil was in proper water condition. Plot size was 5 m^2 while plant to plant distance was 10 cm and row to row distance was 18 cm. Seed drill method with 5 cm depth with seed rate of 100 kg/ha was used. Fertilizer treatment was 150 kg N + 60 kg P_2O_5 + 40 kg K_2 O/ha. Weeding was done when required by manual method in between the intervals while 2.4-D @ 500 g ai/ha in 700 liters of water was applied at 35-40 days after sowing.

Data was recorded by taking 5 samples from each replication on days to germination, 1st leaf stage, 2nd leaf stage, 3rd leaf, booting, half boot, heading, fertilization leaf length and width, plant height, peduncle length, 1000 g seed weight and seed plant^{-1} were determined for principal component analysis.

SSR markers (Primer Invitrogen) were used for finding out rust genetic diversity on molecular basis. DNA extraction was done by commercially available kit (Fermintas) and Polymerase Chain Reaction (Applied Biosystems 96 well USA, model 9902) was carried out by the protocol as mentioned in (Begum et al., 2014). Twenty-two (11 each monomorphic and polymorphic) previously reported DNA markers XGWM35 (200&225 bp), WMC419, XGWM120, GWM174, XGWM140, XWMC170, XWMC405, XWMC348, XWMC407 (*Figure 1*), GWM 148 (190&200 bp), Barc 86 (200&210 bp), WMC 773, BARC 114 (105&200bp), CSLV34 (150&190bp), PSP3000 (300&350bp), XGWM493 (150&300bp), XGWM153 (100&300bp), XGWM111, XGWM44 with 120,285,500 & 700bp (*Figure 2*), BARC4 (90&200bp), XGWM125 (150&190bp) and XGWM410 were employed to amplify PCR products (*Table 2*). PCR products were resolved in gel tank (Cleaver scientific LtD, made in UK serial Number MS 130711) on 4% agarose gel and were stained with Ethidium Bromide. The fragments were visualized under UV light in the gel documentation system (Syngene) at NIGAB for identification. For proper assessment of data all data was compiled with recommended guidelines necessary for statistical analysis. The data were analyzed using Web-based software using SaaS application (McNee, 2007) statistical packages "R" version 4.0.2 (R Core Team, 2014).

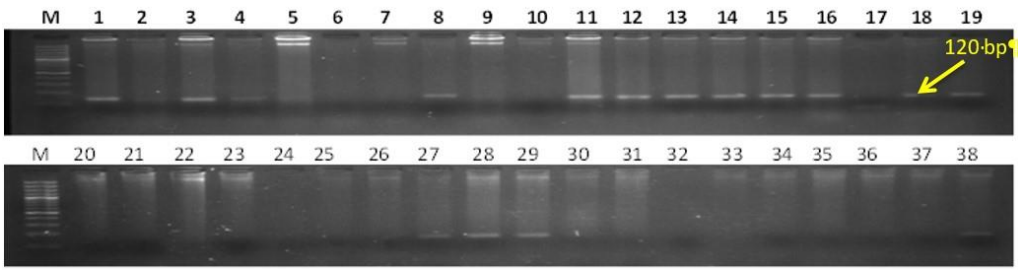

Figure 1: *PCR confirmation of 1-38 accessions with XWMC 407 SSR marker(monomorphic) indication presence of 120 bp bands size*

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

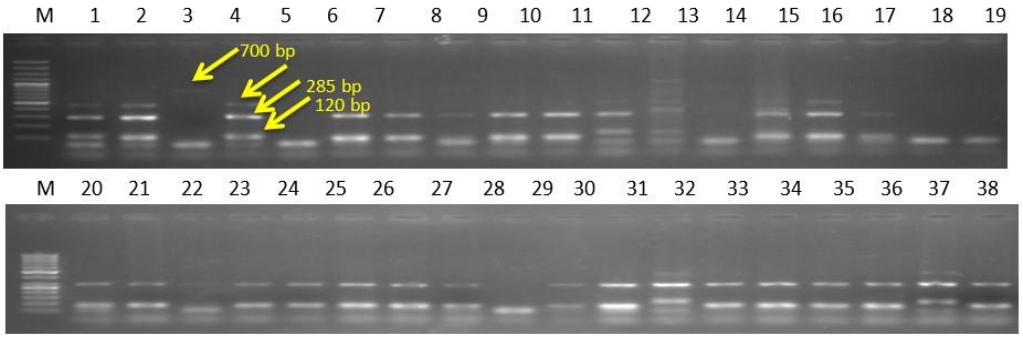

Figure 2: *PCR confirmation of 1-38 accessions with XGWM 44 SSR marker (polymorphic) indication presence of 120-700 bp bands size*

Table 2: *List of applied markers with band size and target site used to detect rust resistance genes*

S.No	Marker	Allelic variant (Band size)	Target Gene/site	Polymorphism	Reference
1	Barc 86	200 & 210	Yr-26	Yes	(Wang et al., 2008)
2	WMC773	298	Yr-26/Lr 38		(Mebrate et al., 2008)
3	BARC114	105 & 200	4D chromosome	Yes	(Båga et al., 2007)
4	XGDM125	150 & 190		Yes	(Tékeu et al., 2017)
5	XGWM35	200 & 225		Yes	(Abbasabad et al., 2017)
6	GWM174	220			(Li et al., 2005)
7	CSLV34	150 & 190	Yr-18/Lr34	Yes	(Begum et al., 2014)
8	PSP3000	300 & 350	Yr-10	Yes	(Begum et al., 2014)
9	WMC419	200	Yr-29		(Begum et al., 2014)
10	XGWM140	120	YrH52		(Peng et al., 2000)
11	XGWM44	120,285,500 &700	Lr-19/Yr18	Yes	(Li et al., 2005)
12	XGWM410	140	YrCN19		(Luo et al., 2006)
13	XGWM120	150	Yr-5		(Begum et al., 2014)
14	xwmc 170	170	2A/stripe rust		(Chhuneja et al., 2008)
15	XWMC405	220	7D		(Suenaga et al., 2003)
16	XWMC348	130	Lr 49		(Bansal et al., 2008)
17	XWMC407	120	Lr17		(Wang et al., 2010)
18	XGWM493	150 & 300	3B/stripe rust	Yes	(Börner et al., 2000)
19	XGWM153	100 & 300	YrP138	Yes	(Yue et al., 2010)
20	GWM148	190 & 200	Lr60	Yes	(Carter et al., 2009)
21	XGWM111	185	Yr33/ yr 26		(Dawit et al., 2019)
22	BARC4	90 & 200	Lr34/Yr18 & Lr46/Yr29	Yes	(Lillemo et al., 2008)

Results

Analysis revealed highly significant variation for many of the traits, furthermore genotype by location interaction were also highly significant (*Table S1-S8*). For genotype results of days to germination, 1st leaf emergence, 2nd leaf, leaf width, days to plant maturity, height and peduncle length showed highly significant results. While significant results were observed for traits like 2nd leaf, days to booting, days for half opening of spike, days to heading, days to fertilization, leaf length and seeds spike^{-1}. Moreover, traits like spike length, number of spikelet spike^{-1}, infertile spikelet spike^{-1}, awn length, spike plant^{-1} (fertile tillers) and seeds plant^{-1} showed non-significant variation. Similarly for genotype by location interaction results showed that highly

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significant variation were observed due to the environment for 1st leaf emergence, 3rd leaf, days to booting, half opening of spike, days to heading, days to fertilization, days to plant maturity and plant height traits, while non-significant variation were reported for traits like days to germination, 1st leave emergence, 2nd leaf, leaf length, leaf width, peduncle length, spike length, number of spikelet spike^{-1}, infertile spikelet spike^{-1}, awn length, seeds spike^{-1}, spike plant^{-1} (fertile tillers) and seeds plant^{-1}.

All the significant environmental interactions are further explained in order to get the exact picture of genotypes performance for the selected locations. It's often difficult for a plant breeder to explain the extent of variation among large number of accession when assessed together. In order to effectively and reliably explain the extent of variation we have used statistical methods like PCA which will not only help us to explain the extent of variation but will help in the grouping of these accessions based on similarities and dissimilarities.

Islamabad region

The PCA was performed to find out the genetic base of accession (*Figures 3, 4* and *Table S9*). Analysis revealed that PC1 was covering 96.7% variation while PCA component 2 (PC2) was only accommodating 2.1% variation (*Figure 3*). PCA distributed all the lines in to three major clusters based on genetic diversity. Cluster III comprised of only one line (2), cluster II was having two lines (6 and 46), and the rest of the accessions (97%) were clustered in cluster I based on their performance for the studied traits. Accessions 69, 7, 80, 29, 30, 91, 83, 92, 75, 81, 56, 82, 96, 93, 5, 65, 84, 27, 82, 96, 93, 73, 95, 19, 94, 31, 11, 97, 62, 54, 100, 18 and 4 are all in quadrant (Plot C) and contained high diversity level.

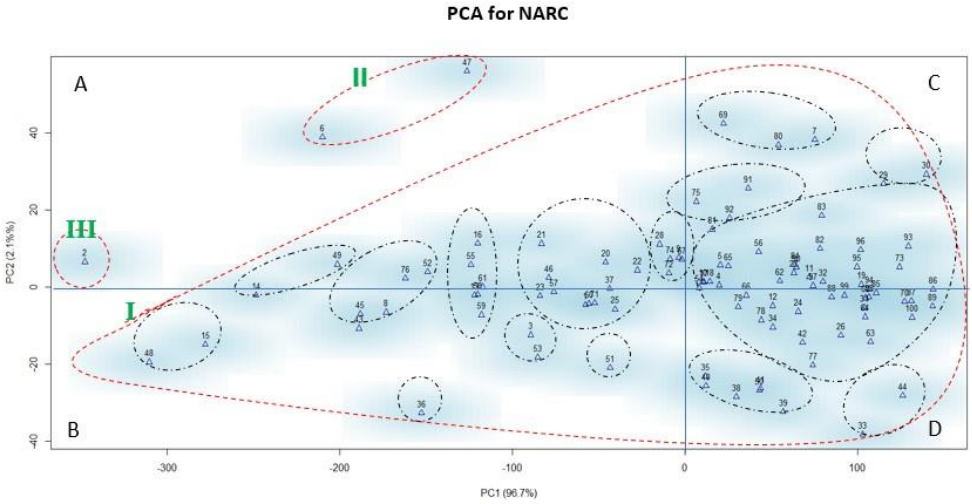

Figure 3. *PCA analysis of yield related traits for100 indigenous lines for Location A (NARC Islamabad field)*

Peshawar region

Analysis revealed that PC1 was explaining 80.1% variation while component 2 (PC2) was having 15.6% variation (*Figure 4*). PCA grouped all lines into five clusters. Cluster IV and V comprised of only one line i.e. 36 and 47 respectively, cluster III was

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

having two lines (52 and 92), cluster II was having 8 accessions (6, 7, 30, 69, 80, 91, 95 and 93), while the rest of the accessions (87%) were clustered in cluster I based on their performance for the studied traits.

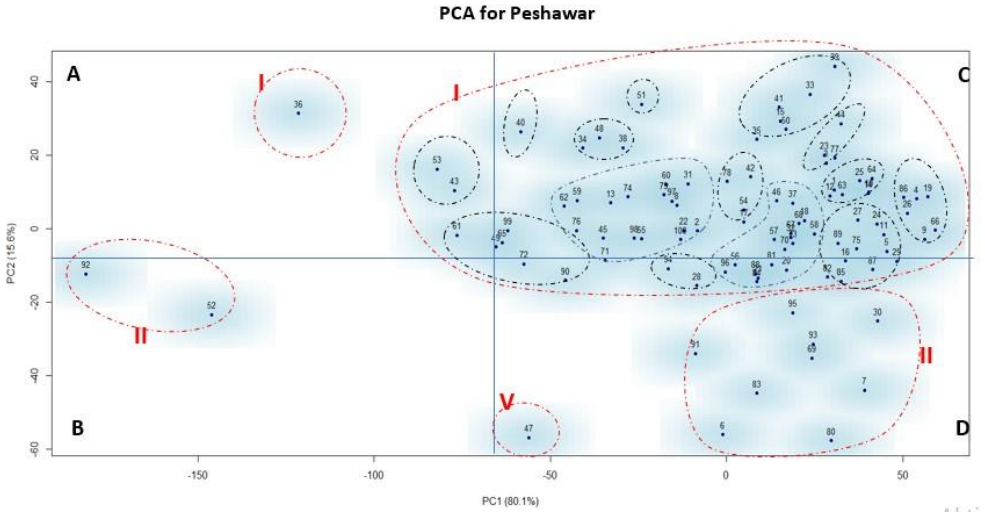

Figure 4. *PCA analysis of yield related traits for 100 indigenous lines for Location B (Peshawar)*

Furthermore, PCA analysis at both locations showed diversification of accession along with random pattern confirming broad genetic base and least similarity among the accessions with desired performance for most of the accessions in 3rd quadrant (Plot C) along with high genetic diversity compared to other quadrants.

Most of the accessions were found in positive quadrant (C) proving them to have broad genetic base with ideal performance for all traits except those where lower mean values are preferred. Each quadrant accessions were different from other quadrant. Remaining germplasm had random pattern confirming broad genetic base. The germplasm in plot B and D also exhibited the diversity were poor in performance for studied traits where higher mean value is required. Moreover, accessions were randomly arranged in quadrant B and C containing some outliers as well proving that more data of these particular accessions are required in order to link them clearly to quadrant C.

Cluster analysis based on SSR

A total of 22 SSR (mono/polymorphic) were used to detect rust resistance genes/alleles. A total of 36 reproducible bands were reported. Data was documented on the bases of detection of specific band sizes (*Tables S9* and *S10*). Bands frequency ranged from 5-21. A total of 23 land races were identified with the presence of nearly 50% bands frequency for SSR markers. Accessions 4, 8, 12, 26, 49, 68, 83 and 84 were having 17 score able bands out of 36. Similarly accessions 2, 6, 11, 14, 22, 27, 30, 31, 32, 38, 51 and 98 were having 18 score able bands, while accessions 24, 25 and 29, 52 had 19 and 21 score able bands respectively. A dendrogram was constructed on the basis of coefficient of dissimilarity to find out genetic diversity in indigenous germplasm. High level of diversity was observed i.e. two main groups were revealed (A and B). Group A was only having 36% of the total accessions while the rest of the accessions were grouped in B shown in (*Table 3*). Both groups were dissimilar with

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Euclidian value of 10. Both the groups were divided into two sub-groups i.e. A-1, A-2 and B-1 and B-2 respectively (*Table 3* and *Figure 5*). A-1 and A-2 were 80 percent dissimilar (Euclidian value 0.8) while sub-groups B-1 and B-2 were 90% distinct. Apart from sub-group A-1, all were subdivided into 2 clusters each.

Table 3: *Distribution of 100 accessions into groups and clusters by cluster analysis on the basis of 22 SSR (mono/polymorphic) markers*

S.No	Groups	Clusters/Subcluster		Accessions number	Total (%)
1	A	A-1*		81, 84, 48, 50	4
		A-2	A-2-1	79, 92, 75, 33, 91, 35, 80, 77, 21, 34, 44, 78, 88, 89, 55, 25, 36	17
			A-2-2	28, 85, 32, 87, 94, 26, 23, 30, 95, 7, 93, 16, 19, 27, 57	15
2	B	B-1	B-1-1	61, 66, 99, 100, 11, 8, 98, 97, 73, 59, 63, 62, 65, 83, 20, 10, 22, 29, 60, 68, 86	21
			B-1-2	4, 56, 9, 74, 69, 18, 17, 13, 1, 5, 15, 14, 2, 12, 96, 31, 37, 76, 6, 58, 64, 3, 38, 82, 72, 24, 70, 67, 71	29
		B-2	B-2-1	43, 39, 90, 40, 41, 46	6
			B-2-2	49, 53, 51, 42, 45, 52, 47, 54	8

**Grouping of accessions are based on similarity in terms of the presence of same band sizes for each SSR marker
*Cluster A-1 has no sub-cluster due to least of all accessions

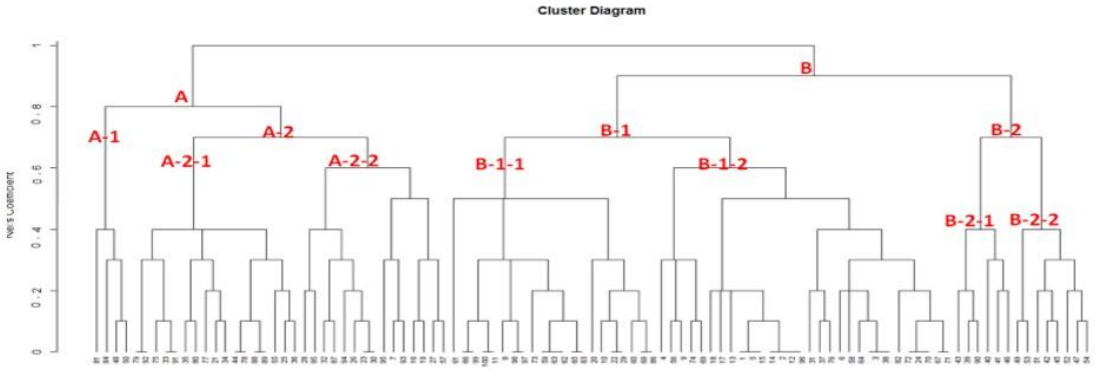

Figure 5: *Dendrogram showing Distribution of 100 accessions in to groups and clusters on the basis of 22 SSR markers*

Cluster B-1-1, B-1-2 and B-2-2, B-2-2 were almost 70% dissimilar to each other. B-1 was the largest subgroup containing 50% of the total accessions. Similarly cluster B-1-1 was the biggest of all with 29 accessions. The cluster grouped different accessions in terms of the presence of multiple alleles detected by 24 SSR markers for rust resistance (*Figure 5*).

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

Discussion

Most agronomic traits are affected by the varying environment, due to which genetic basis dissection becomes very difficult (Wang et al., 2017). Genetic diversity is partially depleting as the breeding only focuses on yield related parameters (Ren et al., 2013; Mengistu et al., 2016b) which is of serious concern as narrowing the genetic base of wheat could be disastrous in fighting climate change and other yield related parameters or disease related issues. Present study was performed in order to find out genetic diversity on morphological traits as wells as for rust resistance genes. Similarly results effect due to location, varieties and G×E was also addressed for the indigenous population. Pooled ANOVA for RCBD showed highly significant variations in all three compartments (location, varieties and G×E) proving the locations and germplasm diversity. Principal Component Analysis can be used for multi-location trials effectively for genetic diversity (Bhanupriya et al., 2014; Mengistu et al., 2016a; Devesh et al., 2019). The PCA results are a bit different from Mengistu and Dvesh as their results showed 80% variation by PC compartments. PC plot proved high genetic diversity but it also suggested that the landrace lot of variability structure and consisted of exceedingly admixed lineages. Results of Gordon et al. (2019) and Meena et al. (2014) were slightly different as PC1 to PC7 showed 79.85% of the total variation. Though PCA explained the variation among germplasm in both the studies the difference in the detected variation depends on the source of the dataset used in the study. Similarities or dissimilarities among germplasm can be identified by cluster analysis on the basis of genetic distance between groups and clusters. All the accessions within one cluster are genetically similar while genetically dissimilar to other cluster. Results of our study are also in line with Chen et al. (2012) and Islam et al. (2012) who found different clusters and subgroups according to geographical zones of ninety wheat accessions with 269 SSR. Rust species specific alleles in crop can be easily recognized by comparing their lethality or resistance to different pathotypes. The accessions that are having more resistance gene combination must be explored further in breeding germplasm with available rust markers (Sadiq, 2019). In order to increase the biodiversity of Pakistani germplasm, present study was design to broaden the genetic base of our existing varieties by identifying accessions with rust resistant genes.

Yellow rust resistance

Rust resistance gene *Yr5* is present on 2BL chromosome and was transferred to bread wheat from spelt wheat. *Xgwm120* marker, that is present at a 12 cM distance from *Yr5* (Begum et al., 2014), showed a 150-bp fragment in 54% of wheat land races. Similarly, *Yr10* (stripe rust resistance gene) is dominant as well as race specific and was mapped on 2BS chromosome of wheat. At present *psp3000* marker is the only one available for *Yr10.* The distance between both marker and gene is 1.2 cM (Begum et al., 2014). Amplification of PCR product produced polymorphic band i.e. 300bp and 350 bp fragment in 43% of population. Moreover, the co-dominant nature of this marker is considered ideal for *Yr10* gene identification in segregating population. Australian germplasm was also successfully screened by this marker (Bariana et al., 2002).

Locus *Yr18* and *Lr34* confer slow rusting to stripe and leaf rust (Begum et al., 2014). The *csLV34* marker (Lagudah et al., 2006) amplified 150bp and 190bp fragment in 16% of the genotypes proving presence of *Yr18* gene, whereas a 215bp size was associated with the nonappearance of *Yr18* was successfully able to amply in 53% of total

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population. The rest of land races didn't amplify any of the bands. This marker is co-dominant which makes them suitable for use in early segregating generations. The frequency of this gene is quite low in Pakistani population as previously reported (Begum et al., 2014) hence there is dire need to broaden the race-nonspecific resistance for yellow rust. Similarly, results were also observed previously (Begum et al., 2019) where all advanced wheat lines were checked by CSLV34 for presence of Lr34/Yr18 that yielded a PCR product of 150 bp amplification in 43% of the accessions.

Two markers Barc 86 (200bp) and Wmc 773 (298bp) were used to detect the presence of *Yr26* gene. Former showed its presence in 94 accessions while the latter was detected in 74 accessions out of the total population. Wheat genotypes with *Yr26* gene were found resistant to majority of *Pst* races in virulent tests (Wang et al., 2008). However, virulence against this gene has recently been detected in Australia. Pyramiding of this gene with other *Yr* genes may be helpful to broaden the genetic base of wheat against *Pst*. Three other markers were used to detect presence of *Yr* 29 gene. Wmc 419 (200bp) showed its presence in 55% of the population while xgwm 140 (120bp) and xgwm 410 (140 bp) were present in 56 and 45% of the landraces, respectively. Similar results were obtained by Begum et al. (2014) for *Yr26* and 29 and genes. The *Yr* resistance for barc4 (90-100bp) is located on arm 5BS of the chromosome (Law and Worland, 1997), similarly Maccaferri et al. (2015) reported that it is associated with genetic resistance for yellow rust but Lillemo et al. (2008) was able to find a significant Quantitative trait locus (QTL) for Powdery mildew resistance on 5BS,close to the SSR marker Xbarc4. STS markers *Xgwm111* with a band size of 185bp was detected in 70% of the population. This marker is considered more reliable for *Yr* identification (Sadiq, 2019) that is linked to *Yr* 15 and 26 with a distance of 1.9 cM (Ma et al., 2001). This marker has been also reported to be linked with *Yr33* as well (Dawit et al., 2019).

The marker Xgwm148 (190-200 bp) is associated with high-temperature adult-plant resistance to stripe rust resistance in spring wheat, present on 2BS chromosome in between a 16.9 cM region (Carter et al., 2009). Its 190 bp and 200 bp allele was detected in 63% and 13% population respectively in present study. Marker for locus xwmc170-2A is associated to stripe rust resistance. It was maped on chromosome 2A of *T. monococcum* within 3.6 cM distance for Xwmc407 and Xwmc170 marker (Chhuneja et al., 2008). In the present study Xwmc407 was identified in 60 out of 100 land races proving that the majority of germplasm was having resistant genes. Microsatellite marker xwmc348 (130bp) was screened in 31 of the land races confirming the presence of *Lr* 49 in this population. Previously the same was reported by Bansal et al. (2008) as well. Xgwm153 (100bp and 300bp) was present in almost 82% of the population. It is 8.2 cM away from the YrP138 resistance gene. This is quite different from other known resistance as it shows resistance to the prevailing Chinese Pst raceCYR32 at seedling stage (Yue et al., 2010). The marker Xgwm493 is present on the short arm of chromosome 3BS indicating the location of the stripe rust resistance gene (Börner et al., 2000) was able to detect the presence of Sr resistance gene in 57% (150 bp) and 66% (300bp) of land races.

Leaf rust resistance

Microsatellite markers barc 114 is present on chrosome 4D (Båga et al., 2007) amplified 105-200 bp band, xgwm 165 (225 bp), gwm 174 (220bp) and xgwm 125 (150-190 bp) were used to detect this gene. These markers identified 85, 62, 1, 55 land

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

races with *Lr* 34 gene, respectively. Xgwm 125 marker has been previously used for genetic diversity as well (Tékeu et al., 2017). Similarly, marker Xgwm35 has been previously used for genetic diversity as well (Abbasabad et al., 2017). Xgwm44 (185 bp) is closely linked to *Lr* 19 (Li et al., 2005) gene and is broadly used for its detection and is also used for maping of gene (Xing et al., 2006) was able to screen 66% of the population. This marker was also reported to be linked with *Yr18* gene (Imtiaz et al., 2004). Marker Xwmc407 (120 bp) was used to detect *Lr* 17 in 42% of the population used in present study, which is flanked by this marker that is on the short-arm of wheat 2A chromosome (Wang et al., 2010). But it is also widely used to examine the presence of Yr17 gene (Sadiq, 2019). Suenaga et al. (2003) mentioned the linkage between stripe and leaf rust severity detected by the marker Xwmc405 which is present on chromosome 7D. Its allele of 120bp was in 81 land races proving that these lines can be used for developing both leaf and stripe rust resistant varieties. But reports suggest that this marker is also linked to other traits as well (Golabadi et al., 2011).

Conclusion and Recommendations

Results indicated high level of diversity in studied pool of indigenous lines which can be used for creating varieties for any desired traits. Based on marker results, 23 wheat lines ranging 17-21 alleles in combination were identified. Durable resistance in the present study was a bit low making it compulsory that these selected land races should be used in future breeding programs as donor parents to increase gene frequency for broadening the available genetic base of Pakistani varieties that will be newly developed. Over the last three decades, rust has destroyed yield of wheat crop significantly therefore, there is a need to develop lines by pyramiding of gene pool in our varieties. This study will ultimately help in yield enhancement, moreover the study discovered the presence of variability among the tested genotypes for rust resistance genes and confirmed possibility for increasing wheat productivity in the target area by incorporating these diversified lines in future breeding programs. Combined PCA and SSR provides sufficient power in identifying diversity at phenotypic and genetic level, thus providing a better chance to plant breeders and geneticists to select desirable genotypes out of base population with unknown diversity or variability. Our results highly recommended that these land races must be evaluated with more number of markers linked to all available pool of rust resistant genes. Furthermore, gene sequencing of these lines for the marker that showed allelic variations are also recommended for finding our desirable gene combination that will be a great assistance in developing future wheat varieties.

Acknowledgements. The Government of Pakistan funded the study through NIGAB project 'National Institute for Genomics and Advanced Biotechnology'. All the authors are thankful to the National Agricultural Research Centre and Crop Diseases Research Program Islamabad Pakistan for providing seeds and guidance. The authors are obliged to Assistant Professor Dr Sajid Ali (Institute of Biotechnology & Genetic Engineering (IBGE) for assistance in rust validation and Mr. Zabih Ullah (Department of Plant Breeding & Genetics, Faculty of Crop Production Sciences, The University of Agriculture, Peshawar, Pakistan) for assisting statistical analysis in the present study.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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SUPPLEMENTAL DATA

Table S1. *Analysis of variance (RCB design) of Germination for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	%SS	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	3788.85	0.51	3788.85	47.942364	7.71	21.1976895
Reps w/n Loc	4	316.117		79.0292			
Genotypes	99	2181.08	0.30	22.0312	9.2770415	1.28	1.42312627
GxL	99	159.097	0.021	1.60704	0.6767063	1.28	1.42312627
Pool Error	396	940.424	0.127	2.37481			
Total	599	7385.57					

*F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant, F-Cal < F-Tab 0.05= Non-significant

Table S2. *Analysis of variance (RCB design) of Days to heading for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	87865.8	0.669094	87865.8	370.12083	7.71	21.19768
Reps w/n Loc	4	949.591		237.3977			
Genotypes	99	35162.3	0.267759	355.175	27.961729	1.28	1.423126
GxL	99	2312.83	0.017612	23.3619	1.8392072	1.28	1.423126
Pool Error	396	5030.06	0.038304	12.7021			
Total	599	131320.					

*F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant, F-Cal < F-Tab 0.05= Non-significant

Table S3. *Analysis of variance (RCB design) of Days to maturity for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	188659.8	0.823797	188659.8	547.3080002	7.71	21.19768958
Reps w/n Loc	4	1378.82		344.705			
Genotypes	99	29695.4	0.12966	299.953	19.713824	1.28	1.4231262
GxL	99	3253.07	0.01420	32.8593	2.1596064	1.28	1.4231262
Pool Error	396	6025.29	0.02631	15.2154			
Total	599	229012.4					

F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant, F-Cal < F-Tab 0.05= Non-significant

Table S4. *Analysis of variance (RCB design) of spike length for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	0.000417	0.00000028	0.000417	1.70869E-05	7.71	21.19768958
Reps w/n Loc	4	97.54047		24.38512			
Genotypes	99	184.7316	0.126064852	1.865976	0.802466252	1.28	1.423126275
GxL	99	262.2779	0.178984093	2.649272	1.139323862	1.28	1.423126275
Pool Error	396	920.8195	0.628387058	2.325302			
Total	599	1465.37					

F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant and F-Cal < F-Tab 0.05= Non-significant

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

Table S5. *Analysis of variance (RCB design) of Number of Spikelets Spike^{-1} for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	62.08167	0.010075	62.08167	0.44953597	7.71	21.19768958
Reps w/n Loc	4	552.4067		138.1017			
Genotypes	99	904.965	0.146867	9.141061	0.972838307	1.28	1.423126275
GxL	99	921.4183	0.149537	9.307256	0.990525658	1.28	1.423126275
Pool Error	396	3720.927	0.60387	9.396279			
Total	599	6161.798					

F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant and F-Cal < F-Tab 0.05= Non-significant

Table S6. *Analysis of variance (RCB design) of seeds spike^{-1} for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	0.06	8.0795E-07	0.06	2.1501E-05	7.71	21.19768958
Reps w/n Loc	4	11162.25		2790.563			
Genotypes	99	13507.33	0.18188918	136.4377	1.3613606	1.28	1.4231262
GxL	99	9903.94	0.133366	100.0398	0.9981861	1.28	1.4231262
Pool Error	396	39687.75	0.53444	100.2216			
Total	599	74261.33					

F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant and F-Cal < F-Tab 0.05= Non-significant

Table S7. *Analysis of variance (RCB design) of spike plant^{-1} for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	45.375	0.031941	45.375	1.50447612	7.71	21.19769
Reps w/n Loc	4	120.64		30.16			
Genotypes	99	226.4183	0.159384	2.287054	1.052260193	1.28	1.423126275
GxL	99	167.4583	0.11788	1.6914	0.77824854	1.28	1.423126
Pool Error	396	860.6933	0.605872	2.1734			
Total	599	1420.585					

F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant and F-Cal < F-Tab 0.05= Non-significant

Table S8. *Analysis of variance (RCB design) of seeds plant^{-1} for 100 selected accessions with three replications across two locations for environmental interactions*

SOV	DF	SS	SS%	MS	F-Cal	F-Tab @ 0.05	F-Tab @ 0.01
Locations	1	16727.0	0.00321829	16727.04	0.85684897	7.71	21.19768958
Reps w/n Loc	4	78086.2		19521.57			
Genotypes	99	838221	0.16127461	8466.884	0.95298555	1.28	1.423126275
GxL	99	746147	0.14355956	7536.848	0.84830581	1.28	1.423126275
Pool Error	396	3518297	0.67692364	8884.588			
Total	599	5197479					

F-Cal ≥ F-Tab 0.01= Highly Significant, F-Cal ≥ F-Tab 0.05= Significant and F-Cal < F-Tab 0.05= Non-significant

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

Table S9. *Combined morphological data for 13 parameters used for PCA*

S.No	Acc.	Germination	1st leaf (days after germination)	2st leaf (days)	3rd leaf (days)	Booting (days)	Half open (days)	Heading (days)	Fertilization (days)	Leaf length (cm)	Width (cm)	Plant height (cm)	Peduncle (cm)	No of seed / spike
1	11123	12.33	7.33	6.33	4.33	118.00	127.67	131.00	136.00	21.00	1.43	75	24.00	33.00
2	11126	11.67	7.33	6.33	4.33	115.50	124.33	129.00	134.00	20.33	1.43	70	26.33	42.00
3	11144	13.33	8.00	7.00	4.67	125.00	128.33	132.67	140.33	17.33	1.37	90	28.00	49.00
4	11145	11.33	6.33	6.33	4.67	109.00	124.33	128.33	136.67	22.00	1.40	68	22.33	35.00
5	11152	11.00	6.33	6.33	4.33	113.50	121.33	125.00	130.33	18.00	1.43	75	27.00	30.00
6	11154	16.00	8.67	7.67	4.67	97.00	107.33	111.67	118.33	20.33	1.67	69	25.67	46.00
7	11155	10.00	7.00	6.00	4.33	91.00	105.67	110.67	117.67	21.33	1.27	103	25.00	23.00
8	11160	12.33	7.67	6.67	4.33	115.50	128.33	133.00	140.00	21.33	1.27	79	28.33	42.00
9	11161	7.00	6.67	6.00	4.33	109.00	120.33	124.67	131.00	19.33	1.30	77	27.67	34.00
10	11162	12.00	7.00	6.33	4.00	113.00	123.33	128.00	134.67	21.00	1.37	98	26.00	23.00
11	11163	12.67	7.67	6.67	4.33	111.67	120.67	125.67	134.00	21.00	1.40	75	27.67	32.00
12	11164	12.67	7.33	6.33	4.33	117.00	126.00	130.00	135.67	18.67	1.43	72	24.00	30.00
13	11166	12.00	7.00	6.67	4.00	117.33	125.00	128.67	136.00	20.67	1.40	78	29.67	46.00
14	11168	13.33	7.33	6.67	4.33	114.67	125.33	130.33	138.00	19.67	1.30	105	32.33	65.00
15	11170	12.67	7.33	7.00	4.33	125.33	132.33	136.00	143.67	16.33	1.40	75	28.67	48.00
16	11171	13.33	7.33	6.67	4.33	111.67	121.00	125.00	129.67	21.67	1.43	68	26.67	33.00
17	11173	13.33	7.67	6.67	4.67	114.33	122.33	127.00	134.67	16.00	1.47	68	26.67	44.00
18	11174	13.33	7.00	6.33	4.33	114.00	122.00	127.33	135.00	19.00	1.63	65	24.67	38.00
19	11177	14.67	7.00	7.00	4.67	114.33	122.33	127.33	133.33	17.67	1.43	72	27.33	23.00
20	11178	14.67	6.67	6.67	4.33	113.33	121.33	125.33	131.33	19.00	1.37	109	21.67	43.00
21	11179	13.00	6.33	7.00	4.67	110.33	118.67	123.67	130.00	20.00	1.17	75	25.00	37.00
22	11181	11.00	6.33	7.00	4.67	114.00	122.33	126.33	132.00	23.67	1.33	67	26.33	46.00
23	11183	13.67	6.33	6.67	4.33	117.67	125.00	129.00	136.00	22.00	1.63	73	32.00	31.00
24	11184	16.00	6.67	7.33	5.00	117.67	126.33	130.00	136.33	24.67	1.53	108	25.00	22.00
25	11185	14.33	7.67	6.67	4.67	116.33	125.33	129.67	136.67	23.33	1.43	85	30.67	33.00
26	11186	12.33	7.67	6.67	4.67	119.33	127.67	132.00	138.33	23.33	1.33	96	28.67	19.00
27	11187	14.67	7.33	7.00	5.00	114.00	121.67	127.00	131.67	19.67	1.37	97	32.33	21.00
28	11188	12.00	7.00	6.00	4.00	110.67	118.00	122.33	128.00	20.33	1.40	92	34.33	34.00
29	11189	12.67	7.00	6.33	4.33	101.00	109.67	114.00	121.00	22.67	1.33	89	32.00	23.00
30	11190	11.33	7.00	6.00	4.00	98.00	108.67	113.00	121.33	23.67	1.40	98	28.00	21.00
31	11192	12.33	7.00	6.00	4.00	115.00	125.67	129.67	135.00	19.67	1.40	84	29.00	26.00

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32	11193	13.33	7.33	7.00	5.00	110.00	122.67	127.00	134.00	26.33	1.50	69	28.33	24.00
33	11194	13.67	7.67	6.67	4.33	132.67	140.00	143.67	148.67	27.00	1.30	85	27.67	31.00
34	11195	13.00	7.33	6.33	4.33	118.00	127.33	131.00	138.67	26.33	1.53	87	27.33	38.00
35	11197	13.67	8.00	6.00	5.00	128.67	134.00	138.00	142.33	26.67	1.27	78	45.67	33.00
36	11198	12.67	7.33	6.33	4.33	132.67	138.33	142.67	146.67	27.33	1.33	103	32.00	45.00
37	11200	13.67	7.67	6.67	4.67	118.00	125.33	129.33	133.00	25.67	1.33	75	28.33	19.00
38	11202	12.33	7.33	6.33	4.33	128.67	135.67	139.67	144.00	23.67	1.50	81	27.00	50.00
39	11205	11.67	7.33	6.33	4.33	132.33	140.00	143.67	148.67	24.33	1.43	74	31.00	31.00
40	11207	14.33	8.00	7.00	5.00	127.33	134.67	139.00	143.67	25.00	1.43	88	32.67	45.00
41	11208	12.67	7.67	6.67	4.67	127.00	134.67	138.33	144.67	22.00	1.30	83	34.33	39.00
42	11209	11.33	7.67	6.67	4.67	122.67	129.33	133.33	138.33	21.33	1.30	86	24.00	32.00
43	11210	10.67	7.00	6.00	4.67	125.67	130.00	133.67	138.67	21.33	1.10	78	22.33	40.00
44	11211	12.00	7.67	6.67	4.67	127.33	135.67	140.00	145.67	26.00	1.37	75	22.67	25.00
45	11214	12.00	7.33	6.33	4.33	118.67	128.67	133.33	140.33	24.33	1.27	75	22.33	39.00
46	11215	7.00	7.00	6.00	4.67	113.33	122.33	127.33	133.00	21.00	1.30	91	35.67	38.00
47	11216	11.33	7.67	6.67	4.67	88.67	98.33	103.00	109.33	22.67	1.40	77	33.67	43.00
48	11217	8.00	7.00	6.00	4.67	128.00	135.00	138.67	143.67	22.67	1.53	88	28.67	47.00
49	11218	5.67	6.33	5.33	4.67	113.00	122.67	127.33	133.00	23.00	1.67	83	30.00	39.00
50	11221	11.00	7.67	6.67	4.67	126.33	134.67	139.67	145.33	23.67	1.43	86	29.00	34.00
51	11222	10.33	6.67	5.67	4.33	129.33	131.67	136.33	142.00	23.33	1.37	82	27.67	39.00
52	11223	9.00	6.67	5.67	4.33	111.00	122.67	127.00	133.33	26.00	1.40	96	36.33	44.00
53	11224	10.33	7.00	6.00	4.67	125.67	133.00	136.67	141.33	18.33	1.27	86	27.33	38.00
54	11225	9.67	7.00	6.00	4.67	114.33	124.33	128.00	133.00	23.00	1.20	84	30.33	34.00
55	11226	10.00	7.00	6.00	4.67	112.33	122.00	125.67	131.67	22.33	1.37	81	31.00	56.00
56	11227	13.00	7.67	6.67	4.67	110.00	119.67	124.00	128.67	21.00	1.50	89	26.33	35.00
57	11228	11.67	6.67	6.67	4.67	114.00	125.00	129.33	137.00	22.67	1.50	73	25.00	41.00
58	11229	14.33	8.00	7.00	5.00	115.00	125.33	129.67	135.33	24.00	1.43	86	31.33	42.00
59	11231	13.33	7.00	6.67	4.67	119.67	128.00	132.00	139.33	23.00	1.33	55	31.33	52.00
60	11233	13.67	6.67	7.33	5.33	117.33	125.00	129.33	135.33	24.67	1.70	91	33.33	52.00
61	11236	14.67	8.00	7.00	5.00	114.00	123.33	129.33	136.00	20.00	1.47	81	28.33	53.00
62	11237	10.67	7.00	6.00	4.00	113.33	121.33	126.67	132.00	22.33	1.53	85	30.00	44.00
63	11239	13.33	7.67	6.67	4.67	119.00	127.33	132.67	140.00	30.33	1.60	90	32.00	34.00
64	11240	11.67	7.67	6.67	4.67	118.00	126.00	131.33	137.67	21.67	1.30	93	20.33	32.00
65	11242	17.67	7.67	6.67	4.67	109.67	120.67	124.67	132.67	20.67	1.33	86	28.00	38.00
66	11243	11.33	7.33	6.33	4.33	113.00	123.67	129.00	136.00	22.33	1.60	85	26.67	29.00

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67	11244	11.00	7.33	6.33	4.33	111.67	120.33	124.67	130.33	20.67	1.37	84	25.33	27.00
68	11246	11.00	7.00	6.00	4.00	115.67	124.00	128.00	133.33	22.00	1.27	84	30.67	25.00
69	11248	12.67	7.33	6.33	4.33	93.33	104.33	109.33	117.00	21.67	1.33	72	22.67	24.00
70	11249	12.33	7.33	6.33	4.33	114.33	125.67	129.67	135.33	22.67	1.57	77	29.33	21.00
71	11250	11.33	7.33	6.33	4.33	115.00	124.67	129.00	136.00	28.67	1.63	96	36.00	45.00
72	11252	12.00	7.33	6.33	4.33	112.67	122.33	127.33	133.67	22.67	1.40	73	24.33	34.00
73	11253	16.33	8.33	5.33	5.33	111.67	121.33	125.33	133.00	19.00	1.20	63	22.00	22.00
74	11255	18.00	9.00	5.33	5.33	112.67	119.33	123.33	130.67	21.67	1.60	76	31.00	39.00
75	11256	13.67	7.00	5.67	4.67	102.67	113.33	117.00	126.00	22.67	1.50	72	21.33	36.00
76	11259	11.67	7.67	6.67	4.67	114.67	123.33	127.67	137.00	22.00	1.33	72	25.67	50.00
77	11261	11.33	7.33	6.33	4.33	124.33	132.67	137.33	142.67	21.33	1.37	93	32.33	19.00
78	11265	12.00	8.00	6.00	5.00	118.67	128.33	132.00	136.67	21.33	1.23	72	29.00	26.00
79	11272	18.33	9.00	5.00	6.00	117.00	124.33	128.33	136.67	21.33	1.17	76	27.67	46.00
80	11274	12.33	7.67	5.67	4.67	93.67	106.00	109.67	119.33	26.00	1.43	85	29.33	25.00
81	11275	12.33	7.67	5.67	4.67	107.33	116.67	120.67	126.33	21.67	1.37	87	27.33	33.00
82	11278	13.33	8.00	6.00	5.00	107.67	117.33	122.67	129.67	19.67	1.40	94	30.00	35.00
83	11288	8.67	7.33	5.33	5.00	104.00	114.33	119.00	126.67	25.33	1.47	72	23.00	34.00
84	11290	12.00	8.00	6.00	5.00	113.33	121.33	125.33	129.00	17.33	1.40	72	31.00	32.00
85	11292	12.00	8.00	5.33	5.00	114.33	124.00	128.00	135.33	19.33	1.33	72	23.67	22.00
86	11293	12.33	7.33	6.33	4.33	114.67	123.33	127.00	134.00	17.33	1.30	77	29.33	16.00
87	11294	11.33	7.33	6.00	4.33	116.00	125.33	129.00	133.67	19.33	1.57	80	25.33	23.00
88	11295	10.67	7.67	5.67	4.67	113.33	124.67	129.33	136.67	19.33	1.60	96	28.00	19.00
89	11296	11.00	7.67	5.67	4.67	116.33	125.33	130.00	136.00	19.33	1.40	72	27.33	9.00
90	11297	10.33	7.67	5.67	4.67	112.33	121.67	125.33	130.00	20.00	1.53	80	25.00	35.00
91	11299	11.67	7.67	5.67	4.67	101.00	111.67	115.67	122.67	16.67	1.33	78	28.33	39.00
92	11304	12.67	7.67	5.67	4.67	104.67	115.00	120.00	127.67	19.67	1.37	79	25.33	31.00
93	11317	15.33	8.00	6.33	5.00	107.67	117.00	121.67	129.00	22.33	1.50	82	27.33	28.00
94	11553	12.33	7.67	6.00	4.67	111.00	122.67	127.33	134.67	21.00	1.33	86	32.33	29.00
95	11558	18.00	9.00	5.67	5.00	110.33	120.33	125.00	132.67	20.33	1.37	76	23.33	24.00
96	12087	12.00	7.67	6.67	4.67	108.00	119.33	123.33	129.00	22.00	1.43	82	31.33	28.00
97	12100	13.67	8.00	7.00	5.00	114.33	122.00	126.67	132.67	20.00	1.90	81	26.67	44.00
98	12231	12.00	7.33	6.33	4.33	116.00	124.33	128.67	133.67	20.33	1.43	82	28.00	25.00
99	18668	13.00	7.67	6.67	4.67	117.00	123.67	127.33	133.00	23.67	1.50	87	28.67	26.00
100	24740	13.33	7.67	7.00	4.67	118.00	126.67	130.33	136.67	21.00	1.37	101	25.67	13.00

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

Table S10. *PCR confirmation of 22 SSR along with band size for indigenous germplasm*

Acc No.	XGWM35 225bp	XGWM35 200bp	WMC419 200bp	XGWM120 150bp	GWM174 220bp	XGWM140 120bp	XWMC170 200bp	XWMC405 220bp	XWMC348 130bp	XWMC407 120bp	GWM 148 190bp	GWM148 200bp
1	0	0	1	0	0	1	0	0	0	1	1	0
2	0	0	1	0	0	1	0	1	0	1	1	0
3	0	0	1	0	0	1	1	1	0	1	1	0
4	0	0	1	1	0	1	1	0	0	1	1	0
5	0	0	1	0	0	1	0	0	0	1	1	0
6	1	0	1	0	0	1	1	1	0	0	1	0
7	1	0	0	0	0	0	0	1	0	0	1	0
8	1	0	1	1	0	1	0	1	0	1	1	0
9	0	0	0	1	0	1	1	0	0	0	1	0
10	0	0	1	1	0	0	1	1	0	0	1	0
11	1	0	1	1	0	1	1	1	0	1	1	0
12	0	0	1	0	0	1	0	1	0	1	1	0
13	0	0	1	0	0	1	0	0	0	1	1	0
14	0	0	1	0	0	1	0	1	0	1	1	0
15	0	0	1	0	0	1	0	1	0	1	1	0
16	0	0	0	0	0	0	0	1	0	1	1	0
17	0	0	1	0	0	1	0	0	0	0	1	0
18	0	0	1	0	0	1	0	0	1	1	1	0
19	0	0	0	0	0	0	0	0	0	1	1	0
20	0	0	1	1	0	0	1	1	1	0	1	0
21	0	0	1	0	0	0	0	1	1	0	0	0
22	0	0	1	1	0	0	1	1	0	0	1	0
23	0	0	0	0	0	0	1	1	1	0	1	0
24	0	0	1	0	0	0	1	1	0	0	1	0
25	0	0	1	1	0	0	0	1	0	0	1	0
26	0	0	1	0	0	0	1	1	1	0	1	0
27	0	0	0	0	0	0	1	1	1	1	1	0
28	0	0	0	1	0	0	1	0	1	1	1	0
29	0	0	1	1	0	0	1	1	1	1	1	0
30	0	0	0	0	0	0	1	1	1	0	1	0
31	0	0	0	0	0	1	1	1	1	0	1	0
32	0	0	0	1	0	0	1	1	1	0	1	0
33	0	0	0	0	0	0	1	1	0	0	0	0

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

34	0	0	1	0	0	0	1	1	1	0	0	0
35	0	0	0	0	0	0	0	1	1	0	0	0
36	0	0	1	1	0	0	0	1	0	0	0	0
37	0	0	0	0	0	1	1	1	0	0	1	0
38	0	0	1	0	0	1	1	1	0	1	1	0
39	0	0	0	0	0	0	0	1	0	1	0	0
40	0	0	0	0	0	1	0	1	1	1	0	1
41	0	0	1	1	0	1	0	1	0	1	0	1
42	0	0	0	1	0	1	1	1	1	1	0	1
43	0	0	0	1	0	0	0	1	0	1	0	1
44	0	0	0	1	0	0	0	1	0	0	0	0
45	0	0	0	1	0	1	1	0	1	1	0	1
46	0	0	1	1	0	0	0	1	1	1	0	1
47	0	0	0	0	0	1	1	1	1	0	0	1
48	1	0	0	1	1	0	1	1	1	0	0	0
49	0	0	1	1	0	1	1	1	1	0	0	1
50	1	0	0	1	0	0	1	1	1	0	0	0
51	0	0	0	1	0	1	1	0	1	0	0	1
52	0	0	0	1	0	1	1	1	0	0	0	1
53	0	0	1	1	0	1	0	1	1	0	0	1
54	0	0	0	1	0	1	1	1	1	0	0	1
55	0	0	1	1	0	0	0	1	0	0	0	1
56	0	0	1	1	0	0	1	0	0	1	1	0
57	0	0	0	0	0	0	1	1	0	1	1	0
58	0	0	1	0	0	1	1	1	0	0	1	0
59	0	0	1	1	0	1	1	1	0	0	1	0
60	0	0	1	1	0	0	1	1	0	1	1	0
61	0	0	0	1	0	1	1	1	0	1	1	0
62	0	0	1	1	0	1	1	1	0	1	1	0
63	0	0	1	1	0	1	1	1	0	0	1	0
64	0	0	1	0	0	1	1	1	0	1	1	0
65	0	0	1	1	0	1	1	1	0	1	1	0
66	0	0	0	1	0	1	1	1	0	1	1	0
67	0	0	1	0	0	0	1	1	0	1	1	0
68	0	0	1	1	0	0	1	1	0	1	1	0
69	0	0	1	0	0	1	1	0	0	1	1	0
70	0	0	1	0	0	0	1	1	0	0	1	0

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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

71	0	0	1	0	0	0	1	1	0	1	1	0
72	0	0	1	0	0	0	1	1	0	0	1	0
73	0	0	1	1	0	1	1	1	0	0	1	0
74	0	0	1	1	0	1	1	0	0	0	1	0
75	0	0	1	0	0	0	1	1	0	0	0	0
76	0	0	1	0	0	1	1	1	0	0	0	0
77	0	0	1	1	0	0	0	1	1	0	0	0
78	0	0	0	1	0	0	0	1	0	0	0	0
79	0	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	1	0	0	0	1	1	0	0	0
81	1	1	1	1	0	0	0	1	1	0	0	0
82	0	0	1	0	0	0	0	1	0	0	1	0
83	0	0	1	1	0	0	0	1	1	1	1	0
84	1	1	0	1	0	0	1	1	1	0	1	0
85	0	0	0	1	0	0	1	0	1	0	0	0
86	0	0	1	1	0	0	1	0	0	0	0	0
87	0	0	0	1	0	0	1	1	1	0	0	0
88	0	0	0	1	0	0	1	1	0	0	0	0
89	0	0	0	1	0	0	1	1	0	0	0	0
90	0	0	0	1	0	0	0	1	0	1	0	0
91	0	0	0	0	0	0	1	1	0	0	0	0
92	0	0	0	0	0	0	0	0	0	0	0	0
93	1	0	0	1	0	0	0	1	0	0	1	0
94	0	0	0	0	0	0	1	1	0	0	1	0
95	1	0	0	0	0	0	1	0	0	0	1	0
96	0	0	0	0	0	1	0	1	0	0	1	0
97	0	0	1	1	0	1	0	1	0	0	1	0
98	1	0	1	1	0	1	0	1	0	1	1	0
99	0	0	0	1	0	1	0	1	0	1	1	0
100	0	0	0	1	0	1	0	1	0	1	1	0
Acc. No.	**Barc 86 200bp**	**Barc 86 210bp**	**WMC773 298bp**	**BARC114 105bp**	**BARC 114 200bp**	**CSLV 34 215bp**	**CSLV 34 190bp**	**CSLV 34 150bp**	**PSP 3000 350bp**	**PSP 3000 300bp**	**XGWM493 150bp**	**XGWM 493 300bp**
1	1	0	0	1	0	1	0	0	0	0	0	0
2	1	0	1	1	0	1	0	0	1	0	0	0
3	1	0	1	1	0	1	0	0	1	0	0	0
4	1	0	1	1	0	1	0	0	1	0	0	0
5	1	0	1	1	0	1	0	0	1	0	0	0

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6	1	0	1	1	0	0	1	0	0	0	0	1
7	1	0	1	1	0	1	0	0	1	0	0	1
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DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

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Acc No.	**XGWM 153 100bp**	**XGWM 153 300bp**	**XGWM 111 185bp**	**XGWM 44 120bp**	**XGWM 44 285bp**	**XGWM44 500bp**	**XGWM44 700bp**	**XBARC4 90bp**	**XBARC4 200bp**	**XGDM 125 150bp**	**XGDM 125 190bp**	**XGWM 410 140bp**
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_80918118

FLORISTIC STRUCTURE AND VEGETATION DIVERSITY OF THE WADI OTHYLAN PROTECTED AREA IN SAUDI ARABIA

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(Received 7th Jul 2020; accepted 7th Oct 2020)

Abstract. Wadi Othylan is one of the most important protected areas in the central region of Saudi Arabia supporting a high level of plant diversity. The current study aims to examine the vegetation and floristic diversity of wadi Othylan protected area and the relation between such diversity and soil characteristics. A total of 78 plant species belonging to 69 genera distributed among 27 families were collected from the studied stands. TWINSPAN, and detrended correspondence analysis (DCA) analyses classified the plant cover data into the following four plant communities: VG I: *Fagonia bruguieri-Haloxylon salicornicum*, VG II: *Acacia ehrenbergiana-Echinops spinosissima*, VG III: *Acacia ehrenbergiana-Acacia gerardii* and VG IV: *Acacia ehrenbergiana-Calotropis procera*. Plant species present in wadi Othylan may be an indicator for the low human impact on this area. A significant variation in soil texture, pH, EC and organic matter and mineral contents were observed which was correlated with different vegetation groups.
Keywords: *soil characteristics, TWINSPAN, organic matter, pH, EC*

Introduction

Wadi Othylan is a protected area located in Al-Kharj in the central region of Saudi Arabia. The central region of Saudi Arabia is characterized by highly diverse terrain ranging from small mountains to desert plains including hillocks and plateaus (Alfarhan, 2001; Shaltout and Mady, 1996). This area belongs to the Saharo-Arabian phytogeographical zone (Alatar et al., 2012). The wadi (valley) ecosystems are considered as one of the most important plant diversity centers in the central region of Saudi Arabia; however, there has been great lack of the studies considering floristic structure and vegetation diversity of these ecosystems until now (Chaudhary, 2001; Alatar et al., 2012). Furthermore, wadi ecosystems are significant for socioeconomic development considering their ecological importance for environmental gradients and physiographic variation.

The central region of Saudi Arabia is characterized by high plant diversity; more than 600 plant species are reported in the central region out of 2,243 plant species in the whole country (Collenette, 1985, 1998, 1999; Chaudhary, 2001). Several studies have been conducted to examine the vegetation of the central region including studies concerning wadi ecosystems e.g. Wadi Al-Jufair (Alatar et al., 2012) and Wadi Hanifa (Taia and El-Ghanem, 2001; El-Ghanim et al., 2010). Other studies examined the vegetation diversity of different protected areas in Saudi Arabia including Harrat Al-Harrah (El-Sheikh et al., 2019), Huraimla (Alatar et al., 2015), and Thumamah Nature Park (El-Sheikh et al., 2013). However, the present study is the first to examine the vegetation of wadi Othylan protected area, Saudi Arabia.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81198130

The majority of soils of the natural habitats in the central region of Saudi Arabia are classified among Aridisols with very low amount or without any accumulation of organic matter and clay (Al-Nafie, 2008). On the other hand, soils in wadi basin are deep with fine texture and mixed with different sizes of rocks supporting the growth of different vegetation (Batanouny, 1987). Since wadi Othylan is one of the most important wadi habitats in the central region of Saudi Arabia and the richest wadis in vegetation and floristic diversity as it is one of the protected areas. We conducted this study aiming to examine the vegetation and floristic diversity of wadi Othylan protected area and the relation between such diversity and soil characteristics.

Materials and methods

Study area

Wadi Othylan (23°785.702'N and 46°926.531'E) is one of the most important wadis in Al-Kharj area in the central region of Saudi Arabia. It is located about 90 km southern Riyadh, the capital city of Saudi Arabia, with an altitude about 519 m above sea level with a total size of approximately 45 km^2 (15 km × 3 km). It is characterized by its high diverse vegetation ranged from trees to shrubs, perennials and annual plants. Wadi Othylan was considered as a protected area and national park in Saudi Arabia on 1982. The study area is considered as one of the main features of the large plateau located in the central region of Saudi Arabia known as "Najd". This area is characterized mainly by distribution of large wadis emerging from the western mountains toward the eastern region of Najd; however, the majority of these wadis are not continuous as they covered by the sand dunes of Dahna desert. During winter rainy days, seasonal springs emerge in these wadis forming shallow pools characterized by high plant diversity. It is thought that these areas had a wetter climate in the past as remnants of their vegetation could be found along their stretch (Al-Nafie, 2008).

Plant biodiversity in wadi Othylan is significantly affected by topography factors as the area examined in this study could be separated into three different habitats i.e. depressions, rocky hills and sand dunes. Wadi Othylan is generally characterized by harsh environment; however, a great biodiversity could be found among different habitats forming the ecosystem of this wadi. In general, the dominant plant life forms during winter season are trees, shrubs and annual herbs classified mainly as mesophytes and representing different plant communities.

The study area could be separated into three different physiographical parts i.e. wadi bed, slope and plateau. The wadi bed (basin) is generally characterized by low sand dunes. On the other hand, plateau is dominated by shallow drainage runnels and notches due to the flat rocky surfaces with little amount of soil distributed among them. The slope is separated into top, middle and lower parts. The upper parts are very steep without any soil cover supporting only cliff vegetation. The middle parts are less steep than the upper parts covered with a mix of different sizes of rocks and shallow soil supporting vegetation different types of vegetation mainly chasmophytic shrubs and grasses. The lower parts are characterized by deep soil accumulated by run-off water and high vegetation density.

Meteorological data of the study area shows that it is characterized by hot dry weather in the summer and cold weather in the winter. Average maximum air temperature ranges from 20.3 °C in January and 44 °C in July, while the minimum air temperature ranges from 7 °C in January and 30 °C in July and August. The annual rainfall in the study area ranges from 2 to 22 mm. The mean wind speed ranges from 4 to 7 km/h. The average

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relative humidity is 49% with lowest values occurs in June and July (11 – 12%). The average evaporation value is around 10.35 mm/day (Al-Nafie, 2008).

Sample stands

A total of 20 study stands were selected to represent different habitats in the study site. Each stand area was 2500 m^2 (50 m × 50 m). *Figure 1* shows the study area with the location of the selected sample stands. Collection of samples was performed in the spring season as the majority of the species expected to exist. To analyze the vegetation of the study area, all plants collected from the different stands were identified and classified. Plant species, chorotypes and life forms (Shelter and Skog, 1978) were recorded. Identification of plant species depends mainly on the available references regarding the vegetation of Saudi Arabia (Collenette, 1985, 1998, 1999; Chaudhary, 2001). The line intercept method was used to calculate the plant cover parameters (Canfield, 1941).

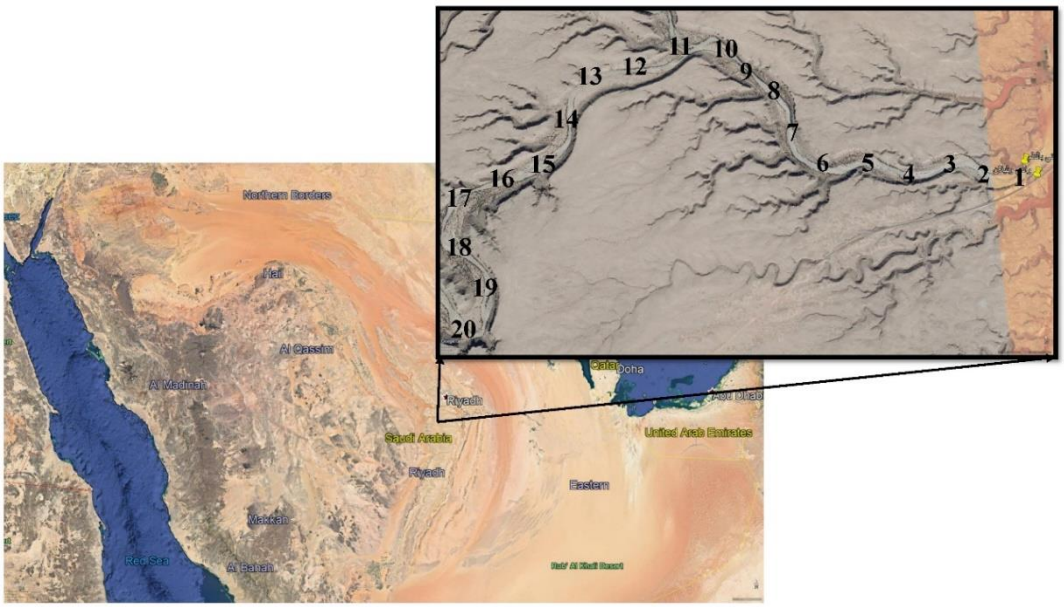

Figure 1. *Study area map showing the whole map of Saudi Arabia with focus on the study area (Wadi Othylan). The numbers on Wadi Othylan map indicate the locations of the selected sample stands*

Soil analysis

For soil analysis purpose, 50-cm depth soil samples were collected using soil spades from the studied stands (3 replicates each) and pooled together in one composite sample. Hydrometer method was used to examine soil texture (Allen, 1989). Mass loss after ignition at 450 °C was used as indicator for total organic matter content. An amount of 100 g of air-dried soil were suspended in 500 ml of distilled water to prepare the soil extract (1: 5). The extract was used for further chemical analysis. Soil pH and electrical conductivity (EC) was measured. Content of nutrient elements (N, P, K, Ca, Mg, Na and Fe) in the soil were analyzed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP MSEOS 6000 Series, ThermoFisher Scientific) method (Allen, 1989).

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DOI: http://dx.doi.org/10.15666/aeer/1806_81198130

Data analysis

Multivariate analysis using TWINSPAN software (Hill, 1979b) and detrended correspondence analysis (DCA) using DECORANA software (Hill, 1979a) were applied for the cover estimates calculated for all collected plants from the 22 studied stands. The relative cover (p_i) of each stand was used to calculate Simpson index following the equation $C = \sum_{i=1}^{s} p_i^2$ as an indicator for the relative dominance concentration and Shannon-Wiener index following the equation $\widehat{H} = -\sum_{i=1}^{s} p_i \log p_i$ as an indicator for the relative evenness where s is the total number of collected species (Pielou, 1975; Magurran, 1988). Pearson's simple linear correlation coefficient (r) was used to examine the relationships among the ordination axes and the soil and community variables. The variation in the species diversity, stand traits and soil variables in relation to plant community were assessed via one-way analysis of variance using SPSS 18.0.

Results

Floristic structure

A total of 78 plant species belonging to 69 genera distributed in 27 families were collected from the studies stands (Appendix 1). As shown in *Fig. 2*, the most represented families in the study area were Compositae represented by 14 species (18%), Poaceae represented by 10 species (13%), Cruciferae represented by 7 species (9%) and Leguminosae represented by 6 species (8%). The most prominent life forms (*Fig. 3a*) were annual herbs (45%), perennial herbs (30%) and shrubs (22%). On the other hand, the dominant chorotypes (*Fig. 3b*) were the Saharo-Arabian region (49%) followed by the Saharo-Arabian-Irano-Turanian (13%) and the Sahelian-Somali-Masai (8%). Some rare species were identified e.g. *Abutilon pannosum*, *Aristida adscensionis*, and *Trigonella hamosa*. Two endangered species were identified in the study area, namely *Astragalus* sp. and *Senecio* sp.

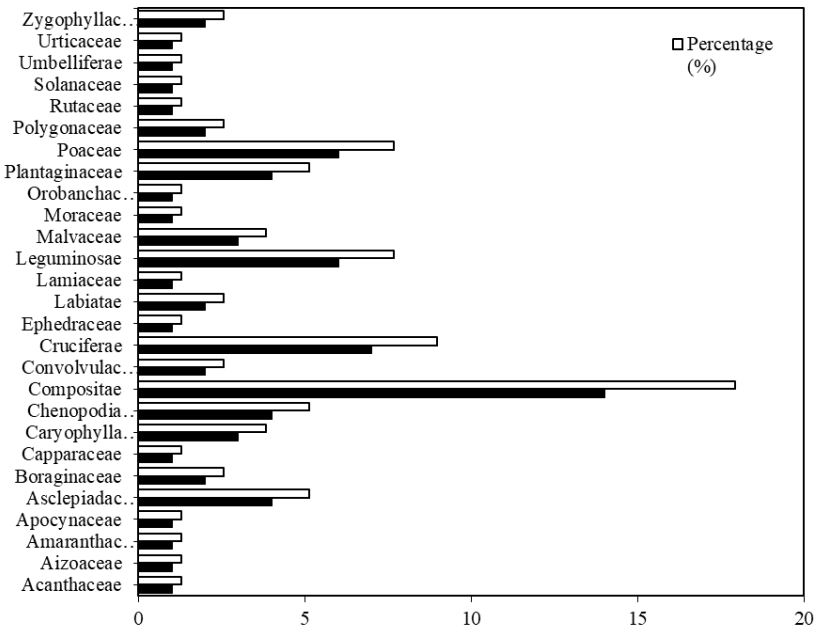

Figure 2. *Families of the recorded species in Wadi Othylan*

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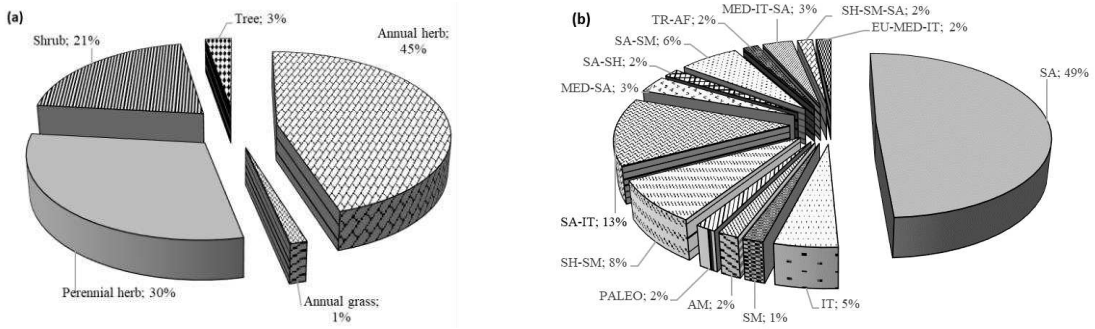

Figure 3. *Life forms (a) and chorotypes (b) of the recorded species in Wadi Othylan (SA: Saharo-Arabian; SA-IT: Saharo-Arabian-Irano-Turanian; SH-SM: Sahelian-Somali-Masai; SA-SM: Saharo-Arabian-Somali-Masai; Med-SA: Mediterranean- Saharo-Arabian; Med-IT-SA: Mediterranean-Irano-Turanian-Saharo-Arabian; AM: American; SM: Somali-Masai; PALEO: Paleotropics; SA-SH: Saharo-Arabian- Sahelian; TR-AF: Tropical African; SH-SM-SA: Sahelian-Somali-Masai-Saharo-Arabian; EU-Med-IT: Euro-Siberian-Mediterranean-Irano-Turanian)*

Multivariate analysis

The dataset formed in this study consisted of 20 studied stands and 78 collected species. TWINSPAN analysis of this dataset generated a dendrogram divided it into four vegetation groups (plant communities) at the second level. Characterization and nomination of these groups were assigned based on the dominant and subdominant species in each group as follows: vegetation group (VG) I: *Fagonia bruguieri-Haloxylon salicornicum*, VG II: *Acacia ehrenbergiana-Echinops spinosissima*, VG III: *Acacia ehrenbergiana-Acacia gerardii* and VG IV: *Acacia ehrenbergiana-Calotropis procera* (*Table 1*). Further detrended correspondence (DCA) analysis confirmed the separation of these plant communities generated by TWINSPAN and reveals a great relationship between topographic aspects and environmental gradients in Wadi Othylan (*Fig. 4a,b*; *Appendix 1*).

Table 1. *Different vegetation groups nominated in the study site*

Group	Stand No		Habitat (%)	Dominant species	P%	C%
	Stand	Total				
VG I	11,12,14, 15,16,17,19	7	A=28.5* B=43 C=28.5	*Haloxylon salicornicum*	42.86	1.57
				Fagonia bruguieri	71.43	2.86
				Rumex vesicarius	71.43	1.71
VG II	1,2,3,4	4	A=25 B=50 C=25	*Acacia ehrenbergiana*	100	6.5
				Echinops spinosissima	100	7.5
				Farsetia longisiliqua	75	5
VG III	5,8,9,10, 13,18,20	7	A=0 B=14 C=86	*Acacia ehrenbergiana*	57.14	5.71
				Acaia gerrardii	42.86	5.43
				Plantago ovate	100	12.57
VG VI	6,7	2	A=0 B=0 C=100	*Acacia ehrenbergiana*	50	3
				Calotropis procera	50	4.5
				Schismus barbatus	100	4

*A: Terrace, B: Slope, C: Wadi bed, P: presence (%), C: cover (%)

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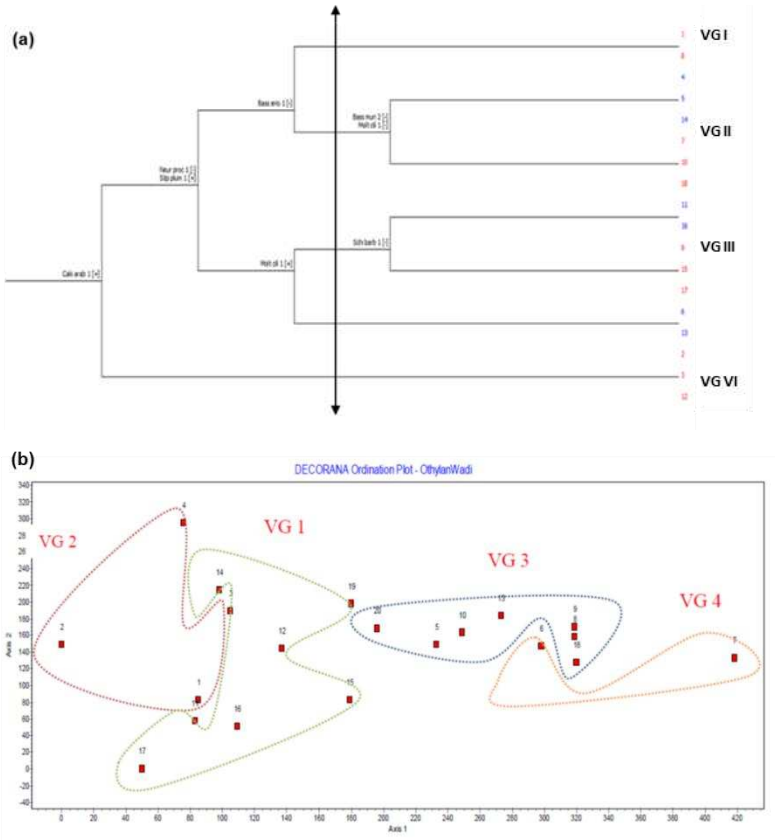

Figure 4. *Relationships among the four plant communities as generated by TWINSPAN (a) and DECORANA (b) software. VG I: Fagonia bruguieri-Haloxylon salicornicum, VG II: Acacia ehrenbergiana-Echinops spinosissima, VG III: Acacia ehrenbergiana-Acacia gerardii and VG IV: Acacia ehrenbergiana-Calotropis procera*

Soil characteristics, plant diversity and plant community relationships

Table 2 shows that number of species was negatively correlated with pH of soil solution (r = -0.571, $P \leq 0.01$). Species richness had a negative correlation with pH of the soil solution (r = -0.637, $P \leq 0.01$); however, it was positively correlated with soil Na content (r = 0.461, $P \leq 0.05$). Similarly, Shannon index had a strong negative correlation relationship with the soil solution pH (r = -0.650, $P \leq 0.01$). On the other hand, Simpson index showed a positive correlation with pH of soil solution (r = 0.481, $P \leq 0.05$).

Table 3 shows that the *Acacia ehrenbergiana-Echinops spinosissima* vegetation group (VG II) had the highest number of species (21.25), species cover (73.00 m-100 m^{-1}) and species richness (4.72). *Fagonia bruguieri-Haloxylon salicornicum* vegetation group (VG I) showed the highest species evenness (0.93) with the lowest species richness (2.73). *Acacia ehrenbergiana-Acacia gerardii* vegetation group (VG III) showed the highest Simpson index (0.14) and the lowest species evenness (0.85). The habitats of *Acacia ehrenbergiana-Calotropis procera* community (VG VI) occupied the soil characterized by the highest sand content (77.00%). On the other hand, the habitats of *Fagonia bruguieri-Haloxylon salicornicum* community (VG I) characterized by the highest silt (24.86%) and organic matter (10.61%) contents. *Acacia ehrenbergiana-Echinops spinosissima* community (VG II) occupied the habitats characterized by the highest soil clay content (7.50%).

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Table 2. *Pearson correlation coefficients between species diversity and soil characteristics*

Variable	No. of species	Species cover (m-100 m^{-1})	Species richness	Species evenness	Shannon index	Simpson index
Bulk soil (%)						
Sand	0.22	0.276	0.158	-0.374	0.103	0.26
Silt	-0.26	-0.326	-0.184	0.392	-0.138	-0.26
Clay	0.02	0.023	0.006	0.207	0.09	-0.204
OM	-0.244	-0.28	-0.204	0.391	-0.113	-0.22
Soil						
pH	-0.571**	-0.392	-0.637**	-0.148	-0.650**	0.481*
EC (mS/cm)	-0.114	-0.166	-0.083	0.214	-0.03	-0.124
Mineral content (ppm)						
N	0.011	-0.153	0.116	0.336	0.144	-0.35
P	-0.364	-0.308	-0.318	0.303	-0.258	-0.12
K	-0.183	-0.193	-0.164	0.197	-0.105	-0.077
Mg	-0.037	-0.145	0.017	0.227	0.057	-0.184
Ca	-0.043	-0.115	-0.01	0.239	0.039	-0.168
Fe	-0.065	-0.095	-0.05	0.198	0.006	-0.126
Na	0.375	0.133	0.461*	0.142	0.42	-0.315

*: P≤0.05, **: P≤0.01

Table 3. *Soil variables and diversity indices of different vegetation groups in the study site. Values are shown as mean ± standard deviation*

VG*	VG 1	VG 2	VG 3	VG 4	Total	F-Value
Diversity indices						
Number	9.14±1.21	21.25±4.11	14.14±5.96	17.00±1.00	14.24±5.81	8.286***
Cover	20.29±5.15	73.00±15.60	50.29±30.87	36.33±1.53	42.62±26.76	6.61**
Richness	2.73±0.35	4.72±0.78	3.43±0.97	4.45±0.23	3.59±1.02	8.67***
Evenness	0.93±0.06	0.92±0.03	0.85±0.08	0.91±0.00	0.90±0.07	2.76
Shannon	0.89±0.09	1.22±0.10	0.94±0.17	1.11±0.02	1.00±0.17	7.72**
Simpson	0.11±0.06	0.06±0.02	0.14±0.05	0.08±0.00	0.10±0.05	2.72
Soil bulk (%)						
Sand	68.00±11.89	74.00±8.16	71.71±9.62	77.00±13.00	71.67±10.35	0.59
Silt	24.86±10.38	18.50±5.74	20.86±8.40	17.00±11.00	21.19±8.91	0.71
Clay	7.14±1.95	7.50±2.52	7.43±1.51	6.00±2.00	7.14±1.85	0.45
OM	10.61±8.34	6.35±3.41	3.89±2.17	2.43±0.18	6.39±5.90	2.54
Soil						
pH	8.10±0.16	7.87±0.05	8.04±0.13	7.74±0.34	7.98±0.20	3.97*
EC	2.35±5.66	0.83±1.41	0.15±0.04	1.21±1.10	1.16±3.30	0.50

* VG: vegetation group, VG I: *Fagonia bruguieri-Haloxylon salicornicum*, VG II: *Acacia ehrenbergiana-Echinops spinosissima*, VG III: *Acacia ehrenbergiana*-Acacia *gerardii* and VG IV: *Acacia ehrenbergiana-Calotropis procera*. *: *P*≤0.05, **: *P*≤0.01, ***: *P*≤0.001

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Discussion

Topography and land forms significantly influence the growth, existence and distribution of different plant life forms in arid and semi-arid regions (Kassas and Girgis, 1964; Zohary, 1973; Shaltout et al., 2010) such the protected area of wadi Othylan; the site under study. The central region dominated mainly be the Najd plateau is characterized by the existence of different wadi (valley) habitats (Mandaville, 1990) including wadi Othylan. Wadi Othylan protected area is one of the richest wadis in vegetation and floristic diversity. The results obtained in this study indicated that the dominant life form in the study site are annual herbs (45%) and perennial herbs (30%) followed by shrubs (20.5%) and trees (2.74%). These results are comparable to the results found by El-Sheikh et al. (2019) as the annual herbs and perennials or subshrubs represented ~96% of plants identified in Harrat Al-Harrah protectorate, Northern Saudi Arabia. In another study, El-Sheikh et al. (2013) found that annual herbs and perennials dominated the identified species in Thumamah Nature Park. Like wise, perennials and annuals dominated the 128 different species identified in wadi Huraimla, Central Saudi Arabia (Alatar et al., 2015). The dominance of annual herbs could be attributed to the abundance of water during rainy seasons supporting the growth of such plants (Schulz and Whitney, 1986; Shaltout and Mady, 1996; Hosni and Hegazy, 1996; Shaltout et al., 2010). However, this water is not adequate to support the growth of perennial herbs; therefore, the existence of such plants may be due to their adaptation to the harsh conditions exist in the study site (Alatar et al., 2012).

The rarity or complete absence of synanthropic species (e.g. *Prosopis juliflora*, *Salsola imbricate*, *Tamarix nilotica*, *Bassia eriophora* and *Cynodon dactylon*) in wadi Othylan indicates low severity of human impact. Indeed, this is clearly true as wadi Othylan is considered as a protected area since more than 30 years ago. Furthermore, the presence of some rare and endangered plants in the study area is considered as another indicator for lower human impact (Taia and El-Ghanem, 2001).

The composition of vegetation life forms and chorotype in the study area showed a typical pattern of the desert flora dominated by xerophytes and chamaephytes. The same pattern of vegetation was observed in different desert habitats among different parts of Saudi Arabia (Collenette, 1985, 1998, 1999; El-Demerdash et al., 1994; Chaudhary, 2001; Al-Turki and Al-Olayan, 2003; Fahmy and Hassan, 2005; El-Ghanim et al., 2010). In either wadi or protected areas in Saudi Arabia, the previous studies showed that xerophytes and chamaephytes are the dominant life forms and chorotypes (El-Sheikh et al., 2013; Alatar et al., 2015; El-Sheikh et al., 2019). In general, there is a great correlation between the plants' life forms and topography of their habitats (Kassas and Girgis, 1964; Zohary, 1973; Shaltout et al., 2010). In this study, the dominant plant chorotype was the Saharo Arabian (49%). The species of Saharo Arabian chorotype are distributed only along the central strip of Saudi Arabia and are more abundant in habitats providing protection and/or habitats characterized by more favorable micro-climate conditions (Zohary, 1973; Hegazy et al., 1998; El-Ghanim et al., 2010; Ghazanfar and Fisher, 2013). The central region of Saudi Arabia covers a wide range of bioclimatic zones and characterized by the existence of different rocky habitats that supports several other chorotypes which were found in the study area beside the Saharo Arabian species e.g. Saharo Arabian Irano Turanian (13%) and Sahelian Somali Masai (8%). Furthermore, the Central region of Saudi Arabia covers the transition zone between the Somalia Masai zone and the Afromontane archipelago-like center (Zohary, 1973; Mandaville, 1990; White and Léonard, 1990). Some parts of the central region cover the Mediterranean and

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Irano Turanian zones (Hegazy et al., 1998; Alfarhan, 1999; Ghazanfar and Osborne, 2010). Species of both zones were found in this study.

The results of correlation analysis in this study showed a positive relationship between species diversity (evenness) and soil organic matter content. In contrast, species diversity was negatively correlated with soil solution pH and EC. Other studies reported the same pattern of correlation in desert habitats of Saudi Arabia (El-Demerdash et al., 1994; Abbadi and El-Sheikh, 2002; El-Sheikh et al., 2010, 2013, 2018).

Conclusion

The current study examined the floristic structure and vegetation diversity in the protected area of Wadi Othaylan, Saudi Arabia. The results howed that this area characterized by high plant diversity and various structure with plants classified into four different vegetation groups. Some rare and endangered plants were found in the studied area indicating the role of protection in keeping vegetation diversity. The limitation of this study were mostly relating to the high danger slopes with high number of snakes and scorpions that hinders the study of some locations, in addition to that based on the sampling areas, adding a complete list of the present species is impossible. Further studies to examine the vegetation diversity of this area and similar protected areas in Saudi Arabia are recommended.

Acknowledgements. This scientific paper contains studies and research results supported by King Abdulaziz City for Science and Technology grant no. (1-17-01-001-0034).

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APPENDIX

Appendix 1. *List of collected plants and their life forms, chorotype, and coverage*

Species	Life form	Chorotype*	VG I		VG II		VG III		VG IV	
			C%	P%	C%	P%	C%	P%	C%	P%
Abutilon pannosum [1]	Shrub		0.71	28.57	0.00	0.00	0.00	0.00	0.00	0.00
Acacia ehrenbergiana	Shrub	SH-SM	0.00	0.00	6.50	100.00	5.71	57.14	3.00	50.00
Acaia gerrardii	Tree	SH-SM	0.00	0.00	0.00	0.00	5.43	42.86	0.00	0.00
Achillea fragrantissima [1]	Perennial herb		0.00	0.00	2.00	75.00	0.00	0.00	0.00	0.00
Aerva javanica [1]	Perennial herb	Subshrub	0.00	0.00	1.25	50.00	0.00	0.00	0.00	0.00
Aizoon canariense [1]	Annual herb	SA	0.00	0.00	0.00	0.00	0.14	14.29	0.00	0.00
Anisosciadium lanatum [1]	Annual herb	SA	0.00	0.00	0.00	0.00	0.00	0.00	0.50	50.00
Anvillea garcinia [1]	Shrub	SA	0.00	0.00	2.00	50.00	0.00	0.00	0.00	0.00
Aristida adscensionis [1]	Perennial grass	MED-IT-SA	0.00	0.00	1.75	50.00	0.00	0.00	0.00	0.00
Astragalus sp. [2]	Annual herb	IT	0.00	0.00	0.00	0.00	0.14	14.29	0.00	0.00
Bassia eriophora [1]	Annual herb	SA-IT	0.00	0.00	0.00	0.00	0.57	42.86	0.00	0.00
Bassia muricata	Annual herb	SA-IT	0.00	0.00	0.00	0.00	0.29	14.29	1.00	50.00
Blepharis ciliaris	Herb	SA-IT	0.00	0.00	0.50	50.00	0.00	0.00	0.00	0.00
Brachypodium distachyon	Annual herb		0.00	0.00	0.50	25.00	0.00	0.00	1.00	50.00
Calendula arvensis	Annual herb		1.00	42.86	0.00	0.00	0.14	14.29	0.00	0.00
Calotropis procera	Shrub	SM	0.00	0.00	0.00	0.00	0.00	0.00	4.50	50.00
Capparis cartilaginea	Shrub		0.00	0.00	1.00	25.00	0.00	0.00	0.00	0.00
Cenchrus ciliaris	Perennial grass	SA	0.14	14.29	1.25	75.00	2.14	14.29	0.00	0.00
Chenopodium murale	Annual herb	PALEO	0.00	0.00	0.00	0.00	1.00	28.57	0.00	0.00
Cistanche tubulosa	Annual herb	SA-IT	0.00	0.00	0.00	0.00	0.00	0.00	0.50	50.00
Convolvulus pilosellifolius	Perennial herb	IT	0.00	0.00	1.00	50.00	0.00	0.00	0.00	0.00
Cuscuta planiflora	Annual herb	AM	0.29	14.29	1.25	75.00	1.14	42.86	0.00	0.00
Diplotaxis harra	Perennial herb	SH-SM	1.43	85.71	2.00	100.00	0.57	42.86	0.00	0.00
Echinops spinosissima	Shrub	SA	0.29	14.29	7.50	100.00	0.57	14.29	0.00	0.00
Emex spinosa	Annual herb	MED-SA	0.14	14.29	0.00	0.00	0.14	14.29	0.50	50.00
Ephedra foliata	Shrub	SH-SM	0.00	0.00	1.00	25.00	0.29	14.29	0.00	0.00
Eremobium lineare	Annual herb	SA	0.00	0.00	0.00	0.00	0.00	0.00	0.50	50.00
Erodium laciniatum	Annual herb	MED-IT-SA	0.00	0.00	0.00	0.00	0.00	0.00	0.50	50.00
Fagonia bruguieri	Perennial shrub	SA	2.86	71.43	3.00	50.00	2.57	85.71	0.50	50.00
Farsetia longisiliqua	Perennial shrub	SA	0.57	28.57	5.00	75.00	1.86	85.71	0.50	50.00
Ficus palmata	Tree		0.00	0.00	0.25	25.00	0.00	0.00	0.00	0.00
Forsskaolea tenacissima	Perennial herb		1.00	42.86	1.50	50.00	0.14	14.29	0.00	0.00
Gymnocarpos decander	Perennial herb	SA	0.00	0.00	0.25	25.00	0.00	0.00	0.00	0.00
Haloxylon salicornicum	Shrub	SA	1.57	42.86	2.50	75.00	0.57	14.29	1.50	100.00
Haplophyllum tuberculatum	Perennial herb	IT	0.00	0.00	0.00	0.00	0.57	28.57	0.00	0.00
Heliotropium bacciferum	Perennial herb	SA-SH	0.00	0.00	0.00	0.00	0.86	57.14	2.00	50.00

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81198130

Species	Life form	Chorotype*	VG I		VG II		VG III		VG IV	
			C%	P%	C%	P%	C%	P%	C%	P%
Hibiscus micranthus [1]	Shrub		0.00	0.00	2.75	75.00	0.00	0.00	0.00	0.00
Hyparrhenia hirta 1	Perennial grass	SA	0.00	0.00	0.50	25.00	0.00	0.00	0.00	0.00
Launaea angustifolia	Herb	SA	0.71	42.86	1.00	50.00	0.57	28.57	0.00	0.00
Launaea mucronata	Annual herb	SA	0.00	0.00	0.00	0.00	0.57	42.86	0.00	0.00
Leptadenia pyrotechnica	Shrub	SA-SM	0.00	0.00	0.75	25.00	0.00	0.00	0.00	0.00
Lycium shawii	Shrub	SA	0.86	42.86	3.00	75.00	0.00	0.00	0.00	0.00
Malva parviflora	Annual herb	MED-SA	1.43	57.14	1.25	50.00	1.00	28.57	2.00	100.00
Medicago laciniata	Annual herb	SA	0.14	14.29	0.00	0.00	0.00	0.00	0.00	0.00
Morettia parviflora	Perennial herb	SA	0.00	0.00	0.00	0.00	0.29	14.29	0.00	0.00
Notoceras bicorne	Annual herb		0.00	0.00	0.00	0.00	0.29	14.29	1.00	100.00
Panicum turgidum	Perennial grass	SH-SM-SA	0.00	0.00	0.50	25.00	0.00	0.00	0.50	50.00
Pennisetum divisum	Perennial grass	SA	0.00	0.00	2.00	25.00	0.00	0.00	0.00	0.00
Pergularia tomentosa	Perennial herb	SA	0.00	0.00	0.00	0.00	0.14	14.29	0.00	0.00
Periploca aphylla	Shrub	TR-AF	0.00	0.00	3.00	50.00	0.00	0.00	0.00	0.00
Picris babylonica	Annual herb	SA	0.00	0.00	0.00	0.00	1.14	57.14	0.00	0.00
Plantago amplexicaulis	Annual herb	SA	0.71	28.57	0.00	0.00	0.00	0.00	0.00	0.00
Plantago boissieri	Annual herb	SA	0.00	0.00	0.00	0.00	0.57	14.29	0.50	50.00
Plantago ciliata	Annual herb	SA	0.00	0.00	0.00	0.00	0.57	28.57	0.50	50.00
Plantago ovata	Anuual herb	SA-IT	0.00	0.00	0.25	25.00	12.57	100.00	3.00	50.00
Pulicaria glutinosa	Shrub	SA-IT	0.00	0.00	1.50	50.00	0.29	14.29	0.00	0.00
Pulicaria undulata	Shrub	SA-SM	0.00	0.00	0.50	25.00	0.00	0.00	1.00	50.00
Reichardia tingitana	Annual herb		0.00	0.00	0.25	25.00	0.00	0.00	0.00	0.00
Rhanterium epapposum	Perennial herb	SA	0.00	0.00	1.50	25.00	0.00	0.00	0.00	0.00
Rhazya stricta	Perennial herb	SA	0.00	0.00	0.00	0.00	0.71	42.86	1.50	50.00
Rumex vesicarius	Anuual herb	SA	1.71	71.43	4.50	100.00	0.86	57.14	0.00	0.00
Salvia aegyptiaca	Perennial herb		0.29	14.29	0.00	0.00	0.14	14.29	0.00	0.00
Salvia deserti	Perennial herb	SA	0.00	0.00	1.25	50.00	0.00	0.00	0.00	0.00
Savignya parviflora	Annual herb	SA	0.57	42.86	0.00	0.00	0.71	42.86	1.50	100.00
Schismus barbatus	Annual grass	SA-IT	0.00	0.00	0.00	0.00	0.00	0.00	4.00	100.00
Sclerocephalus arabicus	Perennial herb	SA	0.57	14.29	0.00	0.00	0.14	14.29	0.00	0.00
Senecio flavus	Annual herb		1.14	71.43	2.00	50.00	0.29	28.57	0.50	50.00
Senecio sp. [2]	Annual herb		0.00	0.00	1.25	25.00	0.00	0.00	0.00	0.00
Senna italica	Shrub	SH-SM	0.14	14.29	0.00	0.00	0.14	14.29	0.00	0.00
Sisymbrium irio	Anuual herb		0.57	42.86	1.25	75.00	1.71	71.43	1.00	50.00
Spergula fallax	Anuual herb		0.00	0.00	0.00	0.00	0.14	14.29	2.00	100.00
Stipa capensis	Anuual herb	SA-SM	0.43	28.57	0.25	25.00	0.43	14.29	0.50	50.00
Tetrapogon villosus	Perennial herb	SA-IT	0.00	0.00	0.50	25.00	0.29	14.29	0.00	0.00
Teucrium polium	Perennial herb	SA-IT	0.00	0.00	0.00	0.00	0.43	14.29	0.00	0.00
Tribulus terrestris	Annual herb	EU-MED-IT	0.14	14.29	0.00	0.00	0.14	14.29	0.00	0.00
Trichodesma africanum	Perennial herb	SA	0.86	14.29	1.25	75.00	0.14	14.29	0.00	0.00
Trigonella hamosa [1]	Anuual herb	SA	0.00	0.00	0.00	0.00	0.00	0.00	0.50	50.00
Tripleurospermum auriculatum	Annual herb	SA-SM	0.00	0.00	0.00	0.00	1.14	28.57	0.00	0.00

[1] rare species, [2] endangered species, *SA: Saharo-Arabian; SA-IT: Saharo-Arabian-Irano-Turanian; SH-SM: Sahelian-Somali-Masai; SA-SM: Saharo-Arabian-Somali-Masai; Med-SA: Mediterranean- Saharo-Arabian; Med-IT-SA: Mediterranean-Irano-Turanian-Saharo-Arabian; AM: American; SM: Somali-Masai; PALEO: Paleotropics; SA-SH: Saharo-Arabian- Sahelian; TR-AF: Tropical African; SH-SM-SA: Sahelian-Somali-Masai-Saharo-Arabian; EU-Med-IT: Euro-Siberian-Mediterranean-Irano-Turanian

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81198130

BACTERIAL COMMUNITY STRUCTURE AND DIVERSITY IN THE SOIL OF THREE DIFFERENT LAND USE TYPES IN A COASTAL WETLAND

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(Received 8th Jul 2020; accepted 7th Oct 2020)

Abstract. The effect of land use on soil bacterial community structure and diversity was studied in three typical land use types in the Yancheng National Nature Reserve, China. These represented long-term arable land use, pristine wetland, and long-term forest land. Physico-chemical parameters of the soils were determined, and high-throughput sequencing of V3 fragments of the bacterial 16S rRNA amplicons was applied to study the community structure of soil bacteria and explore relationships with soil properties. The results showed that in all three soil types the dominant bacterial phyla were Acidobacteria, Proteobacteria, and Actinobacteria, whose abundance differed with land use patterns. Combined with Chao index and ACE index, we found that the bacterial richness of arable land was significantly higher than that of forest or wetland soil. The Simpson and Shannon indices were similar in the three soils, indicating that there was no significant difference in bacterial community diversity. Acidobacteria were most abundant in wetland soil, forest soil was rich in Actinobacteria, and arable soil contained Proteobacteria and Firmicute at high abundance. Correlation analysis identified that soil acidity and moisture content were important driving factors affecting the microbial community composition and structure.
Keywords: *land reclamation, high-throughput sequencing, bacterial community, soil pH, diversity, wetlands*

Introduction

Different land use patterns cause changes in the terrestrial ecology and in the local biogeochemical cycles, leading to changes in soil properties and land productivity (Sun et al., 2015). Changes in land use patterns can fundamentally change the soil quality and cycling of nutrients (in particular C, N, and P) that affect the soil microbial communities, for arable land, forests, and wetlands (Guo et al., 2016; Mganga et al., 2016). Soil microbial communities participate in specific biochemical reactions that play an important role in the regulation and cycling of nutrients; as such, they are part of the local ecosystem and have a large impact on soil quality and plant biomass (Hernandez et al., 2016). Jangid et al. (2011) found that transforming a wetland into an arable land resulted in significant changes in the bacterial abundance and species, and that such a change of land use was the main factor determining the microbial community composition. Krashevska et al. (2015) compared the short-term impact of changed land use, from a natural rainforest to a commercial rubber plantation, and reported that the bacterial abundance in the soil significantly decreased as a result. Mendes et al. (2015) revealed that the content of bacteria belonging to the genera of *Acidophilus* and *Chlamydia* was higher in forest soil, while the content of Actinomycetes members was higher in the logging area of a forest land and the content of nitrifying, thermophilic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

bacteria were higher in arable land. Wood et al. (2017) showed that when oil palms were planted where tropical forest had been removed, the bacterial diversity increased; regeneration of the forest eventually resulted in only slight differences in bacterial diversity and community composition compared to the original forest land. We envisage that a comprehensive assessment of land use patterns, soil characteristics, and the interaction between the soil bacterial community structure and its diversity will improve the understanding and management of terrestrial ecosystems.

Jiangsu Province is the dominant commodity grain producing area in China, due to its rich soil that has allowed for a long history of farming. The local soil physicochemical properties and fertility are highly important to establish sustainable agricultural practices (Wu et al., 2017). Secured grain production of local regions directly affects the grain security of the whole province and even of the whole country. Since the 1980s, this region has secured arable land by means of land reclamation to solve the food problems associated with an increasing population. However, over time the crop yield is not stable and when the local production is no longer economically viable, the land is abandoned and new land is reclaimed. This reclamation of land includes primitive wetlands, which are changed into arable land, at the cost of the natural wetland vegetation. Subsequent abandonment of the used arable land exposes the soil, causing serious erosion, and it is difficult to restore the original plant community (Li et al., 2008). In the beginning of the 20st century, the Yancheng country, which lies within the Jiangsu Province, realized the consequences of this practice were serious and caused severe environmental problems, so they transformed a number of wetlands and abandoned lands into forest lands, in an attempt to protect the local fragile ecosystem and promote sustainable and stable economic development (Frederic et al., 2014; Xu et al., 2014).

At present, there are few studies that have investigated the effects of land use changes on the soil microorganisms in the Yancheng county, in particular following decades of use. Here, we tried to fill this knowledge gap. We hypothesized that the conversion of wetland to arable land, or the reclaimed and abandoned arable land to forests, was not only accompanied with diverse and different vegetation, but also with long-term changes in the soil microbial communities, and these in turn would affect the soil physiochemical or biological factors. We expected that the structure and composition of mature microbial communities would depend upon the specific land use and would be associated with the aboveground vegetation. To investigate this, three different land use patterns in the Yancheng county were compared, namely primitive wetland, wetland that had been conversed to arable land decades ago and has since been used mainly for wheat production, and reclaimed land planted with a 30-year old pine forest. The physicochemical properties of the soil and the structure of the soil bacterial community were compared for these plots to characterize the changes induced by long-term land use. This information can provide a scientific basis for the maintenance and cultivation of soil fertility and the protection of soil microbial diversity, as well as for the ecological reconstruction and rational utilization of land resources in this area.

Materials and methods

Description of the research site

The research site was located in the Yancheng National Natural Reserve of Jiangsu Province, China (32°48′47″-34°29′28″N, 119°53′45″-121°18′12″E). The area is located

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

in the transition area between the warm temperate zone and the northern subtropical zone. The local climate is mainly affected by the ocean and continental climate (Wang et al., 2020). The average annual temperature is 13.7–14.6 °C, the average annual rainfall is 1 000 mm, with rainfall concentrated in summer combined with dry winters, and a seasonality with mild temperatures in winter and summer (Wang et al., 2020). Three plots with different land-use patterns were selected for this study, representing primitive wetland, arable land that was reclaimed from wetland some 50 years ago, and forest land. The primitive wetland has a total area of approximately 1000 hm² with the most common vegetation existing of *Echinopsis*, *Myriophyllum* sp. (water milfoil), *Psyllium chinense*, *Gymnema sylvestris* (a climbing perennial), *Carex sibiricum* (a grass species), *Artemisia halodendron* and *Artemisia serrata*. The arable land of approximately 500 hm² in total is mostly used for wheat production and the soil is yearly ploughed mechanically. In spring, 30 kg/mu of compound fertilizer ($N+P_2O_5+K_2O \geqq 45\%$) is typically applied and in the middle of June, 15 kg/mu of top dressing is applied. The forest land has an area of approximately 500 hm² and was planted with a monoculture of *Pinus sylvestris* 30 years ago. Prior to that it had been in use as arable land for an unknown period of time.

Sample collection

In June 2019, three standard 50 m × 50 m plots were selected from the three different lands and soil samples of 0-20 cm depth were obtained by the five-point mixed sampling method. After removing any stones and plant roots, the five samples from each plot were evenly mixed and then passed through a 2 mm sieve. Aliquots of the samples were stored at -80 °C in 15-mL centrifuge tubes in liquid nitrogen for DNA extraction and microbiological analysis. The remaining soil was divided into two parts: one part was stored at 4 °C and the other was dried naturally to determine the soil physicochemical properties.

Determination of physicochemical properties of soil samples

The soil moisture content (MC) was determined for fresh samples. These were weighed in an aluminum box using a scale with an accuracy of 0.01 g, a cover was placed obliquely, and the soil was dried for 8 h at a controlled temperature of 10 °C±2 °C. When the weight was constant the dry weight was determined and the soil MC was calculated. The pH was measured using a pH meter after adding water at a soil-water ratio of 2.5:1 (g:g). The organic matter in the soil was proxied by total organic carbon (TOC) content which was determined using a Vario-TOC instrument (Elementar, Germany). For determination of total nitrogen (TN) 2 g of accelerating agent mixed with zinc sulfate and copper sulfate and 5 mL of concentrated H_2SO_4 were added to 0.25 g soil and this mixture was passed through a 0.149 mm sieve. After digestion, the sample was filtered at constant volume and then measured with a continuous flow analyzer. To determine nitrogen present in the form of nitrate and ammonium, 10 mL of 1 mol/L KCl was added to 6 g of air-dried soil. After shaking for 1 h, the mixture was filtered and the sample was measured using a continuous flow analyzer. Total P (TP) was determined by the Molybdenum colorimetry method with sulfuric acid and perchlorate acid extraction. Soil-available P was determined by prior extraction with 0.5 mol/L $NaHCO_3$. Total K and available K were determined by atomic absorption spectrometry.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

DNA extraction and high throughput sequencing

Genomic DNA was extracted from 0.5 g of fresh soil samples (n = 18) using the Power Soil DNA extraction kit (MoBio Inc., USA) according to the manufacturer's instructions. The extracted DNA was checked by 1% agarose gel electrophoresis. The internal V3 region of the bacterial 16S rRNA gene was amplified by PCR using the universal BAC primer set 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Huse et al., 2008) by a standard protocol using a GeneAmp 9700 amplifier. The amplicons were checked by 2% agarose gel electrophoresis and then recovered from the gel using the AxyPrep DNA gel extraction kit (Axygen Biosciences, USA). The purified amplicons were quantified by QuantiFluor^{TM} (Promega) and their concentrations were adjusted as needed for sequencing. Sequencing was performed by Majorbio Technologies Co., Ltd. (Shanghai, China) on an Illumina MiSeq platform.

Sequence analysis of the 16S rRNA gene

The original fastq sequence file was quality filtered by Trimmatotic (www.kbase.us) and then merged by FLASH (Fast Length Adjustment of Short reads). Sequences were discarded when their length was shorter than 50 bp and the average quality score was below 20, or when more than two nucleotide mismatches were present or base deletions were found; those sequences that passed were merged when they overlapped for more than 10 bp.

Operational taxonomic units (OTUs) were defined using UPARSE (version 7.1, http://drive5.com/uparse/) at a 97% similarity cutoff and chimeric sequences were identified with UCHIME and removed. The RDP classification algorithm (http://rdp.cme.msu.edu/) was used to classify the obtained 16S rRNA fragment sequences based on the SILVA (SSU123) database.

Data analysis

Alpha diversity analysis was performed using parameters for community richness (Chao and ACE indices) and diversity (Simpson and Shannon indices), as calculated by the Mothur software. The R software package was used to visually analyze the beta diversity and calculate the OTU level based on redundancy analysis (RDA). Student's *t*-test was used to analyze the differences of detected bacterial phyla in the different land use types.

Beta analysis was compared by calculation of Bray-Curtis distances, to quantify the compositional dissimilarity between two different sites. The results were summarized in a Principle coordinates analysis (PCoA). One-way analysis of variance (ANOVA) was used to analyze the effect of different land use types on soil properties and microbial diversity. Duncan's test was used to identify significant differences for each index among different land use patterns ($\alpha = 0.05$). Distance-based redundancy analysis (db-RDA) was performed by R software. A result of $P<0.05$ between groups was considered to be statistically significant. The results of all samples are presented as the mean ± standard deviation. The Student's *t*-test, One-way ANOVA, and Duncan's test were performed by SPSS 20.0.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

Results

Changes of soil physicochemical properties under different land use patterns

The physicochemical properties of the soil collected from plots with three different land use patterns are shown in *Table 1*. The pH was slightly acidic for all three soil samples, although soil from forest land was significantly (P<0.05) less acidic than the other two land types. As expected, the soil moisture content differed significantly between the three different land use types, with a three-times higher water content in wetland soil compared to arable land, and forest soil containing twice as much water compared to arable soil. The organic carbon content also differed significantly (P<0.05), with arable land producing the lowest values and wetland soil containing 3.6 times more organic carbon; forest land contained 2.8 times more organic carbon than arable land. The total nitrogen content was highest for wetland soil, followed by arable soil while forest soil had the lowest total nitrogen content. A different trend was observed for total phosphorus, which was lowest for arable soil and highest for soil sampled from wetland. There were no significant differences in the contents of available P between the three soil types (P>0.05).

Table 1. *Physio-chemical properties of the three different land use soil types*

Type	pH	Moisture content (%)	Soil Organic Carbon (g/kg)	Total Nitrogen (g/kg)	Total Phosphorus (g/kg)	Available phosphorus (mg/kg)
Wetland	5.23±0.12^{b}	63.23±0.27^{a}	43.23±0.51^{a}	4.29±2.41^{a}	2.42±0.59^{a}	28.41±1.74^{a}
Forest land	6.76±0.10^{a}	36.43±0.52^{b}	33.51±0.51^{b}	2.03±2.12c	1.23±0.31^{b}	20.21±2.15^{a}
Arable land	5.62±0.11^{b}	12.42±0.82^{c}	12.42±0.08^{c}	3.14±1.05^{b}	0.71±0.02^{b}	23.33±1.10^{a}

Statistical significance is indicated by superscripts per column

Changes in soil microbial alpha and beta diversity under different land use patterns

Based on sequences obtained from amplified 16S rRNA V3 fragments, the alpha diversity indices of the captured soil bacterial communities were calculated and these significantly differed between forest land and arable land (*Table 2*). The highest number of OTUs was obtained from forest land, while arable land produced lower numbers. There was no significant difference in Shannon and Simpson indices for the three soil types (P>0.05). However, the Ace index was significantly (P<0.05) lower and the Chao1 index significantly higher for forest soil than for the other two soil types. In combination the results indicate that there were no extreme differences in bacterial diversity among the three land use patterns, but the bacterial abundance was highest in the arable soil and lowest in the forest soil.

Table 2. *Diversity indices of the soil bacterial communities*

Types	OTUs	Shannon	Simpson	Ace	Chao1
Wetland	1927.00±80.29^{ab}	6.11±0.16^{a}	0.005±0.001^{a}	2365.14±189.09^{a}	2351.63±169.89^{ab}
Forest land	1954.00±365.42^{a}	6.26±0.05^{a}	0.006±0.001^{a}	1899.19±155.58^{b}	1903.56±170.98^{b}
Arable land	1919.00±204.18^{b}	6.13±0.72^{a}	0.021±0.002^{a}	2355.01±172.76^{a}	2371.02±183.54^{a}

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

The beta diversity of the bacterial communities was compared by principal coordinates analysis (PCoA) based on Bray-Curtis distances. The first component represented over 34% of the data and the second component nearly 22% of the data. The differences in bacterial community structure among the different soil types were significant (PERMANOVA: r=0.48, $P<0.01$, while the differences within individual samples of the same soil type were insignificant (*Fig. 1*). Two of the three forest samples were very similar in community structure but the third sample was quite different. Similarly, one of the three arable samples was quite distinct (in the second PC dimension only). Moreover, it can be seen that the bacterial community structures of wetland and arable soil were more similar to each other and notably different to that of forest soil. This indicates that long-term land use types changes can lead to different bacterial community structures, but the results depend on the type of land use. Apparently, the strongest effect resulted from forestry, whereas wheat production had less of an effect on the bacterial community structure of the soil.

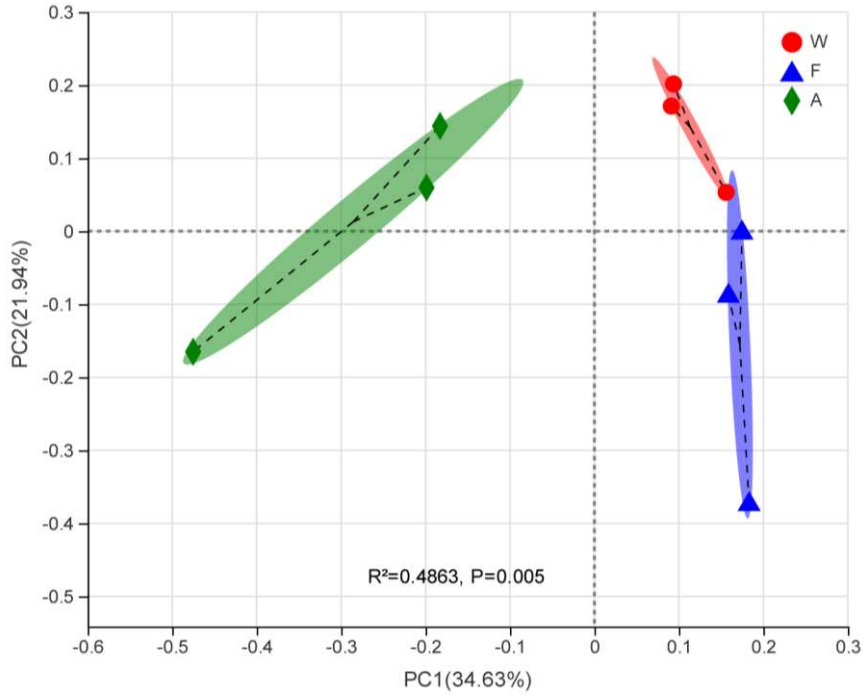

Figure 1. *PCoA of the beta diversity within and between bacterial communities of different land use soil types. Wetland soil samples are shown in red (w), forest soil samples in blue (F), and arable soil samples in green (A)*

Analysis of soil bacterial community structure under different land use types

All obtained sequence reads that could be attributed to an OTU in combination represented 58 bacterial phyla; sequences to which an OTU could not be attributed at the phylum level were uniformly grouped as "others". Of the OTUs obtained from all three soil types combined, Acidobacteria were the most abundant, followed by, Proteobacteria, Actinobacteria, and, as the fourth most abundant phylum, Chloroflexi (*Fig. 2A*). The relative abundances of these highly abundant phyla varied between the soil types. The soil from primitive wetland was relatively rich in Acidobacteria, which

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DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

represented over 41% of all obtained sequence reads (*Fig. 2B*), followed by Proteobacteria and Actinobacteria. Soil from land used for forestry over the past 30 years contained more Actinobacteria and fewer Actinobacteria compared to the wetland soil (*Fig. 2C*). Soil transformed into arable land was relatively rich in Proteobacteria but contained few Actinobacteria. It also contained a strikingly large fraction of Firmicutes (*Fig. 2D*).

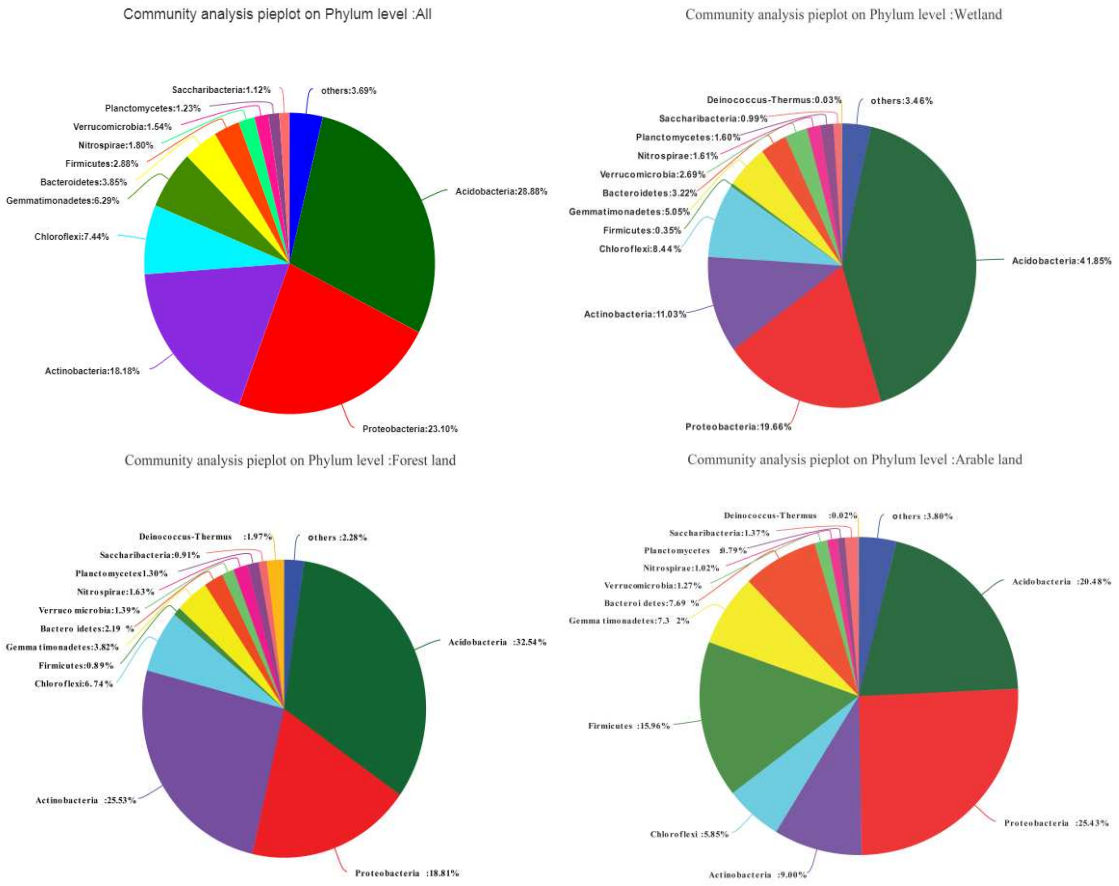

Figure 2. *Bacterial community composition at the level of phyla, for all obtained sequences (top) and for the three different land use soil types*

Differences in relative abundance of bacterial phyla were analyzed for the different soil types using two-sample *t*-tests. Three phyla were significantly different ($P<0.05$) between the wetland and forest soil (*Fig. 3A*), namely Actinobacteria ($P<0.01$), Verrucomicrobia, and Latescibacteria (both $P<0.05$). The latter phylum was found at low abundance but significantly higher in wetland than in forest soil. There were six phyla with significant differences ($P<0.05$ for all) in relative abundance between the wetland and arable soil (*Fig. 3B*): Acidobacteria, Bacteroidetes, Plantomycetes, Latescibacteria, Plantomycetes, and WWE3. Further, there were three phyla with significant differences ($P<0.01$) between the forest and arable soil (*Fig. 3C*), namely Actinobacteria, Bacteroidetes, and low-abundant WWE3 members, plus Plantomycetes that differed at a level of $P<0.05$ (*Fig. 3C*).

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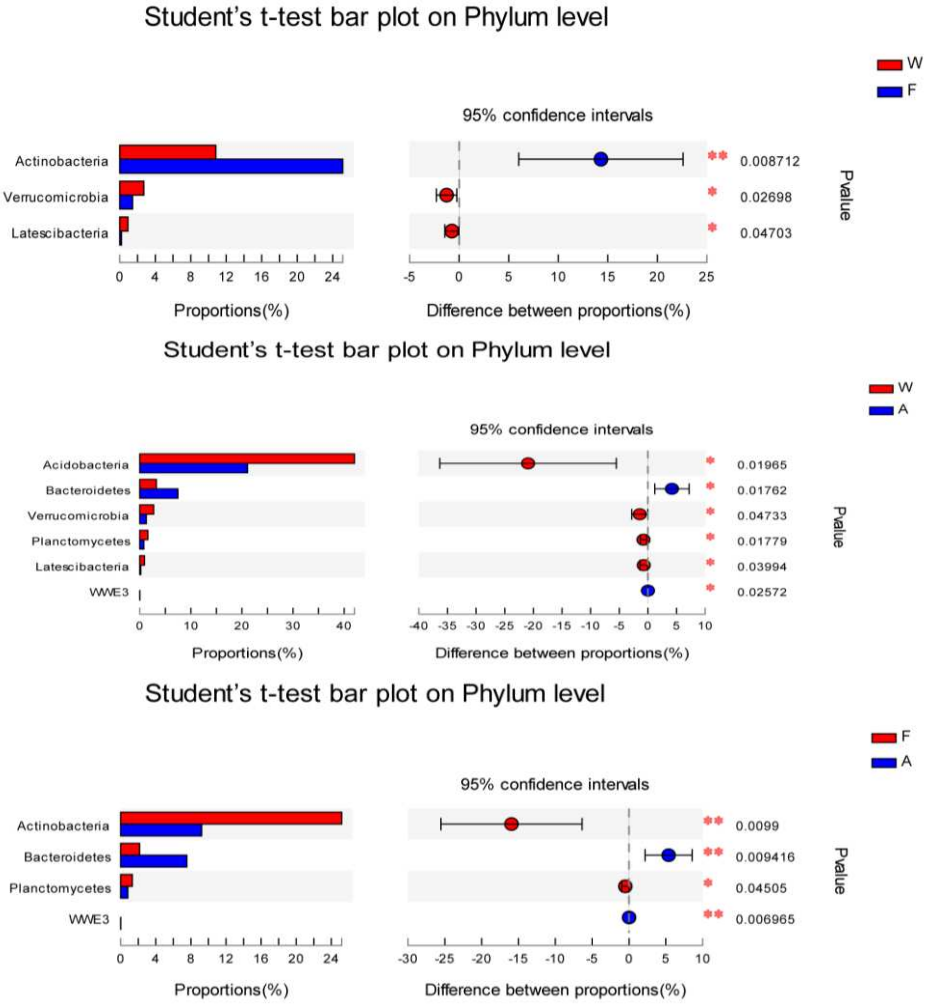

Figure 3. *Student t-test evaluating pairwise differences in bacterial abundance at the phylum level in the soil types, with W: wetland; F: forest land; A: arable land. Significance is represented with * at P<0.05 level and with ** at P<0.01*

Redundancy analysis of the soil bacterial community and physicochemical properties of the different land use soil types

Distance-based redundancy analysis (db-RDA) was conducted to determine relationships between the soil physicochemical properties and the bacterial community compositions at the phylum level (*Fig. 4*). The first axis explained 48.89% of all information and the second axis explained 7.37%; thus, in combination these capture 88.68% of the variability. Associations between the physico-chemical parameters deviating from the origin are indicated by arrows. Longer arrows such as for pH, MC, and to a lesser extent TP indicate that these parameters had a stronger impact on the bacterial community composition, while the shorter arrows of SOC, AP and TN indicate that these parameters had less impact. AP and TP values are associated (which is to be expected) and negatively correlate with the other determined parameters. The bacterial community composition of wetland soil correlated with lower pH and the three wetland samples correlated with MC, as could be expected. Two of the three forest soil samples also correlated with MC and to a lesser extent to AP.

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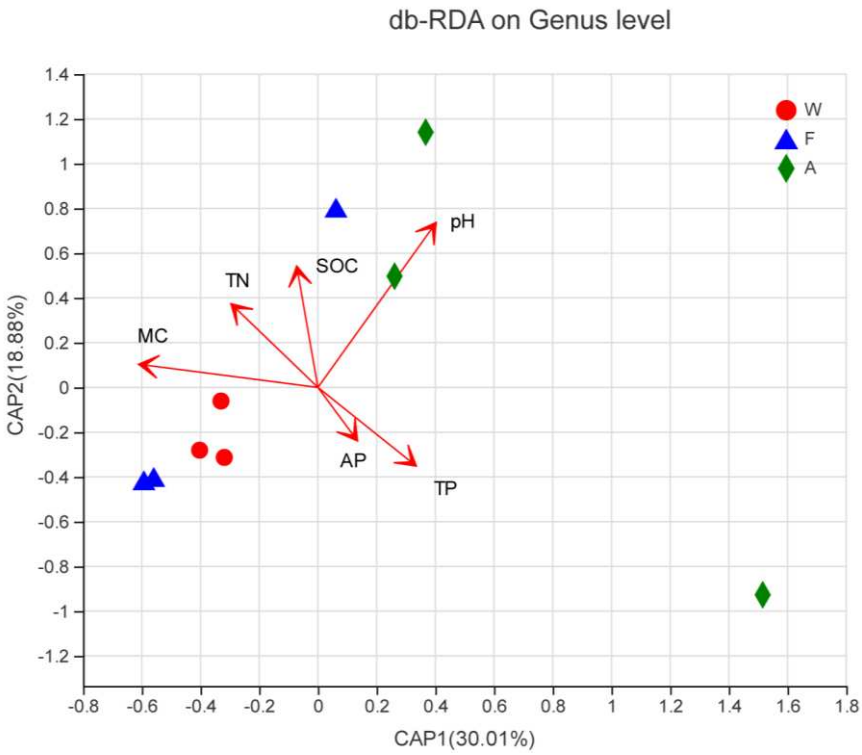

Figure 4. *Distance-based redundancy analysis of the soil bacterial community structure at phylum level and the determined soil physicochemical properties. W: wetland; F: forest soil; A: arable land soil; SOC: soil organic carbon; TN: total nitrogen; MC: moisture content; TP: total phosphor; AP: available phosphor, pH, acidity (the arrow indicates high pH)*

Discussion

Effects of land use types on the composition and diversity of the soil microbial community

Changes in the soil microbial community diversity can reflect the quality and health a soil ecosystem (Wang et al., 2013). The interference of human activities on natural ecosystems is the most important direct driving force that influences and changes a local ecosystem, in which the microbial soil community has important functions. Studying the changes of bacterial community structures and local diversities can reveal differences of the Yancheng wetlands under different land uses (Liu et al., 2006a,b). Here we confirm that different land use significantly changed the ACE and Chao1 indices of the detected soil bacterial communities, with significant changes in relative abundance of members of the bacterial community. Soil collected from arable land and forest land resulted in higher bacterial community abundances compared to relatively pristine wetland. Decades ago these arable and forest lands had been wetlands. The change in land use did not alter the three dominant phyla detected in the soil, which were (in order of decreasing abundance) Acidobacteria, Proteobacteria, and Actinobacteria. These three phyla accounted for more than 80% of the total bacterial community in the samples, which is consistent with the results of previous studies on the structure of soil microbial communities (Zhang et al., 2016; Zeng et al., 2017). However, the relative abundance of the dominant phyla changed significantly. In arable land, Firmicutes were much more abundant, overtaking acidobacteria as the third-most

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abundant phylum, while this phylum was relatively moderate in numbers detected for wetland or forest soil. Compared with the primitive wetland, in forest soil the relative abundance of Actinobacteria was higher; Proteobacteria were more abundant in arable land than in the other two soil types, and Acidobacteria were particularly abundant in wetland.

Previous studies have shown that in particular Proteobacteria and Actinomycetes (a family within the Actinobacteria phylum) are involved in the decomposition of organic matter (Li et al., 2016). Furthermore, Proteobacteria and Acidobacteria are often used as indicators of the soil nutritional status due to their lifestyles (Hartman et al., 2008). For instance, previous studies have reported that the relative abundance of Proteobacteria is positively correlated with soil carbon content, and that their abundance increases with increasing organic matter, especially in nutrient-rich soil (McCaig et al., 1999; Bardhan et al., 2012). Members of Proteobacteria are considered to be mainly responsible for utilization of higher carbon sources and may represent the most common bacterial phylum in the world. Acidobacteria contain many acidophilic species that are widely distributed in different soil environments and their relative abundance can relate to the acidity of the soil (Wei et al., 2016; Dang et al., 2017). Indeed, the lowest pH of the soils investigated in this study was recorded for wetland soil, which was rich in Acidobacteria (*Table 1, Figure 2, Figure 4*). Acidobacteria also negatively correlate to soil nutrition as they are generally more abundant in nutrient-poor soils. This fits with the observed higher abundance in the forest and wetland soil compared to the nutrient-rich arable soil. Actinomycetes are spore forming bacteria that dominate in poor soil conditions and they can decompose more refractory organic carbon by extending their hyphae into plant tissues (Xiao et al., 2014; Yu et al., 2016). In this study, Actinobacteria were the third-dominant group behind Acidobacteria and Proteobacteria for forest and wetland soil (they were fourth for nutrient-rich arable soil). Generally speaking, Proteobacteria are more abundant in soil with a less acidic pH and in near-neutral soil they can outcompete other bacteria by their efficient use of more complex organic carbon sources, while organic carbon with low bioavailability in acidic soil is beneficial to Acidobacteria.

The observed differences in abundance of bacterial phyla is likely caused by the change of soil structure and nutrient supply related to decade-long human agricultural and forestry practices (Wang et al., 2013). Ploughing practiced on the arable lands causes frequent disturbance of the soil (Liu et al., 2009), making the distribution of soil nutrients more uniform and adding aeration. This likely explains the higher diversity of the microbial community compared to the primitive wetland. It is also possible that the higher moisture content of the wetland results in lower oxygen contents, which may also reduce soil bacterial community diversity. Compared with the primitive wetland, the soil from arable and forest land produced significantly higher abundances of soil bacterial communities.

Correlations between soil physicochemical properties and the composition of the soil microbial community

We found that total P and available P were important factors correlating to the bacterial community structure (*Fig. 4*). These results are similar to those of Wang et al. (2013). Land use types determine the vegetation type and soil management practices, subsequently affecting soil nutrients (Zhang et al., 2016; Li et al., 2016). Changes in soil nutrients likely affect the microbial community structure, and the addition of different

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nutrient amounts will increase the microbial community structure and its functional diversity (Hartman et al., 2008). Bacteria are the most diverse microbial group in soil and they are extremely sensitive to environmental variation (McCaig et al., 1999). Like all other living organisms bacterial need phosphor, the bacterial cell wall contains a large amount of phosphonic acid, especially in Gram-positive bacteria, where it can account for about 50% of the dry cell weight (Bardhan et al., 2012). In this study, the content of total P but not available P was significantly lower in the cultivated and forest soil than in the wetland soil (*Table 1*), which may be because the forest canopy intercepts an in water, thereby decreasing surface runoff, which enables retention of the soil surface organic matter and mineral nutrition. This could allow the soil nutrient content in the forest land to be significantly higher than that in cultivated land; however, subsequent artificial fertilization of the cultivated land could compensate the nutrition of the soil (Dang et al., 2017). However, the wetland has no external nutrient supplementation, and vegetation growth utilizes the absorbed P in the soil, resulting in low total P and available P contents. Soil P is not only an energy source for the soil bacterial community, but also an important resource for plant growth (Dang et al., 2017). The special black soil habitat of the Yancheng wetland increases the amount of P adsorption (Wei et al., 2016), but the main source of soil P is the soil parent material, which is a single source. In addition, at the peak of vegetation growth in the black soil area of the Yancheng wetland, the soil P under different land use patterns may be redistributed within the plant-soil-microbial system, which makes the P content an important factor affecting the soil bacterial community variance (Xiao et al., 2014).

Different soil fertility and environmental conditions will alter the soil microbial population to varying degrees (Rani et al., 2009; Niu et al., 2011; Dhal et al., 2011). Studies have shown that the contents of soil organic carbon, total N and other nutrients depend on different land uses, and that the relative richness of dominant bacteria at the phylum and class level are also different. It is likely that changes in the soil nutrient content can lead to changes in soil bacterial composition and community structure (Wang et al., 2013; Xu et al., 2014). In primitive wetlands, soil pH, together with the water content and the degree of aeration, are important parameters affecting the metabolism and growth of plants and microorganisms alike; thus, changes in these parameters will inevitably lead to changes in the microbial community structure. Previously it has been shown (Dang et al., 2017) that soil pH exhibits an excellent correlation with microbial community diversity, because different bacteria have different environmental pH requirements, with even slight changes having a significant impact on the microbial community structure (Niu et al., 2017). In addition, soil nutrients are an important factor affecting the soil microbial community structure (Wei et al., 2016). Under eutrophic conditions, the limiting effect of P on the original microbial community is greatly reduced and the metabolic activities of the microorganisms change, which may change their species composition (Dang et al., 2017). Reclamation of primitive wetlands into arable land can change the pH, water content, and nutrients in the soil, thus affecting the microbial community structure.

Conclusions

After long-term changes in land use patterns of what was originally wetland, changes of the soil physicochemical properties mainly manifested as a decreased soil moisture content and an increase in pH, which was observed for both arable land and forest soil,

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together with an increased total and available P, indicating that the change in soil physicochemical properties has an impact on the structure of the soil microbial community.

The abundance of Proteobacteria, which is related to carbon utilization, was significantly higher in the arable land soil than that in the forest and wetland soils, while Acidobacteria, which is adapted to more acidic conditions, was significantly higher in the forest land and in particular in wetland soils. In the future we are planning to a comprehensive study to determine the interactions between soil microbial diversity and communities and the different land use soil types in combination with different aboveground plant compositions in the Yancheng wetland to further investigate the local ecosystems and formulate recommendations for the conservation of the wetlands.

Acknowledgements. This research were supported by Open Foundation of Shanghai Key Laboratory of Plant Functional Genomics and Resources (PFGR201803); Jiangsu Agricultural Industry Technology System (JATS-2020-3400); The Natural Science Foundation of the Jiangsu Higher Education Institutions of China (18KJB220011); Basic Research on Agricultural Application in Suzhou (SNG2018052); Suzhou Polytechnic Institute of Agriculture Young Teacher Research Capacity Improvement Program (19QN1010); Suzhou Polytechnic Institute of Agriculture Technology Cultivation Project(PY2003).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81318144

FERTILIZER MANAGEMENT FOR AMARANTH (*AMARANTHUS* SPP.) CULTIVATION ON DARK-RED SOIL IN OKINAWA, JAPAN

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(Received 16th Jul 2020; accepted 7th Oct 2020)

Abstract. *Amaranthus* is a promising food crop with unique nutritional compositions. Fertilizer treatments including 0 (Control), N, P, K, N + P (NP), N + K (NK), P + K (PK) and N + P + K (NPK) were evaluated based on growth, yield and quality of edible red leaf-amaranth (*Amaranthus* spp.) cultivated on dark-red soil (pH 6.6) to understand fertilizer management. N, P and K were applied at 50 g m^{-2} according to the treatments in one of the experiments. Effects of the fertilizer NPK (N:P:K = 1:1:1) at 0, 10, 20, 30 and 40 g m^{-2} were evaluated on red stem-amaranth and red leaf-amaranth in the same soil during another experiment. P and K fertilizers applied alone did not promote growth parameters and yield of amaranth, whereas N alone did. The fertilizer K promoted the function of N slightly but P did the same significantly regarding amaranth growth. Growth parameters and yield were the highest with the combined fertilizer NPK followed by NP. Yield and L-ascorbic acid content of amaranth were the highest with fertilizer NPK at 40 g m^{-2}. This study indicates that fertilizer N and P are more effective, and combined fertilizer NPK at 40 g m^{-2} is better for higher yield and quality of amaranth on dark-red soil in Okinawa.
Keywords: *growth characteristics, L-ascorbic acid, nutritive-value, vegetable crop, yield*

Introduction

Growth, yield and quality of plant species differ with soil nutrient status and fertilizer management (Hossain and Ishimine, 2005; Akamine et al., 2007; Chowdhury et al., 2008; Hossain et al., 2011). Different plant species respond differently to fertilizer combination and rates, and plant species requires balanced fertilizer to maximize growth, yield and quality (Hossain et al., 2004; Akamine et al., 2007; Chowdhury et al., 2008; Shimray et al., 2019). The major nutrients N, P and K individually or in combination maintain growth, yield and quality of plants (Ivony et al., 1997; Nakano and Morita, 2009; Barbara et al., 2011). Nitrogen, the principal element of chlorophyll, influences stomatal conductance and photosynthetic efficiency, which is responsible to 26-41% of crop yield (Maier et al., 1994; Ivony et al., 1997). Potassium regulates activities of various minerals and promotes N uptake efficiency of plants. Insufficient K causes shoot yellowing, poor growth and low resistance to cold and drought in plants (Oya, 1972). Phosphorus enhances absorption of other nutrients and promotes plant growth when applied with other fertilizers together (Akamine et al., 2007).

Amaranthus is a promising food crop for its resistance to heat, drought, diseases and pests, as well as high nutritional value (Sreelathakumary and Peter, 1993; Rastogi and Shukla, 2013; Longato et al., 2017; Maurya and Arya, 2018; Soriano-García et al., 2018). Several *Amaranthus* species are popularly being cultivated as vegetable and grain in Africa, Bangladesh, Caribbean, China, Greece, India, Nepal and South Pacific Islands (Prakash and Pal, 1991; Dewan et al., 2017; Stallknecht and Schulz-Schaeffer, 1993; Svirskis, 2003). Vegetable amaranth, superior in taste to spinach (*Spinacia oleracea*), has higher carotenoids (90-200 mg kg^{-1}), protein (14-30%), carbohydrate (5.0 g 100 g^{-1}), fat

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DOI: http://dx.doi.org/10.15666/aeer/1806_81458158

(0.1 g 100 g^{-1}), calories (43 Kcal 100 g^{-1}) and ascorbic acid (28 mg 100 g^{-1}) (Abbott and Campbell, 1982; Dewan et al., 2017; Makus, 1984; Prakash and Pal, 1991; Shittu et al., 2006). *Amartanthus* has antioxidant, antimalarial and antiviral properties, which prevent cancer, cardiovascular diseases, diabetes, etc. (Dasgupta and De, 2007; Khandaker et al., 2008; Shukla et al., 2010; Adegbola et al., 2020).

Amaranthus grows very fast in tropical and subtropical areas under a variety of soils and agroclimatic conditions during summer when vegetables are not available (Dewan et al., 2017; Makus, 1984; Singh and Whitehead, 1996). In Okinawa, some amaranth species are found as weed in various crops and vegetables (personal survey) in the major soil types, dark-red soil, red soil and gray soil, and summer vegetables are very limited (Hossain and Ishimine, 2005; Okinawa Prefecture Agriculture, Forestry and Fisheries, 2008). Our previous study has selected some high-quality amaranth lines as summer vegetables in Okinawa (Ohshiro et al., 2015). Shittu et al. (2006) reported that balanced fertilizers in a specific soil provide higher yield and nutrient compositions of amaranth.

In previous study, soil types and fertilizer regimes were evaluated on growth, yield, and quality of some *Amaranthus tricolor* lines (Ohshiro et al., 2016). All the growth parameters and yield of the amaranth lines were the best in gray soil. We have evaluated fertilizer N levels and combined fertilizer NPK on the amaranth lines in three soil types in Okinawa. Nitrogen fertilizer alone increased growth and yield of amaranths in gray soil but not in dark red soil and red soil. The combined fertilizer NPK resulted in the highest growth parameters and yield of amaranths in all soils. Previous study reported that combined fertilizer NPK at 20–40 g m^{-2} is effective for higher yield and minerals of amaranth in gray soil in Okinawa. Gray soil covers only 5% of land, whereas dark-red soil covers 35% of land and red soil covers 60% of land in Okinawa. Most of the dark-red soil field is used for crop cultivation, but only 40% of the red soil is used for crop cultivation in Okinawa. In addition, we did not evaluate separate and combined application of N, P and K on the amaranth lines in dark-red soil and red soil. Therefore, the objectives of this study were to (i) identify the effect of different fertilizer elements and (ii) to evaluate rates of combined fertilizer on growth, yield and quality of two edible amaranth lines to understand fertilizer management practices in dark-red soil in Okinawa.

Materials and methods

Soil collection

Dark–red soil (Shimajiri mahji) was collected from the top 50 cm layer of a field at the Subtropical Field Science Center, University of the Ryukyus, Japan. The content of Na, K, Ca, Mg, Al, Fe, P and Mn in the soil was 1.24, 2.28, 18.88, 2.85, 0.05, 0.23 and 0.07 mg kg^{-1}, respectively. Total N, total C and soil pH was 0.09%, 0.31% and 6.6, respectively. Coarse sand, fine sand, silt, clay and apparent density of the soil was 2.93%, 7.33%, 23.94%, 57.24% and 0.87 g cm^{-3}, respectively (Hossain and Ishimine, 2005).

Amaranth lines

Edible red stem-amaranth (BB line) and red leaf-amaranth (BC line) of *Amaranthus tricolor* selected for higher yield and quality in our previous studies (three experiments conducted from April, 2010 to May, 2011) were evaluated in this study (Ohshiro et al., 2015).

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DOI: http://dx.doi.org/10.15666/aeer/1806_81458158

Experiment 1: Effects of N, P and K fertilizers applied alone or in combination on edible amaranth cultivated during April to June, 2013

A glasshouse experiment was conducted using dark–red soil at the Subtropical Field Science Center of the University of the Ryukyus, from the 5th April to the 15th June, 2013. The experiment consisted of eight treatments with five replications (planters). The fertilizer treatments were 0 (Cont), N, P, K, N plus P (NP), N plus K (NK), P plus K (PK) and N plus P plus K (NPK). Nitrogen (N), P and K at 50 g m^{-2} (5.0 g per planter) were mixed with 13 kg of air-dried soil per planter (size 65E; 0.1 m^2) prior to the seed sowing according to the treatment design. Seeds of *Amaranthus* BC line were sown on soil surface, and covered with 0.5 cm of soil layer. The planters were placed randomly, and the plants were thinned to the 8 healthiest stands per planter at 2- to 3-leaf stage. Water was applied as required (considering soil moisture checked by squeezing the soil sample firmly in hand to form an irregularly shaped "ball", plant size and growth stage, daily weather condition, etc.) for proper seedling emergence and plant growth.

Experiment 2: Effects of N, P and K fertilizers applied alone or in combination on edible amaranth cultivated during September to December, 2013

A glasshouse experiment was conducted at the Subtropical Field Science Center of the University of the Ryukyus, from September 18 to December 10, 2013. The soil type, planter, soil amount per planter, fertilizer rate, seeds of *Amaranthus* BC line, treatment, replication, seed sowing procedure, and other management practices of this experiment were similar to those taken in the previous experiment (experiment 1). The plants were thinned to the 10 healthiest stands per planter at 2- to 3-leaf stage

Experiment 3: Effects of NPK fertilizer rates on edible amaranth cultivated during July to September, 2014

A glasshouse experiment was conducted using dark–red soil at the Subtropical Field Science Center of the University of Ryukyus, from the 15th July to the 3rd September, 2014. Two amaranth lines, BB and BC were evaluated. Each experiment consisted of five treatments with four replications (planters). The fertilizer treatments of the experiment were 0 (Cont), 10 g m^{-2} (1 g $planter^{-1}$), 20 g m^{-2} (2 g $planter^{-1}$), 30 g m^{-2} (3 g $planter^{-1}$) and 40 g m^{-2} (4 g $planter^{-1}$). The fertilizers of N ($CO(NH_2)_2$), P_2O_5 ($CaH_4(PO_4)_2H_2O$) and K_2O (KCl) were applied at a ratio of N:P:K = 1:1:1. The fertilizers were mixed with 13 kg of air dried soil per planter (size 65E) prior to the seed sowing according to the treatment. *Amaranthus* seeds were sown on soil surface and covered with 0.5 cm of soil layer. Water was applied according to the experiment 1 for proper seedling emergence and plant growth. The planters were placed randomly, and the plants were thinned to the 10 healthiest stands per planter at 2- to 3-leaf stage.

Data collection

Leaf-amaranth is usually harvested when 20-35 cm in height; and both the leaf and stem are used as a vegetable. Stem-amaranth is usually harvested at the young stage (20-35 cm) for the use of both leaf and stem, and at the pre-flowering stage (semi mature plant) for only the stem. Plant height and leaf number were recorded up to 43 days after seed sowing (DAS) at a 7-day interval in experiment 1. Five plants were harvested from each planter at 34 DAS, and plant height, stem diameter, internode

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DOI: http://dx.doi.org/10.15666/aeer/1806_81458158

length, leaf number, largest leaf area, total leaf area, and fresh and dry weight of the leaf, stem and shoot were determined. Shoot (leaf plus stem) weight was considered as yield. In experiment 2, five plants were harvested from each planter at 25 DAS and the same growth parameters were measured. In experiment 3, five plants were harvested from each planter at 26 DAS and the same growth parameters were measured. Stem diameter was measured at five cm from the soil surface, and internode length from the third internode from the soil surface.

Determination of SPAD value, leaf area and dry weight of edible amaranth

The SPAD value of the second and third fully expanded leaves from the top was measured with a chlorophyll meter SPAD-502 (Konica Minolta, Inc., Osaka, Japan). Leaf area was measured with an automatic area meter (AAM-8, Hayashi Denkoh Co. Ltd.). Various parts of amaranth plants were dried at 80 °C for 48 h using a forced convection oven (DRLF23WA, Advantec) for dry weight measurement.

Determination of mineral, nitrogen, carbon and pH in soil, and nutrient status and L-ascorbic acid in edible amaranth

Various parts of amaranth plants were dried at 60 °C for 48 h using the same forced convection oven. Soil samples were dried at room temperatures of 25-28 °C for 5 days. The plant parts and soil were ground finely for chemical analysis. Mineral contents of soil and nutrients of amaranth were determined with an Inductively Coupled Plasma Spectrometer (ICPS-8100, Shimadzu Co. Ltd.). Total C and N were determined with a Gas Chromatograph (Soil GS-8A, Shimadzu Co. Ltd., NC-220F Juka analysis center) and Sumigraph (NC-90A, Shimadzu Co. Ltd.). Soil pH was determined with a TOA pH meter (HM-20S, Toa Electronic Ltd.). L-ascorbic acid content in leaves was determined by using a RQ Flex/agrocheck kid small-sized reflecting photometer (Kanto Chemical Co. Ltd.).

Statistical analysis

Average data for each replication was calculated, and then mean and standard deviation (SD) of the replications were determined using analysis of variance. Fishers protected least significant difference (LSD) test at the 5% level was used to compare treatment means. The amaranth lines were analysed separately.

Results

Effects of N, P and K fertilizers applied alone or in combination on cultivated edible amaranth

The plant grown from April to June, 2013 showed that plant height and leaf number increased significantly with the fertilizers of N, NK, NP and NPK compared to that with other treatments (*Fig. 1*). Plant height and leaf number were increased by 300% and 67%, respectively with the NP or NPK as compared to that with N and NK (*Fig. 1*).

Separate application of P and K did not increase the growth parameters of amaranth, whereas N alone did (*Fig. 1; Table 1*). Amaranth grew faster and better with fertilizer NPK and NP than with other fertilizer treatments (*Fig. 1*). Internode length, largest leaf area, total leaf area and leaf weight were significantly and similarly higher with the

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NPK and NP compared to those with the other treatments (*Table 1*). Total leaf area and leaf weight increased by 6-67 and 5-59 times, respectively when cultivated with the combined fertilizer NP or NPK, as compared to those with other fertilizer treatments. Stem diameter was the highest with the NPK followed by NP. Stem weight increased by 12-165 and 8-115 times with the combined fertilizer NPK and NP, respectively, as compared to that with other fertilizer treatments. The fertilizer NPK and NP resulted in 6.5 and 5.5 times higher yield, respectively as compared to the fertilizer NK.

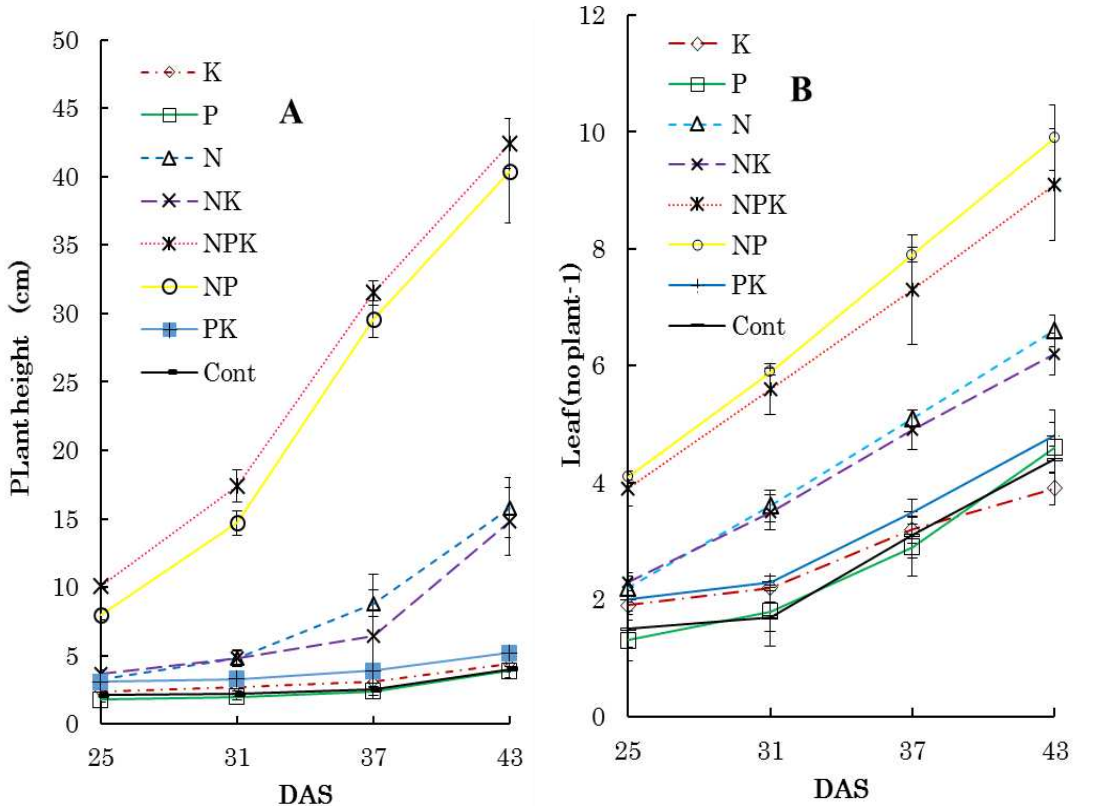

Figure 1. *Effects of N, P and K applied alone or in combination on plant height (A) and leaf number (B) of edible amaranth BC line cultivated during April to June, 2013*

Table 1. *Effects of N, P and K fertilizers applied alone or in combination on growth parameters of edible amaranth BC line cultivated during April to June, 2013*

Treatment	Stem diameter mm	Internode length cm	Largest leaf area cm^2 $leaf^{-1}$	Total leaf area cm^2 $leaf^{-1}$	Fresh leaf weight g $plant^{-1}$	Dry leaf weight g $plant^{-1}$	Fresh stem weight g $plant^{-1}$	Dry stem weight g $plant^{-1}$	Fresh shoot weight g $plant^{-1}$	Dry shoot weight g $plant^{-1}$
K	1.26d	0.29d	1.79c	4.97c	0.11c	0.016c	0.06c	0.004d	0.17d	0.020d
P	1.25d	0.18d	1.76c	4.81c	0.10c	0.017c	0.05c	0.004d	0.15d	0.021d
N	3.81c	1.48b	17.38b	58.57b	1.55b	0.199b	1.00c	0.060c	2.55c	0.259c
NK	3.69c	1.22bc	14.58b	54.46b	1.39b	0.193b	0.97c	0.055c	2.36c	0.248c
NPK	8.05a	3.27a	56.26a	333.04a	9.23a	0.947a	13.9a	0.660a	25.49a	1.607a
NP	7.09b	3.02a	55.55a	326.47a	8.27a	0.896a	9.65b	0.459b	17.92b	1.355b
PK	1.41d	0.29d	1.69c	5.11c	0.14c	0.022c	0.07c	0.007d	0.21d	0.029d
Cont	1.23d	0.16d	1.93c	4.13c	0.09c	0.016c	0.04c	0.004d	0.21d	0.020d

Data were recorded at 34 day after seed sowing. Data with the same letter within each column are not significantly different at the 5% level, as determined by LSD test

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The plant grown from September to December, 2013 showed that plant height and leaf number were the highest with the application of fertilizer NPK followed by NP (*Figs. 2* and *3*). The fertilizer N, NK and PK also resulted in increased plant height and leaf number compared to the control treatment. The plant height was 1.7-2.0 times higher with the NPK or NP compared to that with the N and NK. Individual application of P and K did not increase the growth parameters of amaranth, whereas N alone did (*Table 2*). All the growth parameters were the highest with the NPK fertilizer followed by NP. Total leaf area and dry leaf weight increased by 2-11 and 2-9 times, respectively with the combined fertilizer NPK or NP, compared to those with other fertilizer treatments. Yield was the highest with the NPK followed by NP (*Table 2*). The fertilizer NPK and NP resulted in a 2.2 and 1.8 times higher yield than the fertilizer NK.

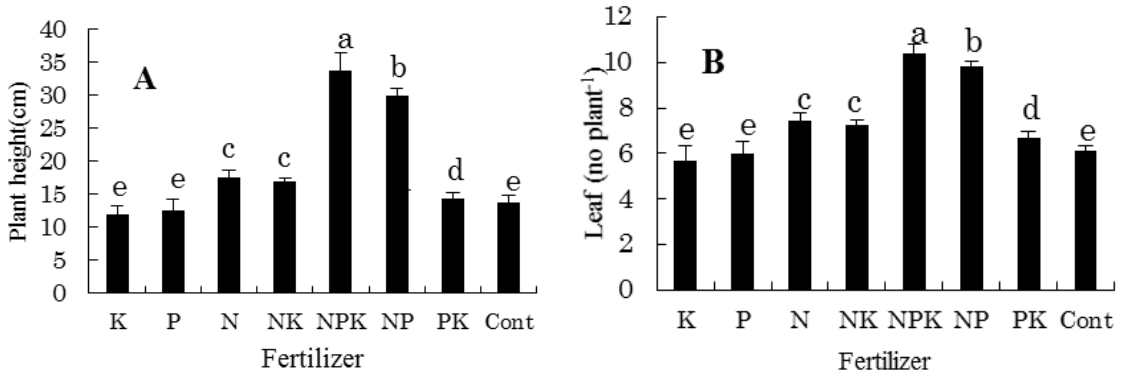

Figure 2. *Effects of N, P and K fertilizers applied alone or in combination on plant height (A) and leaf number (B) of edible amaranth BC line cultivated during September to December, 2013. Bars with the same letter are not significantly different at the 5% level (LSD test)*

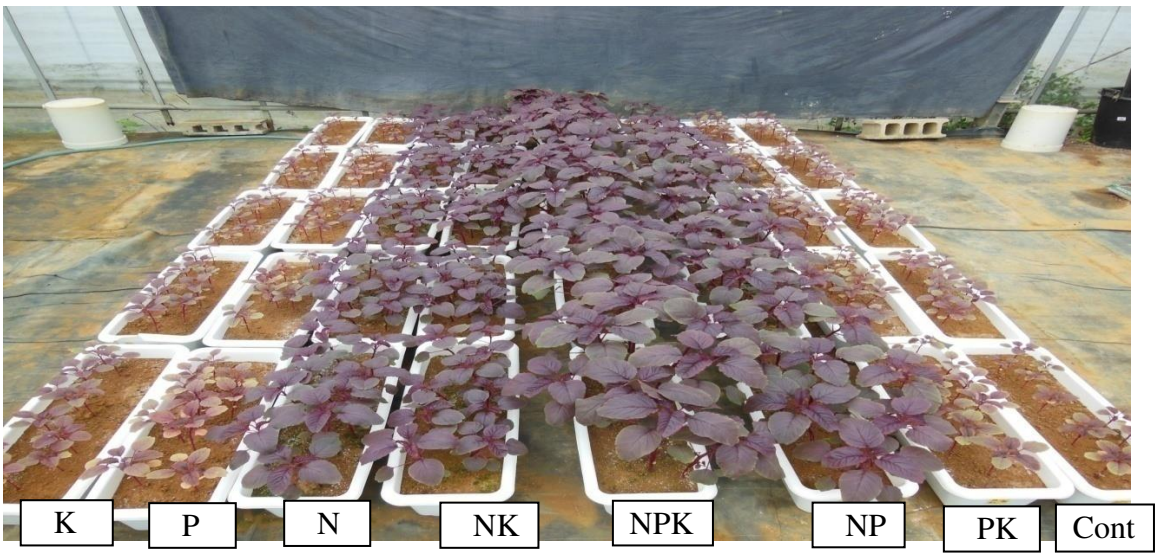

Figure 3. *Effects of N, P and K fertilizers applied alone or in combination on growth of edible amaranth BC line cultivated during September to December, 2013. Photo taken at 34 day after planting. Alone K (K), alone P (P), alone N (N), N plus K (NK), N plus P plus K (NPK), N plus P (NP), P plus K (PK), Control (Cont)*

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Table 2. *Effects of N, P and K fertilizers applied alone or in combination on growth parameters and yield of edible amaranth BC line cultivated during September to December, 2013*

Fertilizer treatment	Stem diameter mm	Internode length cm	Largest leaf area cm^2 $leaf^{-1}$	Total leaf area cm^2 $plant^{-1}$	Fresh leaf weight g $plant^{-1}$	Dry leaf weight g $plant^{-1}$	Fresh stem weight g $plant^{-1}$	Dry stem weight g $plant^{-1}$	Fresh shoot weight g $plant^{-1}$	Dry shoot weight g $plant^{-1}$
K	2.68d	0.56d	9.15d	34.82d	0.97d	0.13d	0.48d	0.04e	1.45d	0.17d
P	2.92d	0.51d	9.33d	37.12d	1.09d	0.18d	0.58d	0.07de	1.67d	0.25d
N	5.04c	2.00c	33.02c	162.34c	4.06c	0.52c	3.05c	0.18c	7.11c	0.70c
NK	5.25c	2.16c	33.86c	168.61c	4.33c	0.48c	3.28c	0.18c	7.61c	0.66c
NPK	8.17a	4.16a	59.42a	393.64a	11.05a	1.15a	12.71a	0.61a	23.77a	1.76a
NP	6.57b	2.84b	52.94b	356.77b	8.72b	0.99b	9.62b	0.47b	18.34b	1.46b
PK	2.77d	0.70d	10.48d	52.55d	1.33d	0.21d	0.79d	0.09d	2.13d	0.30d
Cont	2.98d	0.63d	11.30d	50.20d	1.21d	0.19d	0.71d	0.08d	1.92d	0.27d

Data were recorded at 25 day after seed sowing. Data with the same letter within each column are not significantly different at the 5% level, as determined by LSD test

Effects of fertilizer rates on growth and yield of amaranth BB and BC lines

SPAD value increased with all the fertilizer rates in both amaranth lines (*Table 3*). The SPAD value was the highest with the fertilizer at 40 g m^{-2} than with other fertilizer rates in the BB line, but same with all the fertilizer rates in the BC line. Plant heights of both BB and BC lines increased with the increasing rate of fertilizer (*Figs. 4* and *5*). The leaf number of the BB line was the highest with the fertilizer NPK at 40 g m^{-2} followed by 30 g m^{-2}. The BC line obtained similarly higher leaf number when cultivated with the fertilizer rates of 20, 30 and 40 g m^{-2} (*Fig. 5*). Stem diameter, internode length, largest leaf area, total leaf area, leaf weight, stem weight and shoot weight of both amaranth lines were increased with the increasing rate of fertilizer (*Table 3*). The yield of the BB and BC lines was the highest when cultivated with the fertilizer rate at 40 g m^{-2}.

Table 3. *Effects of NPK fertilizer rates on SPAD value, growth parameters and yield of edible amaranth BB and BC lines cultivated during July to September, 2014*

Amaranth lines	Fertilizer rates g m^{-2}	SPAD value	Stem diameter cm	Largest leaf area cm^2 $plant^{-1}$	Total leaf area cm^2 $plant^{-1}$	Fresh leaf weight g $plant^{-1}$	Dry leaf weight g $plant^{-1}$	Fresh stem weight g $plant^{-1}$	Dry stem weight g $plant^{-1}$	Fresh shoot weight g $plant^{-1}$	Dry shoot weight g $plant^{-1}$
BB	Cont	19.92b	0.89d	0.75d	2.24d	0.05d	0.02c	0.03d	0.01d	0.08d	0.03d
	10	33.85a	3.00c	13.56c	48.75c	1.59c	0.64b	0.81c	0.21c	2.41c	0.85c
	20	32.87a	3.95b	23.00b	86.37b	2.95b	0.90b	1.86b	0.41b	4.80b	1.31bc
	30	34.85a	4.38a	23.25b	94.18b	3.16b	1.01b	2.63b	0.52b	5.78b	1.53b
	40	33.50a	4.69a	35.14a	151.97a	4.89a	1.60a	4.07a	0.70a	8.93a	2.30a
BC	Cont	30.90c	1.28d	1.21d	3.93d	0.12d	0.08d	0.09d	0.03d	0.18d	0.15d
	10	33.22b	4.29c	12.60c	53.90c	1.88c	1.00c	1.57c	0.52c	3.45c	1.52c
	20	34.20b	4.93bc	20.31b	94.42b	3.46b	1.59b	3.33b	0.84b	6.79b	2.43b
	30	34.77b	5.72b	20.78b	102.49b	4.16b	1.84b	4.75b	1.15b	8.90b	2.99b
	40	37.57a	6.99a	31.42a	162.07a	5.83a	2.35a	7.85a	1.55a	13.68a	3.89a

Data were recorded at 26 day after seed sowing. Data with the same letter within each column for each amaranth line are not significantly different at the 5% level, as determined by LSD test

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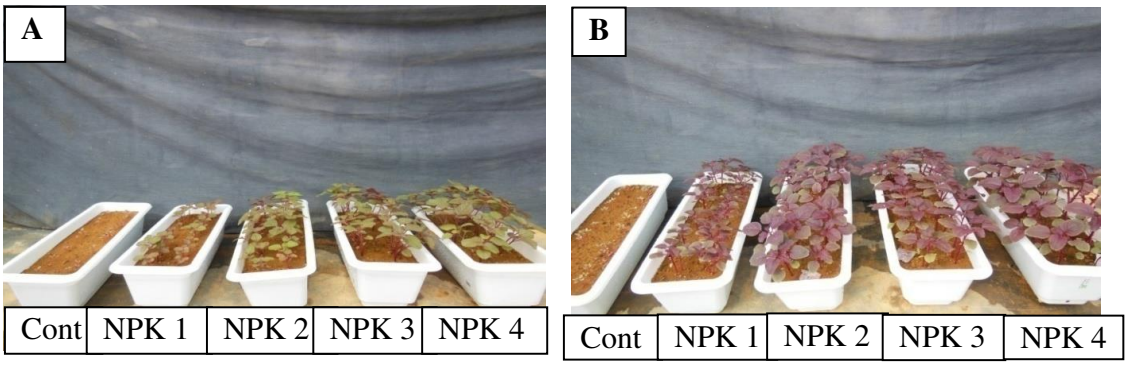

Figure 4. *Effect of NPK fertilizer rates [(Cont (0 g), NPK 1 (10 g m^{-2}), NPK 2 (20 g m^{-2}), NPK 3 (30 g m^{-2}), NPK 4 (40 g m^{-2})] on growth of amaranth BB (A) and BC (B) lines at 26 DAS*

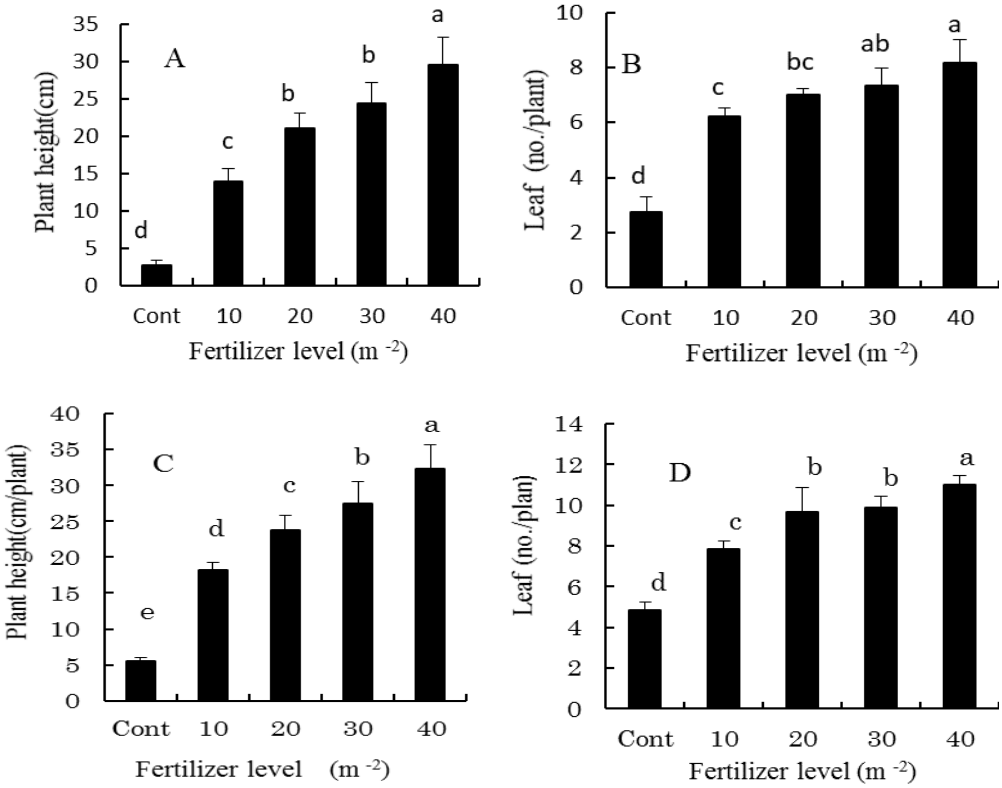

Figure 5. *Effects of NPK fertilizer rates on plant height and leaf number of amaranth lines (A and C: BB line, C and D: BC line). Bars with the same letter are not significantly different at the 5% level (LSD test)*

Effects of N, P and K fertilizers applied alone or in combination on mineral, nitrogen and carbon content of amaranth

The experiment conducted from April to June, 2013 showed that the Na content of amaranth was the highest with the fertilizer NP, and K was the highest with the fertilizer NPK (*Table 4*). The Ca content in the plants decreased, but Mg content increased with all the fertilizers, except fertilizer K, compared to those with the control treatment. Al content decreased with all the fertilizers, except fertilizers P and PK, whereas Fe content

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did not differ clearly with the fertilizers, except fertilizer P. The P content in amaranth was the highest with the fertilizer P followed by fertilizer PK, NP and NPK. Total N and C content in the plant was not significantly influenced by the fertilizers, however N in the plant was lower with the fertilizer NPK.

Table 4. *Effects of N, P and K fertilizers applied alone or in combination on minerals, total N and total C in amaranth BC line cultivated during April to June, 2013*

Fertilizer treatment	Na mg g^{-1}	K mg g^{-1}	Ca mg g^{-1}	Mg mg g^{-1}	Al mg g^{-1}	Fe mg g^{-1}	P mg g^{-1}	TN %	TC %
K	2.16d	12.80d	10.94d	9.48c	0.67b	0.17cd	5.72c	-	-
P	3.80b	27.86b	15.16bc	15.68a	1.23a	0.97a	20.34a	-	-
N	3.38b	31.14a	13.38cd	15.28a	0.12c	0.02d	7.56c	4.46a	39.53a
NK	2.14d	30.20ab	13.42cd	13.18b	0.17c	0.01d	6.14c	4.71a	40.26a
NPK	3.10bc	35.06a	15.64bc	12.62b	0.15c	0.03d	11.58b	3.63a	39.57a
NP	7.32a	19.28c	15.16bc	16.50a	0.08c	0.22bc	12.62b	4.16a	40.21a
PK	2.98c	25.34b	16.96ab	16.98a	1.06a	0.26c	18.68a	-	-
Cont	3.76bc	26.40b	18.78a	8.12c	1.32a	0.42b	9.86c	-	-

Data were recorded at 34 day after seed sowing. Data with the same letter within each column are not significantly different at the 5% level, as determined by LSD test. —, data not recorded due to poor growth

The experiment conducted from September to December, 2013 showed that Na and K content was the highest with the fertilizer NP and NPK, respectively (*Table 5*). The Ca content was the lowest with the fertilizer NP, and Mg content was lower with all the fertilizer treatments, except fertilizer K. The P content increased when the amaranth plant was cultivated with the fertilizers P, NP, PK and NPK. The content of Al and Mn was not clearly influenced by fertilizers. The Zn content in the plant increased with the fertilizer N and NK. Total N and C contents were the highest with the fertilizer NP followed by NPK (*Table 5*).

Table 5. *Effects of N, P and K fertilizers applied alone or in combination on mineral and total nitrogen and carbon in amaranth BC line cultivated during September to December, 2013*

Fertilizer treatment	Na mg g^{-1}	K mg g^{-1}	Ca mg g^{-1}	Mg mg g^{-1}	Al mg g^{-1}	Fe mg g^{-1}	P mg g^{-1}	Mn mg g^{-1}	Zn mg g^{-1}	TN %	TC %
K	7.17d	91.83a	23.98bc	46.23a	0.65ab	0.26d	8.70b	1.57a	0.11b	2.52d	38.54f
P	7.07d	77.17b	23.87bc	37.90bc	0.67a	0.24d	9.52ab	1.36b	0.16b	1.86f	39.51e
N	11.13b	90.83a	23.97bc	31.90cd	0.56abc	0.42ab	7.92b	1.44ab	0.36a	5.26bc	41.25c
NK	8.88c	76.5b	25.77ab	32.83c	0.51bc	0.36bc	7.96b	1.58a	0.34a	5.15c	40.44d
NPK	9.53c	91.25a	20.60cd	26.75de	0.50bc	0.43ab	9.70a	1.47ab	0.09b	5.58b	41.79b
NP	12.73a	87.83a	18.40d	22.60e	0.48c	0.44a	10.16a	1.34b	0.12b	6.13a	43.33a
PK	6.68d	78.33b	25.73ab	35.83bc	0.55abc	0.25d	10.35a	1.47ab	0.09b	2.14ef	39.76e
Cont	7.32d	93.33a	28.93a	39.30b	0.63ab	0.30cd	8.47b	1.51a	0.05b	2.41de	39.89e

Data were recorded at 25 day after seed sowing. Data with the same letter within each column are not significantly different at the 5% level, as determined by LSD test

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Effects of fertilizer rates on L-ascorbic acid, mineral, nitrogen and carbon content of edible amaranth

L-ascorbic acid content of the amaranth increased significantly with all the fertilizer rates in both BB and BC lines, and L-ascorbic acid content was the highest when the amaranth was grown with the fertilizer NPK at 40 g m^{-2} followed by 30 and 20 g m^{-2} (*Fig. 6*).

The Na, K, Ca, Mg, P and Zn content of the amaranth BB line increased with all the fertilizer rates, whereas the Al and Fe content was not clearly influenced with the fertilizer rates (*Table 6*). The Na content of the BC line tended to increase with all the fertilizer rates, and K content increased with the 20-40 g m^{-2} (*Table 6*). The Ca content increased with the 10 and 20 g m^{-2} but not with the 30 and 40 g m^{-2}, compared to that with the control treatment, and the Mg and Zn content was not influenced by the fertilizer rates. The Al, Fe and P content increased with all the fertilizer rates. The Mn content decreased with all the fertilizer rates in both amaranth lines. The total N content was the same with all the fertilizer rates in the BB line, but increased with the increasing fertilizer rate in the BC line. The C content of the amaranth lines was not influenced with the fertilizer rates.

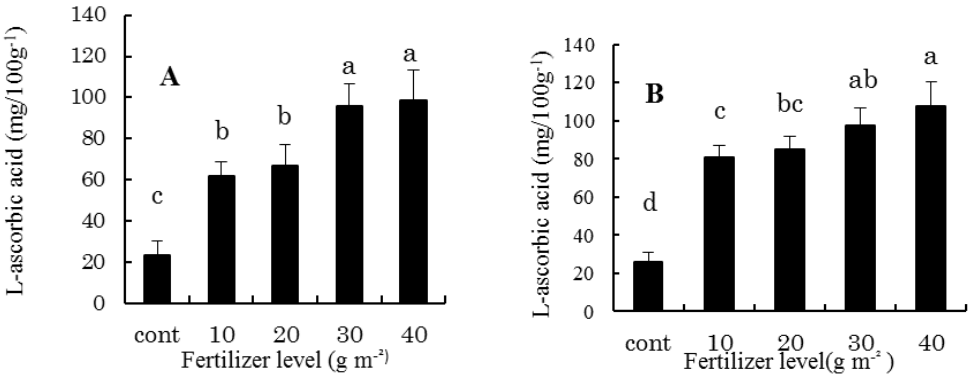

Figure 6. *Effects of NPK fertilizer rates on L-ascorbic acid of amaranth BB (A) and BC (B) lines at 26 DAS. Bars with the same letter are not significantly different at the 5% level (LSD test)*

Table 6. *Effects of NPK fertilizer rates on mineral, total nitrogen and total carbon in amaranth BB and BC lines cultivated during July to September, 2014*

Amaranth line	Fertilizer rates g m^{-2}	Na mg g^{-1}	K mg g^{-1}	Ca mg g^{-1}	Mg mg g^{-1}	Al mg g^{-1}	Fe mg g^{-1}	P mg g^{-1}	Mn mg g^{-1}	Zn mg g^{-1}	TN %	TC %
BB	Cont	5.25c	127.67d	32.33c	24.73b	0.63a	1.02ab	14.03b	2.13a	0.32b	–	–
	10	6.63ab	141.50c	42.40a	27.10a	0.43b	0.89c	39.96a	1.98a	0.35a	3.30a	38.28a
	20	6.36ab	156.50b	40.90a	27.83a	0.64a	0.99b	41.43a	1.39b	0.36a	3.61a	39.40a
	30	6.15b	157.16b	35.70b	26.23a	0.66a	1.02ab	39.53a	1.28b	0.36a	3.47a	39.04a
	40	6.76a	183.33a	36.70b	26.13a	0.65a	1.02ab	41.70a	1.42b	0.35a	3.33a	38.77a
BC	Cont	6.50b	91.10c	24.23b	33.33a	0.42b	0.93c	21.35c	0.71a	0.34a	–	–
	10	6.53b	96.33c	29.13a	30.66a	0.66a	1.10a	24.26c	0.32b	0.37a	1.88c	39.43a
	20	6.95b	114.33b	28.33a	32.33a	0.60a	1.09a	25.63bc	0.36b	0.36a	2.25b	38.90a
	30	6.98b	124.66b	24.33b	29.30a	0.63a	1.08a	28.86b	0.36b	0.35a	2.32b	39.01a
	40	8.30a	152.50a	24.63b	30.86a	0.65a	1.04b	31.56a	0.37b	0.34a	2.90a	38.59a

Data were recorded at 26 day after seed sowing. Data with the same letter within each column for each amaranth line are not significantly different at the 5% level, as determined by LSD test. –, data not recorded due to insufficient sample

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DOI: http://dx.doi.org/10.15666/aeer/1806_81458158

Discussion

Both experiments indicate that P and K applied alone were not effective for amaranth growth, but N was effective. Combined application of NP resulted in significantly higher growth parameters and yield than the combined application of NK. This result indicates that fertilizer P enhanced the function of fertilizer N significantly but K did slightly in all the growth parameters of amaranth. Combined application of NPK resulted in the highest growth parameters and yield followed by the combined application of NP, indicating that fertilizer K enhanced the function of fertilizers N and P for the growth of amaranth plant. Similarly, other studies reported that N is more effective than P and K for vegetative growth of plants (Akamine et al., 2007; Chowdhury et al., 2008; Hossain et al., 2011; Sarker et al., 2002). The content of K and P in the soil was 2.28 and 0.23 mg kg^{-1}, respectively, indicating that K content in the soil was about 10 times higher than P, which was sufficient for amaranth growth. This result also indicates that amaranth needs a higher amount of P than K for proper growth on dark-red soil in Okinawa. The fertilizers N, P and K together provided balanced nutrients, which resulted in higher plant growth parameters and yield. Similarly, other studies reported higher plant biomass in various crop species with the combined fertilizer of N, P and K (Oya, 1972; Akamine et al., 2007; Hao and Papadopoulos, 2004; Hossain et al., 2012).

The growth of the amaranth BB line increased with the increasing rate of fertilizer NPK, whereas, the growth of the BC line was similarly higher with the 20-40 g m^{-2}. Similarly, Ohshiro et al. (2016) reported that fertilizer requirement varies with the plant species or cultivars. All the growth parameters and yield of the amaranth lines were the highest with the fertilizer NPK at 40 g m^{-2} followed by 30 g m^{-2} due to the higher SPAD value which probably contributed to higher photosynthesis (Sarker et al., 2002)

The experiments conducted from April to June, and September to December showed that Na content of amaranth was the highest with the NP fertilizer, while K was the highest with the NPK fertilizer followed by NK (*Tables 4* and *5*). The results indicate that P fertilizer influenced the absorption of K fertilizer by the plant. The Ca content in the amaranth was lower with all the fertilizer treatments, except K and PK. The Mg content in the amaranth decreased with all the fertilizers when cultivated from April to June, but increased when cultivated from September to December, which may be due to the differences in temperature and solar radiation during the cultivation time. The P content increased in the amaranth with the P, NP, PK and NPK fertilizers, which indicates that fertilizer P was the cause of increased P mineral in the amaranth plant. Total N content in the plant was not significantly influenced with the fertilizers when cultivated from April to June, but significantly the highest with the N followed by NK when cultivated from September to December. The total N in the plant with the fertilizer NPK was lower due to the highest biomass production, which is supported by other studies (Akamine et al., 2007; Hossain et al., 2011)

L-ascorbic acid content in the amaranth BB and BC lines was the highest with fertilizer NPK at 40 g m^{-2} followed by 30 g m^{-2}. The Na, K, Ca, Mg and P content of the amaranth BB line increased with all the fertilizer rates, whereas the content of Na, K, Al, Fe and P in the amaranth BC line increased with the fertilizer NPK at 20-40 g m^{-2}. The Ca content was lower in the BC line, and Mg and Zn content was not influenced by the fertilizer rates. The total N content was the same with all the fertilizer rates in the BB line, but increased with the increasing fertilizer rate in the BC line. The results showed that all the minerals were not increased or decreased with the fertilizer rates.

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DOI: http://dx.doi.org/10.15666/aeer/1806_81458158

Similar result was reported in other studies (Ivony et al., 1997; Barbara et al., 2011). Overall results indicate that the fertilizer NPK at 40 g m^{-2} is better for a higher yield and quality of the amaranth plant on dark-red soil.

Conclusions

The fertilizer P and K applied alone were not effective for amaranth growth, because the P and K content in the soil was probably sufficient or there was a lack of N content. Combined application of NP resulted in significantly higher growth parameters and yield than the combined fertilizer of NK, which indicates that P fertilizer influenced the function of N fertilizer significantly but K did slightly in the amaranth. It is also considered that K content in the soil was sufficient for amaranth growth but P was not sufficient. Combined application of NPK resulted in the highest growth parameters, which indicates that fertilizer P and K applied alone were not effective because of insufficient N, but effective when applied together with N. All the growth parameters and yield of the amaranths were the highest with the combined fertilizer NPK at 40 g m^{-2} followed by 30 g m^{-2}. The P, K and N content in the amaranth increased but Ca and Mg content decreased with the fertilizer NPK. However, total amount of the minerals significantly increased. The Na, K, Ca, P, N and L-ascorbic acid content in the amaranth lines increased, and Mg, Al and Fe content was the same with the fertilizer NPK at 30-40 g m^{-2}. Overall results indicate that combined fertilizer NPK at 40 g m^{-2} is better for higher yield, mineral and L-ascorbic acid of amaranth on dark-red soil in Okinawa. However, it is very difficult to clarify the actual effects of individual or combined fertilizers of N, P and K on amaranth cultivation in field soil. In addition, a specific plant species needs a specific ratio and amount of fertilizers N, P and K for proper growth, yield and quality. Therefore, further experiments should be conducted to evaluate ratio and amount of fertilizers N, P and K on amaranth cultivation by using nutrientless soil.

Acknowledgements. The authors acknowledge the technical staffs of CRAC, University of the Ryukyus for supporting chemical analysis.

Conflicts of interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81458158

IMPACT OF SOME TREATMENTS ON SEED GERMINATION AND SEEDLING VIGOUR OF KANGAR (*GUNDELIA* SP. L.)

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(Received 16th Jul 2020; accepted 6th Oct 2020)

Abstract. This research was conducted at the Horticulture Department, College of Agricultural Engineering Sciences, University of Sulaimani, Kurdistan region-Iraq to study the effect of various treatments such as removing fruit layers, soaking seeds in distilled water, GA_3 with 100 and 200 ppm, *Moringa oleifera* leaf extract with various concentrations (0.5, 1.0, 1.5 and 2.0 g/L) for 24 h and subjecting the seeds to freezing temperature at -2 °C for 24 and 48 h on Kangar (*Gundelia sp*) seed germination and growth. Removing the seed coat and submerging seeds in 200 and 100 ppm recorded the highest germination percentages of 50, 46.67 and 33.33%, respectively. Removing the seed coats enhanced leaf proliferation (1.67). Moreover, maximum lateral root number (23.34), leaf length (7.42 cm) and leaf width (1.70 cm) were achieved in soaking seeds in 100 ppm GA_3. Whereas, the highest taproot length was observed after soaking the seeds with 200 ppm GA_3, which reached at 16.6 cm. In addition, the seeds that were treated with moringa leaf extract exhibited the highest chlorophyll and carotenoid concentrations, at 1.5 g/L. In most aspects, removing the seed coat and GA_3 were noticeably more effective for promoting *Gundelia* seed germination.
Keywords: *seed treatment, GA_3, freezing, seed coat removal, plant extract*

Introduction

Kangar (*Gundelia* sp. L.), belongs the sunflower family Asteraceae, is a spiny and thistle-like flowering plant and disseminated wildly in the semi-dried areas of Lebanon, Syria, Palestine, Israel, Jordan, Iraq, Iran, Azerbaijan, Armenia, and Anatolia (Ayoubi and Baradari, 2015). The only distinguished species of this genus is *Gundelia tournefortii,* L. (Firat, 2017), however, recently, many new species of this genus were recorded, such as *G. Anatolica*, *G. Asperrima, G. Cilicica, G. Colemerikensis, G. Dersim*, *G. Glabra*, *G. Komagenensis*, *G. Mesopotamica*, *G. Munzuriensis*, *G. Rosea*, *G. Tournefortii*, *G. Vitekii* (Genç and Firat, 2019). *Gundelia* L. found in Mediterranean regions and was used as food, especially *Gundelia tournefortii* L. (Owies et al., 2004). The youngest stem and head of it are used for food, and many nutritive values (proteins, fibers and minerals) were found in it (Oweis, 2003). Overharvesting, land cultivation and overgrazing threaten of *Gundelia* L. for extinction (Shibli et al., 2009; Yazdanshenas et al., 2016). In this regard, some efforts have been exerted to propagate the important wild species of this genus through seed or *in vitro*.

Seed propagation is the easiest and cheapest method for propagation of plants, at the same time a large number of new plants can be achieved through seeds (Hartmann et al., 2011). The seed of many plant species required specialized treatment to improve

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81598170

germination due to seed dormancy phenomenon. It was found that removing the seed coat enhanced seed gemination because the seed coat acts as a physical barrier for water and oxygen uptake, a mechanical barrier for embryo emergence, or prevents leaching of geminating inhibitors from the embryo (Hopkins and Hüner, 2008). Additionally, soaking seeds in water for a period increases germination percentage of seeds as well, Hossain (2005) found that seed germination of *the Terminalia chebula* (Retz.) was raised by soaking the seeds in water for 48 h. Soaking seed in an optimal concentration of certain phytohormones is also beneficial for germination seeds of some species (Afzal et al., 2005). Stratification of seeds is the common method used to break dormancy in the dormant seeds of many species, Vaisi et al. (2018) reported that stratification improved seed germination in *Gundelia tournefortii* seeds in the combination of scarification and GA_3 treatment. On the other hand, plant extracts, which are safer for human and environment, are used as an alternative for synthetic seed germinating promoters, Phiri and Mbewe (2010) referred that moringa (*Moringa oleifera*) leaf extracts increased germination percentage of cowpea by 4%. The objective of the current study is to study the effect of some treatments on seed germination of *Gundelia* L.

Materials and methods

Plant materials and preparation

This research was conducted at Horticulture Department, College of Agricultural Engineering Sciences, University of Sulaimani, Kurdistan region-Iraq to study effects of some treatments on germination of *Gundelia* seeds and seedling vigorous. The seeds were collected from the wild *Gundelia* plants in middle July 2019, then the seeds placed in the refrigerator at 5 °C until the time of using. The intact and healthy seeds selected on October 23, 2019, 23/10/2019 for the germination experiment. The dry seeds were soaked in distilled water (WS), gibberellin (GA_3) with 100 and 200 ppm (GA_3-100 and GA_3-200), and *Moringa oleifera* leaf extract with concentrations: 0.5 (MO-0.5), 1.0 (MO-1.0), 1.5 (MO-1.5) and 2 g/L (MO-2.0) for 24 h. Moringa leaf extract solutions were prepared by placing moringa leaf in the water bath at 40 °C for 3 h, after that placed in the refrigerator for 24 h, then, in the following day, they were filtered through filter paper and used for the seed treating. The other treatments were applied with the *Gundelia* sp. seeds by subjecting the seeds to freezing at -2 °C for 24 (FR-24 h) and 48 h (FR-48 h), removing the seed coat (DE), while, the untreated seeds were directly sown as control seeds (CO). Seeds coat-removal were achieved manually, after seeds soaking in distilled water for 2 h at 25 °C to simplify removal of the seed coat. Finally, the treated and control seeds were sown in peat moss medium with three replications for each replication 10 seeds were sown. The experiment was performed in a plastic high tunnel, and it was laid out in Complete Randomized Design (CRD). The maximum and minimum temperature of inside the high tunnel was weekly calculated in *Figure 1,* from 23/10/2019, which was the date of starting the experiment, to 31/12/2019. After this period, the measured traits were: germination percentage (GP), number of lateral roots (NLR), taproot length (TPL), number of leaves (NL), leaf length (LL) and leaf width (LW).

Chlorophyll and carotenoid contents

Chlorophylls (CHa and CHb) and carotenoids (CA) were quantified according to Sumanta et al. (2014). The leaf samples were ground in liquid nitrogen, then 0.5 g of

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DOI: http://dx.doi.org/10.15666/aeer/1806_81598170

the sample was homogenized in 10 mL of 80% Acetone, after Centrifuging for 5000 rpm for 30 min at 4 °C, 0.5 mL of the supernatant were remixed with 4.5 mL of 80% Acetone. The solution of the mixture was analyzed spectrophotometrically at various wavelengths 470, 646.8 and 663.2 nm for chlorophyll a (CHa), chlorophyll b (CHb), and carotenoids (CA). The following equations were used for the quantifications.

$$CHa\ (\mu g/mL) = 12.25 \times A663.2 - 2.79 \times A646.8 \quad \text{(Eq.1)}$$

$$CHb\ (\mu g/mL) = 21.50 \times A646.8 - 5.10 \times A663.2 \quad \text{(Eq.2)}$$

$$CA\ (\mu g/mL) = (1000 \times A470 - 1.82\ CH\ a - 85.02\ CH\ b)/198 \quad \text{(Eq.3)}$$

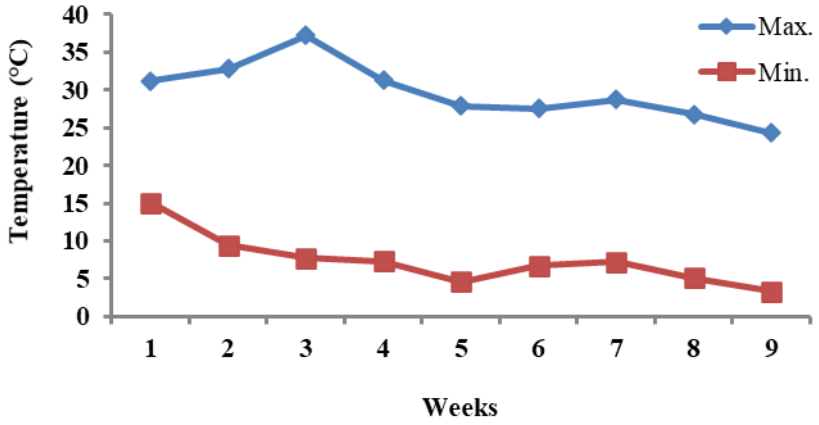

Figure 1. *Weekly maximum and minimum temperatures inside the plastic high tunnel from 23/10/2019 to 31/12/2019*

Statistical analysis

After 9 weeks the experiment was terminated, the parameters of germination percentage and seedling lateral root number, seedling taproot length, seedling leaf number, seedling leaf length, seedling leaf width and pigments concentration (CHa, CHb and CA) were measured. Using XLSTAT software version 2019.2.2, one-way ANOVA-CRD, Duncan's new multiple-range test and principal component analysis (PCA) has been used to determine significant differences between different treatments and relationships between different variables ($p \leq 0.05$). Correlation analysis was conducted by Displayr software.

Results and discussion

Impact of different treatments on germination percentage

The effect of different treatments on seed germination showed that there were no significant differences between all the treatments and the control (*Table 1; Fig. 2*), while the germination percentage (GP) significantly varied among the treatments themselves. Decoated seeds played a significant role in accelerating germination percentage, which it recorded the highest germination percentage (50%), followed by

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DOI: http://dx.doi.org/10.15666/aeer/1806_81598170

the seeds soaked in 100 and 200 ppm GA_3 (33.33 and 46.67%, respectively). The lowest germination percentage (3.33 and 6.67%) was achieved from the seeds that soaked in 0.5 and 1 g/L moringa extracts, respectively. A non-significant variation was observed between the decoated seeds and seed treated by GA_3.

Table 1. *Analysis of variance of germination percentage (GP), number of lateral root (NLR) and taproot length (TPL) of Kangar*

GP					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	6854.55	685.45	2.79*	0.02
Error	22	5400.00	245.45		
Corrected total	32	12254.55			
NLR					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	1406.16	140.62	2.87**	0.01
Error	22	1075.28	48.88		
Corrected total	32	2481.44			
TPL					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	574.40	57.44	3.11**	0.01
Error	22	406.01	18.46		
Corrected total	32	980.42			

*: significant difference among treatments, **: high significant difference among treatments

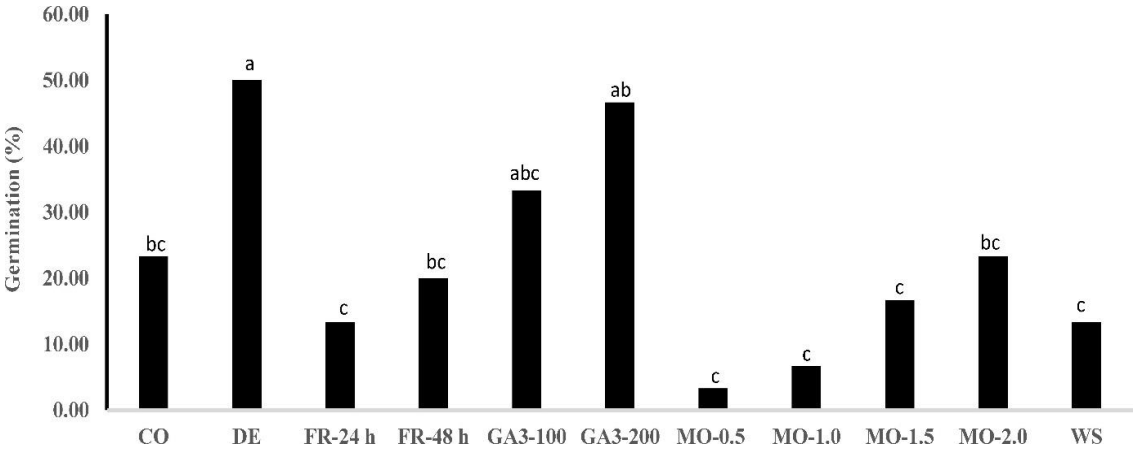

Figure 2. *Effect of the different treatments on germination percentage of Kangar. Each value represents the mean of three replicates. Means sharing a specific letter are not significantly different according to Multiple Duncan Test at $p \leq 0.05$. CO: Control, DE: decoated seeds, FR-24 h: treatment of seeds by freezing for 24 h, FR-48 h: treatment of seeds by freezing for 48 h, GA_3-100: treatment of seeds by gibberellin (GA_3) at concentration of 100 ppm, GA_3-200: treatment of seeds by gibberellin (GA_3) at concentration of 200 ppm, MO-0.5: treatment of seeds by moringa leaf extract at concentration of 0.5 g/L, MO-1.0: treatment of seeds by moringa leaf extract at concentration of 1.0 g/L, MO-1.5: treatment of seeds by moringa leaf extract at concentration of 1.5 g/L, MO-2.0: treatment of seeds by moringa leaf extract at concentration of 2.0 g/L, and WS: treatment of seeds by distilled water*

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DOI: http://dx.doi.org/10.15666/aeer/1806_81598170

Similarly, AbuQaoud and Alkony (1995) found *Gundelia tournefortii* germination seeds were increased by removing the seed coat. Improving seed germination by removing the seed coat could be interpreted as an embryo rescue from the restriction of seed coat, which acts as a mechanical barrier for embryo emergence, and it helps the embryo to reach water and gas easily and quickly; despite these seed the coat prevents leaching of germination inhibitors from the seed, or supplying them to an embryo (Hartmann et al., 2011). Besides, Shibli et al. (2009) reported that soaking *Gundelia tournefortii* seeds in 250 ppm GA_3 gave the highest germination percentage in the greenhouse experiment. Gibberellin stimulates germination via enzymatic weakening of the covering effects of endosperm and seed coat around radicle, storage, food mobilization and embryo cell expansion (Finch-Savage and Leubner-Metzger, 2006). Generally, in this study, moringa leaf extracts did not enhance seed germination compared to control. In this regard, Tahir et al. (2018 and 2020) found that reduction of seed germination as a result of application moringa extracts may due to that moringa extracts contained chemical fractions [eicosane, gamma-sitosterol, l-(+)-ascorbic acid, 2,6-dihexadecanoate, octadecanoic acid, methyl 11,14,17-eicosatrienoate, and octadec-9-enoic acid], which were matched positively with the inhibition of seed germination of wild mustard. In the foregoing experiment, also, they further explained that the above allelopathic compounds minimized hydrolytic enzymes during germination.

Influence of various treatments on root growth

As shown in *Table 1* and *Figure 3,* the results showed significant variations in the number of lateral roots and taproot length among treatments. The highest number of lateral root numbers (23.24 and 20.04) in seeds soaked in GA_3-100 and GA_3-200, respectively, was significantly induced on taproot; afterwards, the decoated seeds gave (18.74) root number, whereas, the lowest root number (2.33) was observed in seeds soaked in MO-1.0 extract (*Fig. 3A*). Moreover, TPL reached the highest value (16.60 cm) in seeds, were soaked in GA_3-200 and to a lesser extent (16.33 cm) in seeds soaked in MO-1.5 extract, however, they were significantly different with control seeds by (8.47 cm), whereas soaking seeds in MO-0.5 and MO-1.0 extracts showed the shortest TPL (3.33 and 4.33 cm, respectively) (*Fig. 3B*). A non-significant difference was stated among the treatments: DE, GA_3-100 and GA_3-200. The elongation taproot in treated seeds with GA_3 may be by reason of that GA_3 induces cell elongation at the sub-apical region of the roots (Parab et al., 2017).

Effect of different treatments on leaf growth

The results of *Table 2* and *Figure 4* explained the significant difference among various treatments in terms of NL, LL and LW traits. DE and GA_3 (both concentrations), MO-1.5, FR-24 and FR-48 h, produced the highest leaf numbers compared to control treatment. The maximum leaf number (1.67) was recorded in the decoated seeds followed by the seeds treated with GA_3 and freezing. The seeds soaked with MO-0.5 and M0-1.0 extracts gave the lowest leaf number (0.33). Additionally, the different treatments resulted in different LL significantly, the longest leaf (7.42 cm) was observed in the seeds soaked in GA_3-100, by contrast, the shortest leaf (0.45 cm) was achieved from soaked seeds in MO-1.0 extract. The seeds soaked in GA_3-100 gave also the broadest leaves (1.70 cm), but the narrowest leaves (0.30 cm) observed from the seeds were soaked in MO-1.0 moringa extract. Removing the seed coat and GA_3

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treatments in this study gave the best leaf traits, that is maybe due to the early initiated germination, which gives more time to the seedlings to produce the higher number of leaves, accelerate the exposure of leaves to sunlight to do photosynthesis, develop more root number for nutrient uptake, and consequently improving leaf growth in term of leaf length and width. Muralidhara et al. (2016) reported that decoated seeds and treatment of seeds with GA_3 led to the improvement of seed germination in mango varieties.

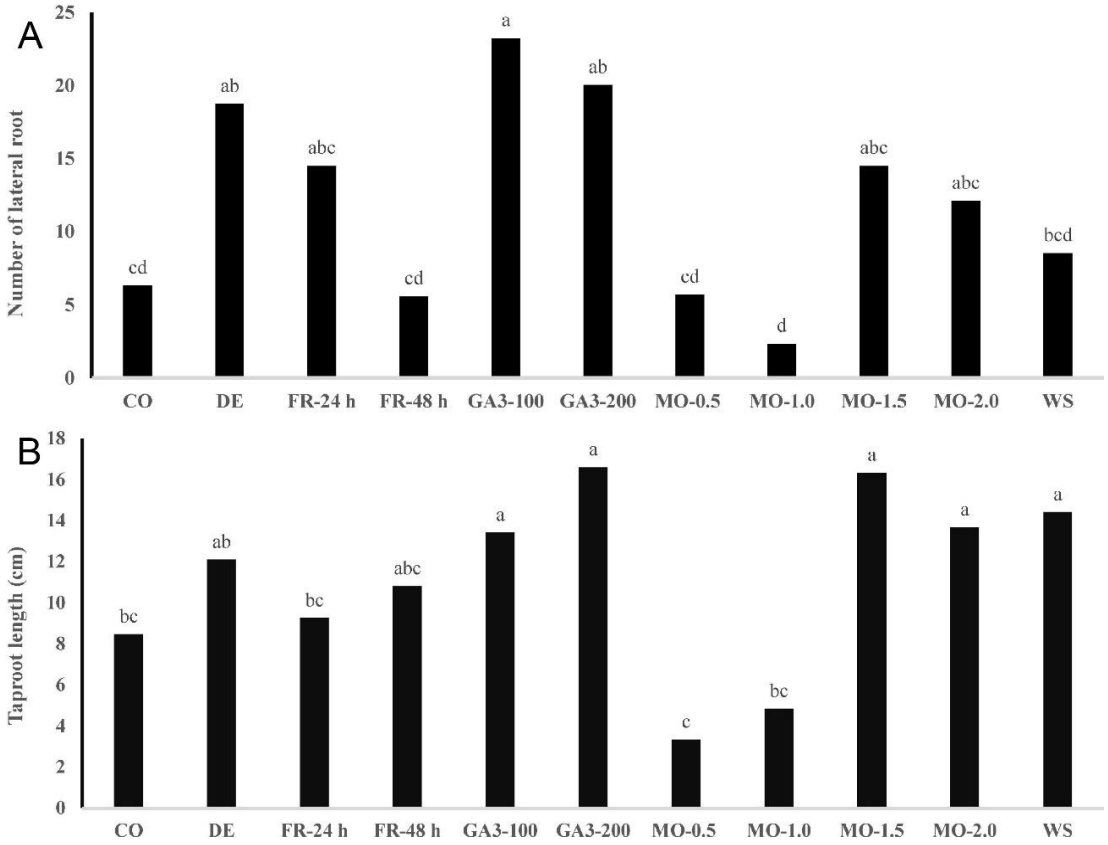

Figure 3. *Impact of the various treatments on the number of lateral roots (A) and taproot length (B) of Gundelia. Each value signifies the mean of three replicates. Means sharing a specific letter are not significantly different according to Multiple Duncan Test at $p \leq 0.05$. CO: Control, DE: decoated seeds, FR-24 h: treatment of seeds by freezing for 24 h, FR-48 h: treatment of seeds by freezing for 48 h, GA_3-100: treatment of seeds by gibberellin (GA_3) at concentration of 100 ppm, GA_3-200: treatment of seeds by gibberellin (GA_3) at concentration of 200 ppm, MO-0.5: treatment of seeds by moringa leaf extract at concentration of 0.5 g/L, MO-1.0: treatment of seeds by moringa leaf extract at concentration of 1.0 g/L, MO-1.5: treatment of seeds by moringa leaf extract at concentration of 1.5 g/L, MO-2.0: treatment of seeds by moringa leaf extract at concentration of 2.0 g/L, and WS: treatment of seeds by distilled water*

Impact of various treatments on pigments content

The obtained results in *Table 3* and *Figure 5* showed that the concentration of chlorophylls and carotenoids significantly impacted by the different treatments. The prominent chlorophyll (CHa and CHb) and carotenoid contents (CA) were measured at MO-1.5 and MO-2.0 extracts, and they were over the control (CO) and other treatments significantly. The highest CHa (1.79 µg/mL) and CA (0.37 µg/mL) contents were stated from the treatment of MO-1.5 moringa extract. Inversely, the seeds were soaked in GA_3-

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100 resulted in the lowest concentrations of CHa (0.98 µg/mL) and CA (0.08 µg/mL). CHb displayed no significant variation among different treatments. Chlorophyll raising in seeds soaked with moringa extracts may be due to the presence of compounds in moringa leaf extract like cytokinin, zeatin, and zeatin, which have the role in the production of chlorophyll (Hala et al., 2017). Otherwise, it was reported that exogenous application of GA_3 increased leaf area, but it affected inversely on the concentration of chlorophyll per leaf unit area (Leite et al., 2003; El-Shraiy and Hegazi, 2009).

Table 2. *Analysis of variance of number of leaves (NL), leaf length (LL) and leaf width (LW) of Kangar*

NL					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	5.40	0.54	2.10ns	0.06
Error	22	5.64	0.26		
Corrected total	32	11.04			
LL					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	149.58	14.96	3.85**	0.00
Error	22	85.31	3.88		
Corrected total	32	234.89			
LW					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	6.73	0.67	2.63*	0.02
Error	22	5.63	0.26		
Corrected total	32	12.36			

*: significant difference among treatments, **: high significant difference among treatments, Ns: non-significant difference among treatments

Table 3. *Analysis of variance of different pigments in Kangar leaf*

CHa					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	0.96	0.10	38.21**	< 0.0001
Error	11	0.03	0.003		
Corrected total	21	0.99			
CHb					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	0.07	0.01	0.54ns	0.83
Error	11	0.13	0.01		
Corrected total	21	0.20			
CA					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr > F**
Treatment	10	0.12	0.01	4.83**	0.01
Error	11	0.03	0.003		
Corrected total	21	0.15			

**: high significant difference among treatments, Ns: non-significant difference among treatments

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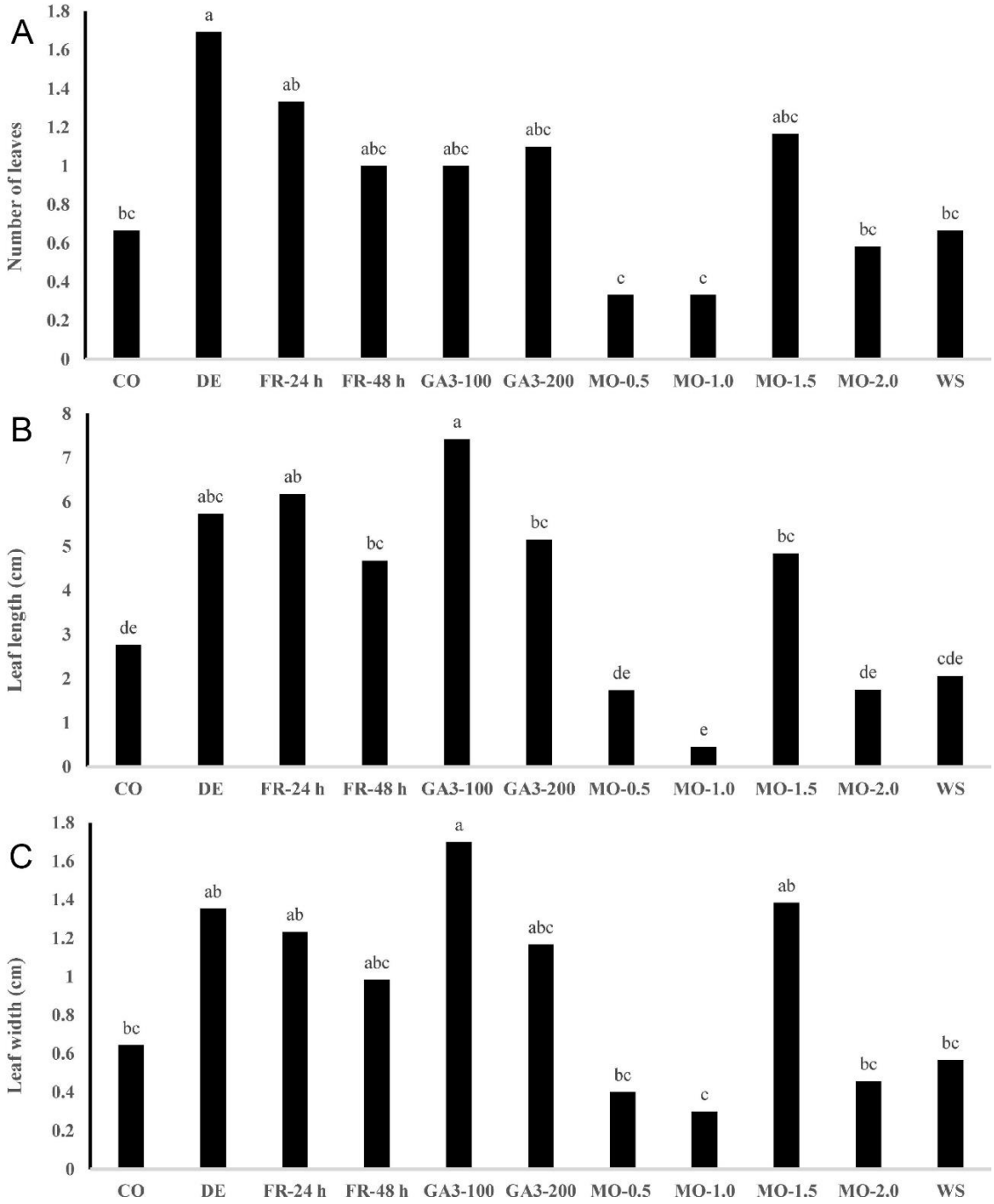

Figure 4. *The influence of the various treatments on the number of leaves (A), leaf length (B) and leaf width (C) of Gundelia. Each value denotes the mean of three replicates. Means sharing a common letter are not significantly different according to Multiple Duncan Test at $p \leq 0.05$. CO: Control, DE: decoated seeds, FR-24 h: treatment of seeds by freezing for 24 h, FR-48 h: treatment of seeds by freezing for 48 h, GA_3-100: treatment of seeds by gibberellin (GA_3) at concentration of 100 ppm, GA_3-200: treatment of seeds by gibberellin (GA_3) at concentration of 200 ppm, MO-0.5: treatment of seeds by moringa leaf extract at concentration of 0.5 g/L, MO-1.0: treatment of seeds by moringa leaf extract at concentration of 1.0 g/L, MO-1.5: treatment of seeds by moringa leaf extract at concentration of 1.5 g/L, MO-2.0: treatment of seeds by moringa leaf extract at concentration of 2.0 g/L, and WS: treatment of seeds by distilled water*

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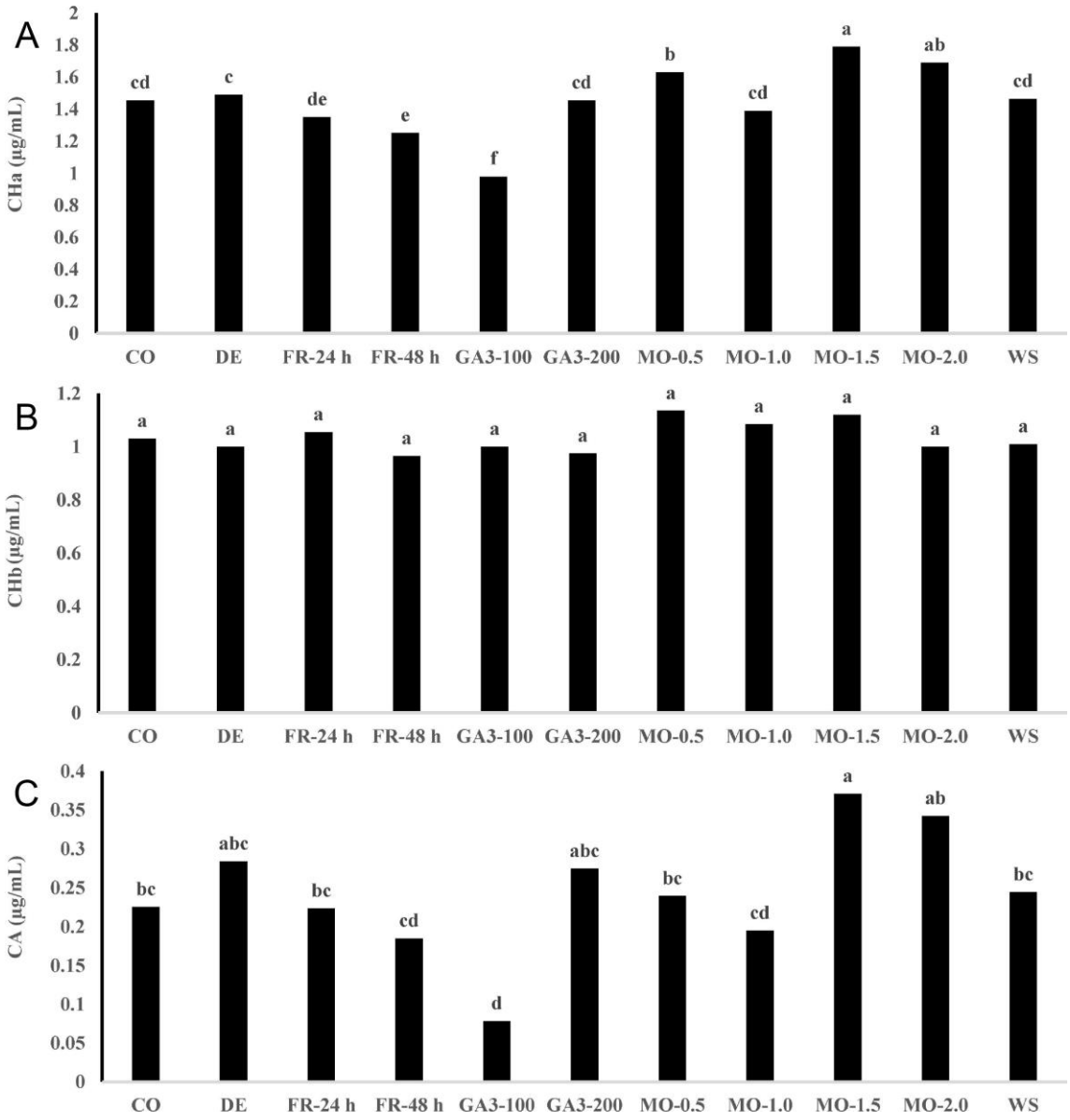

Figure 5. *Effect of the treatments on leaf chlorophylls (CHa and CHb) and carotenoids (CA). Values are the average of three measurements. Average separation within a trait followed by the common letters does not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test. CO: Control, DE: decoated seeds, FR-24 h: treatment of seeds by freezing for 24 h, FR-48 h: treatment of seeds by freezing for 48 h, GA_3-100: treatment of seeds by gibberellin (GA_3) at concentration of 100 ppm, GA_3-200: treatment of seeds by gibberellin (GA_3) at concentration of 200 ppm, MO-0.5: treatment of seeds by moringa leaf extract at concentration of 0.5 g/L, MO-1.0: treatment of seeds by moringa leaf extract at concentration of 1.0 g/L, MO-1.5: treatment of seeds by moringa leaf extract at concentration of 1.5 g/L, MO-2.0: treatment of seeds by moringa leaf extract at concentration of 2.0 g/L, and WS: treatment of seeds by distilled water*

Relationship between different treatments and traits

The principal component analysis was conducted to determine the relationship between the variables (treatments and traits) that account for the observed variance in

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the data. The differential influence of the variables in each main component is calculated by the association between each variable and the main component. The first two main components (PC1 and PC2) clarified together 78.51% of the observed variance and were thus depicted in a two - dimensional space (*Fig. 6*). PC1 plotted on the horizontal axis, clarified the highest proportion of the variance (54.74%), while PC2, plotted on the vertical axis, accounted for a further 23.77% of the total variation. *Figure 6* shows the PCA plot distribution of the treatments and studied characters on two first components. DE and GA_3-200 treatments seemed to be positively correlated with TPL, NL, NLR, GP, LW and LL, positioning themselves on the positive side of the horizontal axis representing PC1. As seen in *Figure 7,* GP was positively and significantly associated with NLR (r = 0.78, p-value = 0.007 and NL (r = 0.68, p-value = 0.021), while negatively linked to CHb (r = -0.67, p-value = 0.024). It also shows that NLR is well correlated with TPL (r = 0.67, p-value = 0.024), NL (r = 0.70, p-value = 0.016), LW (r = 0.82, p-value = 0.002) and LW (r = 0.84, p-value = 0.001). A strong, positive and significant association was observed between LL and LW (r = 0.97, p-value = 0.000) and between Cha and CA. (r = 0.94, p-value = 0.000). On the other hand, CHb, which are very close to zero, shows the negative correlations with MO-0.5, WS and CO, placing themselves on the negative side of the horizontal axis reflecting PC1. PC2 was positively correlated with CA and MO-1.5, while negatively associated with FR-48 h, positioning themselves on the positive and negative sides of the vertical axis of PC2, respectively. As appeared in figure, we can conclude that DE and GA_3-200 had a positive impact on the following traits: TPL, NL, NLR, GP, LW and LL.

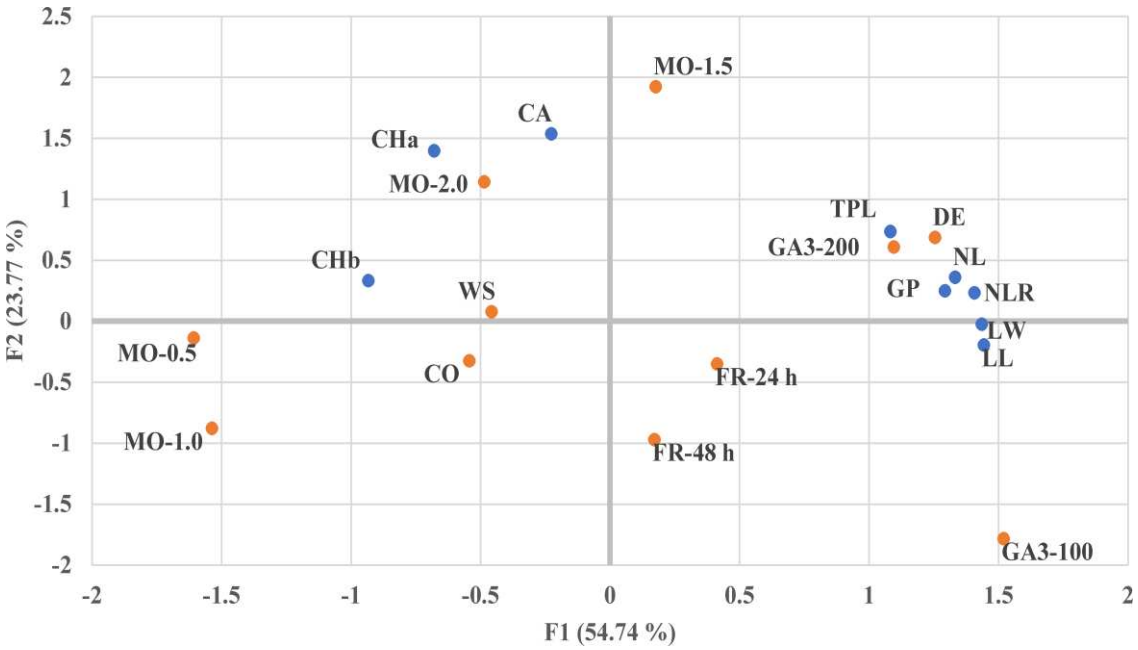

Figure 6. *PCA plot showing the distribution and relationship of different treatments and traits. CO: Control, DE: decoated seeds, FR-24 h: treatment of seeds by freezing for 24 h, FR-48 h: treatment of seeds by freezing for 48 h, GA_3-100: treatment of seeds by gibberellin (GA_3) at concentration of 100 ppm, GA_3-200: treatment of seeds by gibberellin (GA_3) at concentration of 200 ppm, MO-0.5: treatment of seeds by moringa leaf extract at concentration of 0.5 g/L, MO-1.0: treatment of seeds by moringa leaf extract at concentration of 1.0 g/L, MO-1.5: treatment of seeds by moringa leaf extract at concentration of 1.5 g/L, MO-2.0: treatment of seeds by moringa leaf extract at concentration of 2.0 g/L, and WS: treatment of seeds by distilled water*

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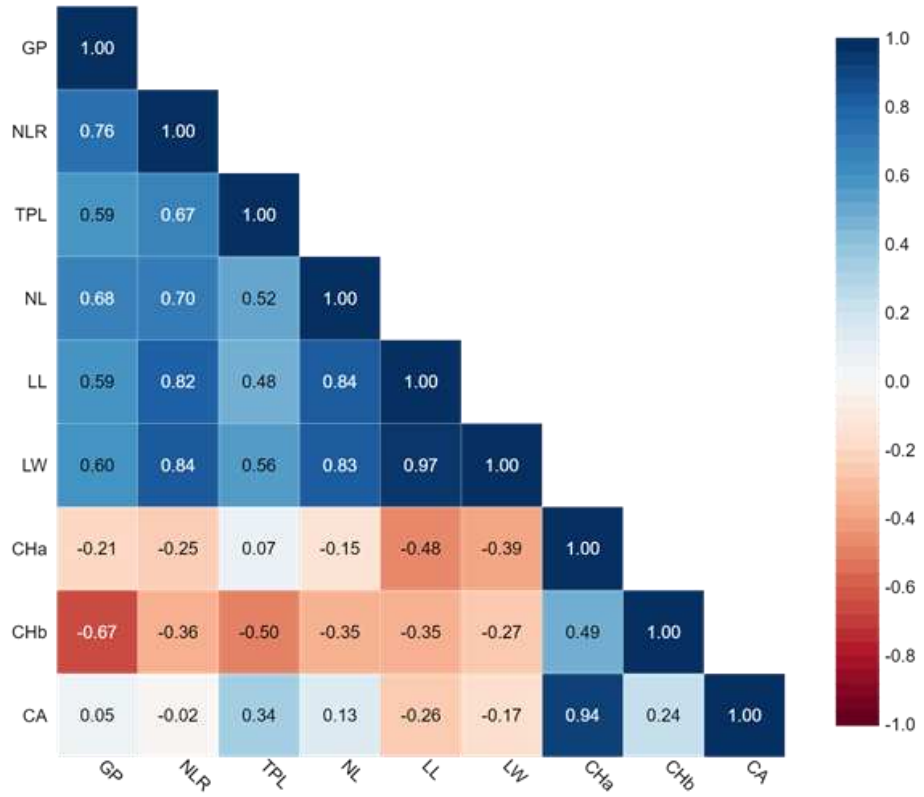

Figure 7. *Pearson correlation analysis of nine studied characters. GP: germination percentage, NLR: number of lateral roots, TPL: taproot length, NL: number of leaves, LL: leaf length, LW: leaf width, CHa: chlorophyll a content, CHb: chlorophyll b content, and CA: carotenoids content.*

Conclusion

Results of this research indicate that removing *the Gundelia* seed coat has improved its germination level and the number of roots. Though, GA_3 has been successful in growing the number of lateral roots, taproot length, leaf length and leaf width. In addition, the moringa extracts increased chlorophyll and carotenoid concentrations. Further analysis of the use of some plant extracts, such as *Glycyrrhiza glabra* and some germination enhancement agent, such as PEG at low concentrations, is advised.

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DOI: http://dx.doi.org/10.15666/aeer/1806_81598170

CHARACTERISTICS AND DIFFERENCES OF POLYPHENOL OXIDASE, PEROXIDASE ACTIVITIES AND POLYPHENOL CONTENT IN DIFFERENT POTATO (*SOLANUM TUBEROSUM*) TUBERS

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(Received 19th Jul 2020; accepted 7th Oct 2020)

Abstract. Potato enzymatic browning is a serious issue during processing. It not only affects the appearance of potato products but also reduces the nutritional value of potato tubers. In the present study, seven different potato cultivars' tubers were evaluated by measuring the browning index (BI) at different times after cutting. Initial PPO and POD activity and total phenol content, which related to enzymatic browning of plant tissues, were also determined. Results showed significant differences in these factors between the different cultivars. There was significant correlation between BI and PPO, POD activities, but no significant correlation with total phenol content. The activities of PPO and POD and the total phenolic content were higher in the epidermis and perimedullary tissues than pith tissues, which is consistent with their phenotypes. Further, qRT-PCR analysis revealed that the PPO genes were induced by wounding and were more highly expressed in browning-susceptible tubers than browning-resistant tubers, suggesting that browning-susceptible cultivars have higher StuPPO gene expression levels than browning-resistant cultivars. In addition, *StuPPO1 and StuPPO2* were the most highly expressed PPO genes in both browning-susceptible/resistant cultivar' tubers, indicating that *StuPPO1* and *StuPPO2* were the major contributors to the increase in PPO activity and the browning degree in potato tubers. This work suggests that the enzymatic browning of potato tubers is positively correlated with PPO and POD activity. *StuPPO1* and *StuPPO2* were the main genes responsible for enzymatic browning in potato tubers.
Keywords: *potato, enzymatic browning, polyphenol oxidase, peroxidase, polyphenol content, PPO genes, correlation, browning index*

Introduction

Enzymatic browning universally occurs in fruits and vegetables. It has a negative impact on the color, flavor, taste, nutritional properties, and shelf life of food products. Browning is considered to be one of the main causes of quality loss during post-harvest handling and processing (Stodt et al., 2014), leading to significant economic losses,

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especially in the agricultural product processing industry. It is estimated that up to 50% of losses for some tropical fruits are due to enzymatic browning (Whitaker, 1995). Clearly, the control and minimization of enzymatic browning is of great importance to agriculture and the horticultural industry.

Browning is a particularly serious issue for potato (*Solanum tuberosum* L.), which is grown worldwide and is the fourth most important crop in terms of food production after rice, maize, and wheat (FAOSTAT, 2018). It is estimated that enzymatic browning of potato tubers during harvest and storage alone lead to losses of up to $300 million annually in the USA and approximately $26 million in the UK (Shepherd et al., 2015). Potato tubers have a short storage life, and processing tubers to potato flour is the best way to prolong postharvest shelf life. Potato flour can also be conveniently processed into a variety of foods. However, because potato easily browns and anti-browning processes, including heating and the addition of food additives, are required, the cost of processing potato flour is much higher than that of processing flour from wheat and rice (Ali et al., 2016). This has limited the popularity of potato flour on the market. Because of its important influence on the post-processing of agricultural products, there is a need to fully understand the mechanism of enzymatic browning of potato tubers.

Numerous researchers have suggested that polyphenol oxidase (EC1.10.3.1; PPO) and peroxidase (EC 1.11.1.7; POD) are responsible for enzymatic browning (Jiang et al., 2004; Fortea et al., 2009; Escalante-Minakata et al., 2018). Both PPO and POD can take phenolic compounds as the reaction substrate. However, POD catalyzes phenol oxidation only in the presence of hydrogen peroxide. Therefore, PPO was thought to be the main enzyme in enzymatic browning due to the low H_2O_2 concentration in fruit and vegetable tissues (Richard-Forget and Gauillard, 1991; Yang et al., 2004). Richard-Forget and Gauillard (1991) indicated that in the presence of PPO, POD enhanced the phenol degradation and quinone forms. In other words, POD can enhance the occurrence of enzymatic browning.

PPO genes are widely distributed in plants, animals, and microorganisms (Mayer, 2006). In plants, most PPOs are predicted to be localized in plastids (Yoruk and Marshall, 2003) except for PtrPPO13 in poplar (*Populus trichocarpa*) and aureusidin synthase (AmAS1) in snapdragon (*Antirrhinum majus*), which are localized in the nucleus (Ono et al., 2006). By contrast, phenolic compounds, which are the substrates of PPOs, accumulate in vacuoles. Therefore, PPOs can act on phenolic compounds only when the cell membranes are broken. In the presence of oxygen, PPOs catalyze the oxidation of phenolic compounds into o-quinones. O-quinones are highly active and can polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments that precipitate on the surface of the wounded tissues, resulting in the loss of quality of fruits and vegetables (Bittner, 2006). Therefore, tissue browning always occurs after cell damage caused by mechanical damage during harvest, transport, processing, or some stress during storage (Li and Thomas, 2014).

PPO genes are usually present in multigene families in most organisms, and the numbers of PPO genes vary significantly among species (Tran et al., 2012). For instance, there are 11 PPO genes in black poplar (*Populus trichocarpa*), two in rice (*Oryza sativa*), eight in sorghum (*Sorghum bicolor*), four in grapevine (*Vitis vinifera*), and 19 in *Salvia miltiorrhiza* (Li et al., 2017). In potatoes, five PPO genes, *POTP1/POTP2* (M95196/M95197), *POT32* (U22921), *POT33* (U22922), and *POT72* (U22923), have been cloned (Hunt et al., 1993; Thygesen et al., 1995). All five of these PPO genes have tissue- and development-specific expression patterns and are induced

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by biological and abiotic stress. *POTP1* and *POTP2* are only expressed in young leaves and flowers and are most highly expressed in flowers (Thygesen et al., 1995; Thipyapong et al., 1995; Chi et al., 2014).

Although a number of studies about the enzymatic browning of potato tubers have been performed, there are few studies on enzymatic browning and the changes of total phenol content in potato tubers after fresh cutting. In the current study, the relationship between PPO, POD, total phenol and enzymatic browning, and its distribution characteristics in potato tuber were studied. A comparison of PPO and POD activity, total phenol content, and PPO gene expression levels between browning-susceptible (BS) and browning-resistant (BR) cultivars was also conducted. Our results will help us to obtain a deeper understanding of the characteristics of enzymatic browning of fresh-cut potato tubers but also have considerable potential in browning-resistant genetic breeding.

Materials and methods

Plant material and observation of the browning phenotype

Four browning-susceptible (BS) potato cultivars (Leshu 1 (L1), Dianshu 6 (D6), Zhongshu7 (Z7), and Zhongshu3 (Z3)) and three browning-resistant (BR) potato cultivars (Zhongshu1 (Z1), Zhongshu4 (Z4), and Xingjia2 (X2)) were randomly selected for this study. The potato cultivars were grown in the Baiyun District Experimental Field, Guangdong Province, China and stored at 4 °C after harvesting. Before treatment, the tubers were kept at room temperature for two days. Tubers of the same size and with no evidence of mechanical wounding were selected as experimental materials, and were washed with tap water to remove soil. Potato tubers were cut transversely (sliced transverse sections) using a sharp stainless-steel knife, and then kept indoors at 25 °C with ~80% relative humidity. At different times after cutting, images were taken with a camera and chromatic values (ΔE^*) were obtained using a colorimeter (NS810, 3nh, Shenzhen, China). All experiments were repeated and data from each experimental time point was derived from at least three separate samples of tubers. All the perimedullary tissues used for further determination below were 0.2-1 cm tissues under the tuber epidermis.

Determination of browning index (BI) and browning degree (BD)

For the manual inspection, ΔE^* of the browning of potato tubers were measured using a colorimeter (NS810, 3nh, Shenzhen, China) calibrated with a standard white plate. The browning index (BI) values of potato tubers were determined using a colorimeter (NS810, 3nh, Shenzhen, China). The ΔE^* was recorded at 0 h, 1 h, 2 h, 3 h, 5 h, 8 h, and 12 h after the tubers were cut transversely. In order to avoid errors caused by uneven browning, ΔE^* was averaged from five randomly selected points on the perimedullary zone of each potato tuber. The relative BI of each potato tuber was calculated as follows:

$$BI_\chi = \Delta E^*_\chi - \Delta E^*_0 \quad \text{(Eq.1)}$$

χ = 0, 1, 2, 3, 5, 8, 12 h after cutting.

The determination of browning degree (BD) was carried out as previously described (Chi et al., 2014) with some modifications. Five grams of fresh tissues including

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perimedullary and pith of tubers were homogenized with 30 ml of precooled distilled water in a mortar on ice, then centrifuged at 10,000 × g for 5 min at 4 °C. The supernatant was collected and incubated in a 25 °C water bath for 5 min. The absorbance was measured using an ultraviolet-visible spectrophotometer (UV-1800, Shanghai Spectrum Instruments Co., Ltd., China) at 410 nm. The absorbance value was used as BD value.

PPO and POD activity test

PPO and POD activities were assayed spectrophotometrically using a method based on that described by Cao et al. (2007) with some modifications. Three grams of fresh tissue from different sections (including epidermis, perimedullary, and pith) of the potato tubers were homogenized with 5.0 ml of 0.1 mol L^{-1} sodium acetate-acetic acid buffer (pH 5.5, 1 mM PEG, 4% (W V^{-1}) PVPP, 1% (W V^{-1}) Triton X-100) on ice. The homogenates were then centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was collected for the PPO and POD activity assays.

PPO activity assay: The reaction cuvette contained 4.0 ml 50 mM sodium acetate-acetic acid buffer (pH 5.5), and 1 ml 50 mM catechol. The reaction mixture was incubated in a 25 °C water bath for 10 min, 100 µl enzyme solution was added, and immediately after mixing, the absorbance at 410 nm was recorded every 10 s for 2 min.

POD activity assay: The reaction cuvette contained 3.0 ml 25 mmol L^{-1} guaiacol, 0.5 ml of the enzyme solution, and 0.2 ml 5 M hydrogen peroxide. Immediately after mixing, the absorbance at 470 nm was recorded every 10 s for 2 min.

An enzyme activity unit (U) was defined as an increase of 0.01 in absorbance per minute per gram fresh weight.

Content of total phenols

The total phenolic content was measured using the Folin-Ciocalteu procedure (Cen et al., 2016) with some modifications. Two grams of fresh potato tuber tissues were homogenized with 10 ml 95% cold alcohol in a precooled mortar. The homogenate was then transferred into a triangular flask and was ultrasonicated (100 W) in an ultrasonoscope (KQ5200, Kunshan Ultrasonic Instrument Co., Ltd., China) for 2 h. The homogenate was filtered into a 100 ml volumetric flask through four layers of gauze, 95% alcohol was added to a final volume of 100 ml, and the sample was thoroughly mixed. Next, 2 ml filtered homogenate, 2 ml 20% sodium carbonate, and 1.5 ml Folin-Ciocalteu reagent were added to a 50 ml volumetric flask, and distilled water was added to a final volume of 50 ml. After incubation for 30 min in a 55 °C water bath, the absorbance of the solution at 765 nm was measured using an ultraviolet-visible spectrophotometer (UV-1800, Shanghai Spectrum Instruments Co., Ltd., China). A standard curve for gallic acid was used to quantify the total phenolic content, which was expressed as gallic acid equivalents per g of fresh weight (mg g^{-1}).

Quantification of hydrogen peroxide

0.1 g of the perimedullary tissues of same tubers were used to determine the H_2O_2 content at different time (0, 10, 20, 30 min) after cut-wounding. H_2O_2 content was colorimetrically measured using a hydrogen peroxide assay kit (Suzhou Comin Biotechnology Co. Ltd, Suzhou, China) and calculated according to the manufacturer's instructions.

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RNA extraction

Potato tubers were cut transversely and kept in an incubator set at 25 °C and 80–90% humidity. Samples were obtained after 0 h, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Total RNA was extracted using the TiangenRNA extraction kit (Tiangen Biotech Co. Ltd, Beijing, China) according to the manufacturer's instructions and digested with RNase-free DNase I. The concentration of RNA was determined using a NanoDrop UV–visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was evaluated by 1% agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg RNA using the Tiangen trans kit (Tiangen Biotech Co. Ltd, Beijing, China) following the manufacturer's protocol and stored at -20 °C for real-time quantitative PCR (qRT-PCR).

qRT-PCR

qRT-PCR analysis was performed with first strand cDNA template and Taq DNA polymerase (TaKaRa, Dalian, China) using gene-specific primers (*Table 1*) (Wang et al., 2019). SYBR Premix Ex Taq^{TM} II (TaKaRa, Dalian, China) and the Bio-Rad CFX96TM qRT-PCR detection system (Bio-Rad, Hercules, CA, USA) were used according to the manufacturers' instructions. The PCR program was as follows: 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curve analysis was done after the PCR program was complete (65 °C to 95 °C, at increments of 0.5 °C). *EF1α* (Gene Bank accession AB061263), a housekeeping gene, was used as the internal control (Nicot et al., 2000). The $2^{-\Delta\Delta Ct}$ method was used to analyze relative mRNA abundance. The expression assay was repeated three times, and each assay was performed with three independent technical replicates. The primers for qRT-PCR analysis were designed using Primer 5 (Lalitha, 2000).

Table 1. *The primer sequences of qRT-PCR used in this study*

Transcript name	Gene name	Genomic sequence length	Primer name	Primer sequence (5' - 3')	Length (bp)	GC %	Tm (℃)
PGSC0003DMT400076054	*StuPPO1*	1770	T054F	TCCGTCCCAATTCTTCGGTG	93	55.0	60.0
			T054R	TGAACCGGGGTATGAGGGAT		55.0	60.0
PGSC0003DMT400048684	*StuPPO2*	1797	T684F	ATATCGCGACTGTTGATTTCC	133	42.9	56.5
			T684R	GTCGCACCTTCAATGGAGATA		47.6	57.8
PGSC0003DMT400048681	*StuPPO3*	1671	T681F	GGGGTACGATTACGCACCAA	121	55.0	60.1
			T681R	CGCAAGTGGGAATACCTCGT		55.0	60.1
PGSC0003DMT400048685	*StuPPO4*	1791	T685F	CCAATGGAAATATTACCTTTCT	119	32.0	59.5
			T685R	CATACTGCAACTGCTACTCTCC		50.0	52.0
PGSC0003DMT400076055	*StuPPO6*	1791	T055F	CTCCTGGTGGTCCAGCAGTT	124	60.0	59.6
			T055R	AGATGAGCAGGGGAACGGA		57.9	60.0
	EF1α		EF1α-F	ATTGGAAACGGATATGCTCCA	101	42.9	60.0
			EF1α-R	TCCTTACCTGAACGCCTGTCA		52.4	64.0

Statistical analysis

All experiments were repeated at least three times form three or more separate samples of tubers collected from twenty potato plants, and each tuber was measured at least twice. The means and standard deviations were calculated. one-way ANOVA was

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performed to ascertain the significance of difference of the mean, and Tukey's test for multiple comparisons for all experimental tests at 0.05 significant level. The correlation analysis between all individual discriminants associated with enzymatic browning was carried out using Pearson's correlation coefficients. Statistical analysis was performed using IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.).

Results

Browning characteristic of potato tubers at different times after cutting

Seven potato cultivars including four browning-susceptible (BS) cultivars (L1, D6, Z3, and Z7) and three browning-resistant (BR) cultivars (Z1, Z4, and X2) were selected to understand the browning process of potato tubers. Enzymatic browning was rapidly observed in BS cultivars after cutting and increased with time (*Fig. 1a*). Changes in browning were most obvious from 0 to 3 h. The D6 tubers were more prone to browning than other BS tubers (L1, Z3, and Z7) because they had obvious browning earlier than the others. Browning was not observed in the BR cultivars (Z1, Z4, and X2) until 8 h after cutting, and only occurred in the part of the tubers close to the epidermis (*Fig. 1a*). Consistent with the enzymatic browning phenotype of tubers, the BI of BS tubers significantly increased with time, while the BI of BR tubers did not have apparent changes (*Fig. 1b*).

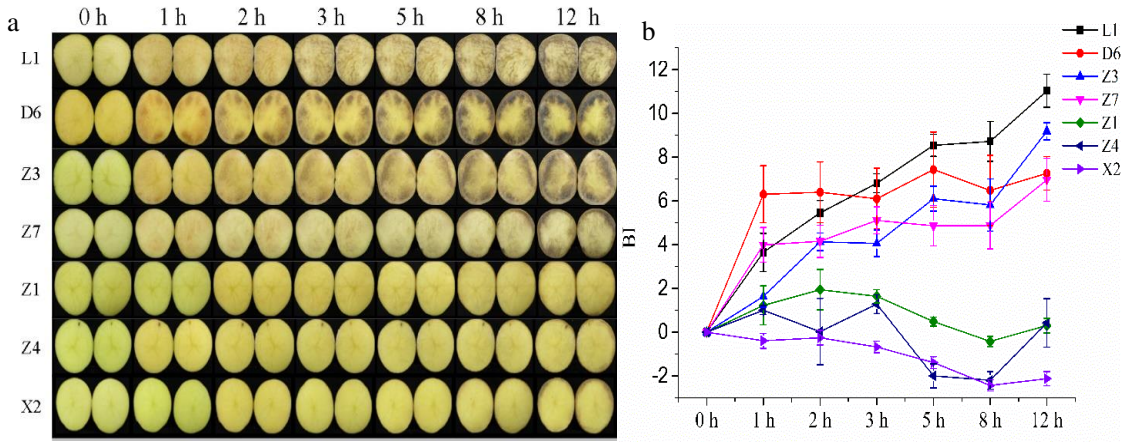

Figure 1. *The enzymatic browning process of potato tubers. a, the enzymatic browning of different potato cultivars' tubers after wounding for 0-12 h; b, the change of enzymatic browning index (BI) of different potato cultivars' tubers after wounding for 0-12 h. Values are means ± s.e. (n = 5)*

Cultivars showing higher browning in fresh-cut potato tubers exhibited higher PPO and POD activity

The PPO and POD activity and total phenol content had significant differences between different cultivars, especially between BS and BR cultivars (*Table 2*). The BS tubers had significantly higher PPO and POD activity than BR tubers ($P < 0.05$) except the POD activity between Z3 and X2 tubers, while there were no significant differences in total phenol content. The PPO and POD activity in BS tubers were 10.5-15.65 U and 15.74-37.6 U, respectively; and 5.77-9.68 U and 5.68-11.91 U in BR tubers. L1 had the

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highest PPO activity; D6 tubers had the highest POD and total phenol content; and X2 had the lowest PPO and POD activity. Though D6 tubers have lower PPO activity compared to other BS tubers, they had earlier browning than others, which may be due to D6 having the highest POD activity and total phenol content. In addition, though Z7 tubers had the lowest total phenol content, they browned easily, which might due to Z7 having higher PPO and POD activity. In contrast, though X2 (a BR cultivar) had higher total phenol content, it had the lowest PPO and POD activity. Those results suggested that higher PPO and POD activity were major contributors to a higher degree of enzymatic browning.

Table 2. *The BI, PPO and POD activity, and total phenol content of potato varieties. Values are means ± s.e. (n = 3)*

	Cultivar	BI	PPO activity (U)	POD activity (U)	Total phenol content (mg g^{-1})
BS	L1	7.00 ± 0.53a	15.65 ± 2.59b	16.82 ± 1.45c	0.87 ± 0.17cd
	D6	6.54 ± 0.31ab	10.50 ± 0.45c	37.60 ± 4.36a	1.21 ± 0.09a
	Z7	5.64 ± 0.96b	15.63 ± 0.99a	25.18 ± 8.82b	0.68 ± 0.07d
	Z3	4.391 ± 0.28c	12.73 ± 0.88b	15.74 ± 4.31cd	1.12 ± 0.09ab
BR	X2	-1.24 ± 0.11e	5.77 ± 0.25d	11.91 ± 0.66cd	0.90 ± 0.05cd
	Z1	1.59 ± 0.08d	9.68 ± 0.52c	8.87 ± 1.19de	0.97 ± 0.11bc
	Z4	1.26 ± 0.04d	6.22 ± 1.03d	5.68 ± 0.44e	0.76 ± 0.06cd

Different letters indicate significant difference between varieties test at $P < 0.05$. BS, browning-susceptible cultivars; BR, browning-resistant cultivars

The correlation analysis also showed that there was a positive correlation between BI_3 with PPO and POD activity, the correlation coefficients were 0.79 and 0.675, respectively. In addition, the cultivars with higher PPO activity also had higher POD activity, the correlation coefficient was 0.528 (*Table 3*).

Table 3. *The correlation analysis between BI, PPO, POD activity, and total phenol content*

	BI_3	PPO	POD	Total phenol content
BI_3	1			
PPO	0.790**	1		
POD	0.675**	0.528*	1	
Total phenol content	0.170	-0.004	0.248	1

*Indicates significance at the 0.05 level; ** indicates significance at the 0.01 level

PPO and POD activity and total phenol content in different sections of potato tubers

During the browning of potato tubers, the perimedullary zones of tubers always have a higher degree of enzymatic browning than pith zones (*Fig. 1a*). The BS cultivar Z3 and the BR cultivar X2 were used to study this further. BD was recorded as browning value. Consistent with the lower browning phenotype of pith tissues, the BD values of the pith tissues of both cultivars were all lower than those of the perimedullary tissues. In addition, the BD values of both perimedullary and pith tissues of Z3 were all

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obviously higher than those of X2 (*Fig. 2*), which is consistent with the more pronounced browning phenotype of Z3.

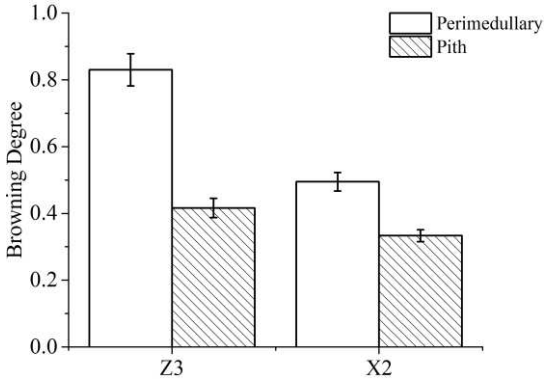

Figure 2. *The browning degree (BD) of perimedullary and pith tissues of potato tubers. Z3 and X2 are browning-susceptible (BS) and browning-resistant (BR) potato cultivars, respectively. Values are means ± s.e. (n = 3)*

Furthermore, the PPO and POD activity and the total phenol content in different sections of potato tubers were investigated (*Fig. 3*). As shown in *Figure 3b,* the PPO activity in the epidermis tissues of both cultivars was obviously higher than that in the perimedullary and pith tissues; PPO activity in the epidermis was 1.5 - 4.7 and 3.7 - 13.8 times higher than in the perimedullary and pith tissues, respectively. The pith tissues of tubers had the lowest PPO activity. Similar differences in POD activity between the three different sections were also observed; the activities were 12.5 - 29.9 and 26.3 - 42.7 times higher in epidermis tissues than in the perimedullary and pith tissues, respectively. In the Z3 tubers, there was a significant difference in POD activity between the perimedullary and pith tissues; while in X2 tubers, POD activity had no significant difference in between the perimedullary and pith tissues (*Fig. 3c*). In both cultivars, the epidermis tissues also had the highest total phenol content (*Fig. 3d*). There was no significant difference in the phenol content between the perimedullary and pith tissues in X2 tubers.

These results indicated that the potato tuber epidermis had the highest levels of PPO and POD activity and polyphenol content, while the pith tissues had the lowest. In browning cultivars, PPO and POD activity and total phenol content in three different parts of tubers were significantly different; in the non-browning cultivars, there were no significant differences in POD activity and total phenol content, but there were significant differences in PPO activity. The epidermis tissue of the BR cultivar X2 had higher PPO and POD activity than BS cultivar Z3, which suggests that the PPO and POD activity in epidermis tissues are not associated with the browning of potato tubers.

Determination of PPO and POD activity and total phenol content at different times in fresh-cut potato tubers

In order to understand the changes of PPO, POD and total phenol content in potato tubers after cut-wounding, and the difference between susceptible- and resistant-browning tubers, we randomly selected a susceptible-browning cultivar Z3 and a resistant-browning cultivar X2 for further study. Changes of PPO and POD activity and total phenol content

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in Z3 and X2 tubers at different times after potato cutting were determined. Overall, the magnitude of the variation of PPO and POD activity of Z3 were large compared to X2, and the magnitude of the variation of perimedullary tissues were small compared to pith tissues (*Fig. 4*). In Z3 tubers, the PPO activities of perimedullary tissues were high at 0 h after cutting, fluctuated until reaching a minimum at 12 h, and increased thereafter; the PPO activities of pith tissues started low and gradually increased until 20 h after cutting (*Fig. 4a*). In X2 tubers, the PPO activity of both perimedullary and pith tissues exhibited a similar trend with pith tissues of Z3 (*Fig. 4b*).

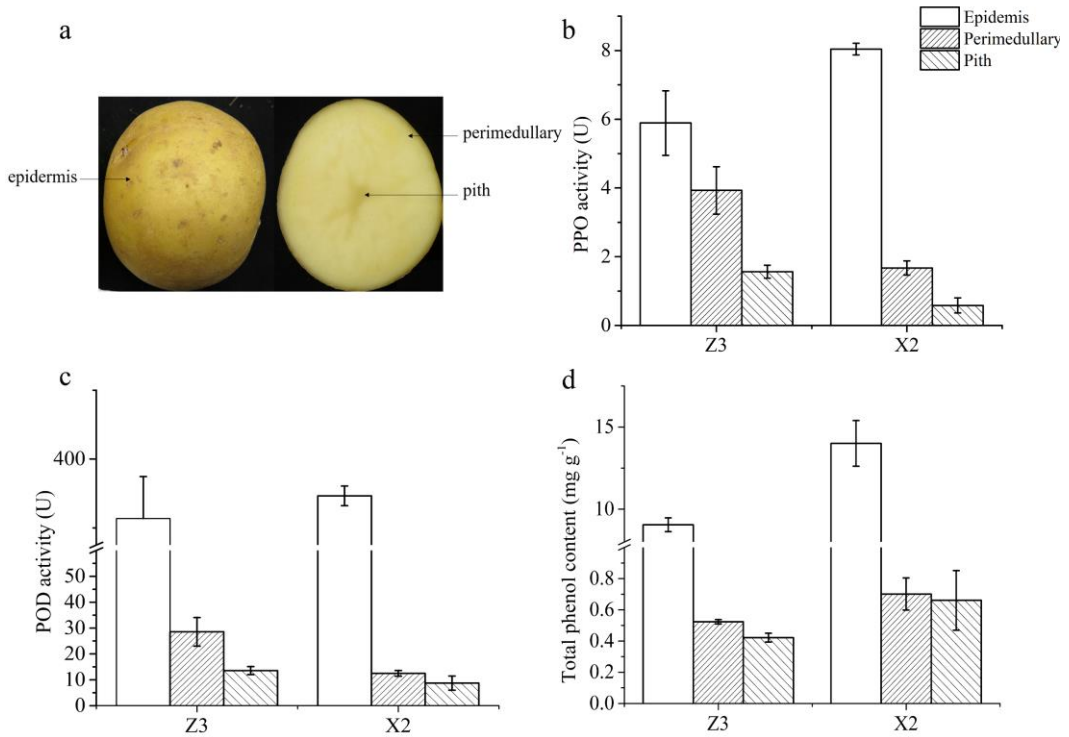

Figure 3. *The diagram of potato tubers' structure (a) and PPO activity (b), POD activity (c), total phenol content (d) in epidermis, perimedullary and pith tissue of potato tubers. Z3 and X2 are BS and BR potato varieties, respectively. Values are means ± s.e. (n = 3)*

The POD activity in perimedullary or pith tissues of Z3 and X2 had similar trends. In perimedullary tissues of Z3 and X2, the POD activity did not exhibit a significant increase until 12 h or 16 h after cutting, and then sharply increased to its maximum at 20 h after cutting (*Fig. 4c* and *d*).

The total phenol content in perimedullary tissues of Z3 gradually declined until 2 h after cutting, then rapidly increased and exhibited the highest level at 4 h. The total phenol content in pith tissues of Z3 and perimedullary and pith tissues of X2 exhibited similar trends. On the contrary, the total phenol content in these tissues increased until 1 h or 2 h after cutting, declined until reaching a minimum at 3 h or 8 h, and increased gradually thereafter (*Fig. 4e* and *f*).

In general, those parameters exhibited similar trends in pith tissues of Z3 tubers and both perimedullary and pith tissues of X2 tubers. The perimedullary and pith tissues of X2 tubers and the pith tissues of Z3 tubers do not brown easily compared to the perimedullary tissues

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of Z3 tubers. Those results suggested that the changes of PPO and POD activity and total phenol content are different in BS tissues and BR tissues after cutting.

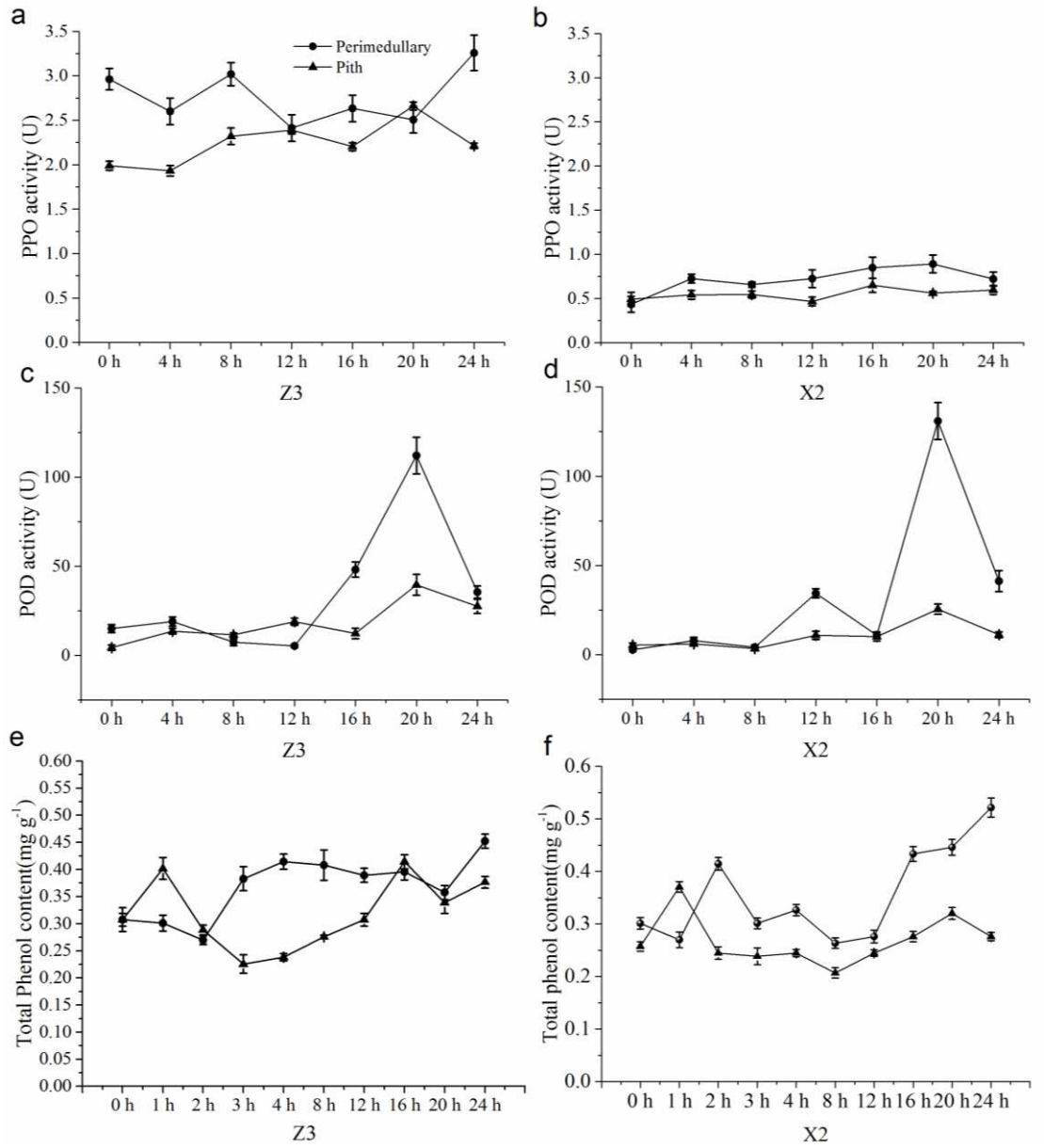

Figure 4. *The changes in PPO, POD activity and total phenol content of potato tubers at different times after wounding. Z3 and X2 are BS and BR potato cultivars, respectively. a and b, PPO activity change of Z3 and X2 tubers after wounding, respectively; c and d, POD activity change of Z3 and X2 after wounding, respectively; e and f, the total phenol content change of Z3 and X2 after wounding, respectively. Values are means ± s.e. (n = 3)*

The production of H_2O_2 in potato tubers induced by wounding

The H_2O_2 content in the perimedullary tissues of Z3 and X2 tubers were tested. Results showed that the highest content of H_2O_2 in potato tubers occur at 20 min after wounding (*Fig. 5*), which indicated that the production of H_2O_2 is induced by cut-wounding.

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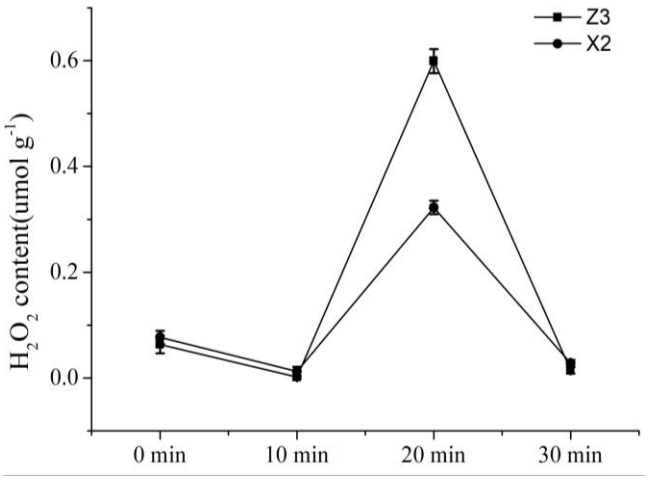

Figure 5. *The production of H_2O_2 in potato tubers after wounding. Z3 and X2 are BS and BR potato cultivars, respectively. Values are means ± s.e. (n = 3)*

StuPPO genes and expression profiles in wounded potato tubers

qRT-PCR was performed to determine the expression patterns of the four StuPPO genes, *StuPPO1-StuPPO4*, at different times after cutting. As shown in *Figure 6,* all four StuPPO genes expressed in potato tubers were up-regulated after cutting, and the highest level of expression was observed 12 h after cutting in Z3 tubers and 24 h in X2 tubers. Overall, the expression levels of all four StuPPO genes were higher in cultivar Z3 than in X2.

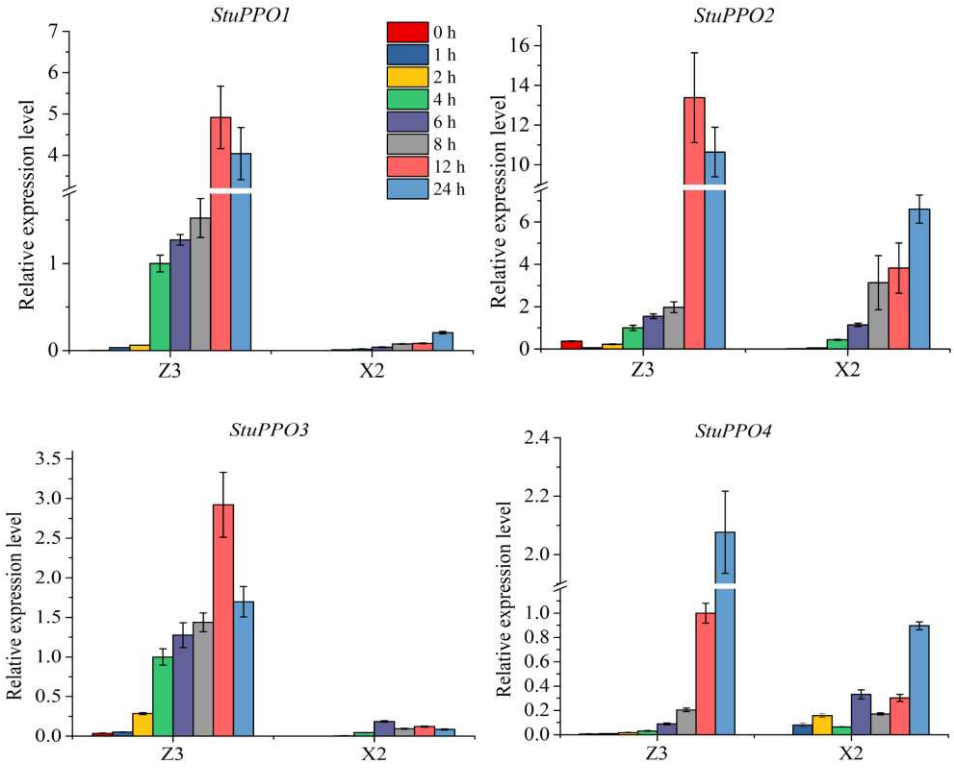

Figure 6. *The relative expression levels of five StuPPO genes in potato tubers at different times after wounding. Z3 and X2 are BS and BR potato cultivars, respectively. Values are means ± s.e. (n = 3)*

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In addition, the results also showed that the expression level of *StuPPO1* in Z3 tubers, and *StuPPO1* and *StuPPO2* in X2 tubers were much higher than the other StuPPO genes (*Fig. 7*), which indicated that *StuPPO1* and *StuPPO2* were more responsible for the enzymatic browning of Z3 and X2 tubers.

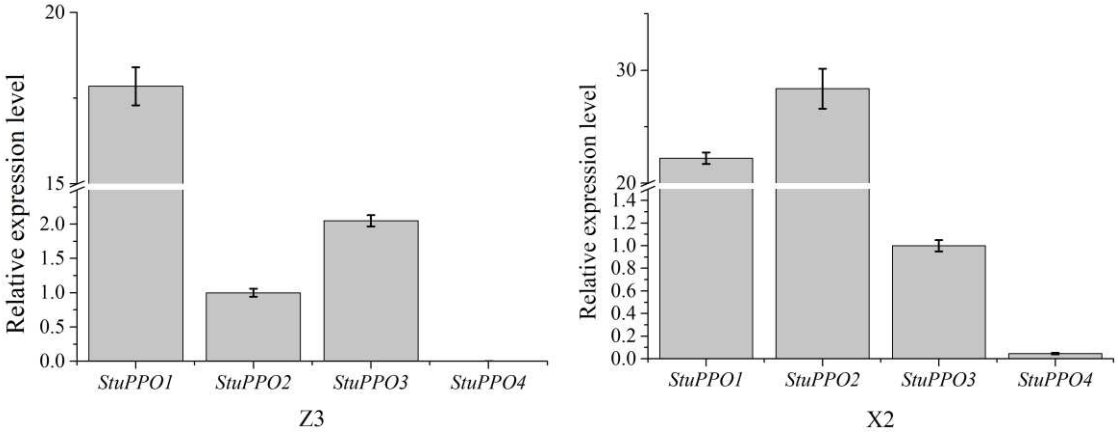

Figure 7. *The expression of StuPPO gene in the potato tubers after wounding for 12 h. Z3 and X2 are BS and BR potato cultivars, respectively. Values are means ± s.e. (n = 3)*

Discussion

In this study, seven potato cultivars, including four BS and three BR cultivars, were chosen to reveal contributions to enzymatic browning of potato tubers. The results showed that the BS tubers always have obvious browning after cutting for 1 h, and the browning deepened over time; while the BR cultivars have no significant browning until 8 h after cutting, and only brown in the tissues close to the epidermis. In general, during the process of tuber browning, changes between 0-3 h were most obvious after cutting (*Fig. 1*). The evaluation of enzymatic browning by a colorimeter has been applied to a variety of fruits and vegetables, which can reflect the degree of enzymatic browning more accurately (Cho et al., 2016; Rana et al., 2019). However, in potato tubers, there was a little starch precipitated on the cut surface over time, which results in deviations of ΔE^* values, especially in BR cultivar tubers, causing negative values of BI (*Table 1*). Similar results were found by Severini et al. (2003). They also believed that for a comprehensive color evaluation, it is advisable to combine image and colorimeter methods.

In our study, the color gauge evaluation is basically consistent with the photo evaluation (*Fig. 1*). The results of Severini et al. (2003) showed that the best hue angle values were obtained at a short time of treatment, which is consistent with our results. Therefore, we used the BI at 3 h (BI_3) after cutting to further analyze the correlation between BI with PPO and POD activity and total phenol content of tubers. The results showed that the BI_3 of potato tubers was significantly correlated with the PPO and POD activity, and has no significant correlation with total phenol content (*Table 2*), which consistent with the BS tubers always having higher PPO and POD activity than BR tubers (*Table 1*). The much higher activity of POD in D6 tubers might the reason for its more obvious browning at 1 h after cutting though it has lower PPO activity compared to other BS cultivars. Though H_2O_2 is required for POD to catalyze phenol compounds, the higher POD activity enhanced the browning occurrence of tubers (Richard-Forget and Gauillard, 1991). PPO was considered works as a promoter for POD activity it

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DOI: http://dx.doi.org/10.15666/aeer/1806_81718187

produces hydrogen peroxide when reacting with phenolic compounds (Tomás-Barberán and Espín, 2001). In our study, cut-wounding induced the production of H_2O_2 in potato tubers, which further promote the reaction of POD catalyze the polyphenol. Gong and Tian (2002) reported that the POD purified partially from litchi fruit peel can rapidly oxidize 4-methylcatechol in the presence of H_2O_2, supporting the involvement of POD in litchi enzymatic browning. Zhang et al. (2005) showed that POD activity in the pericarp increased consistently with skin browning index during storage of litchi fruit. However, Wen et al. (2020) suggest POD activity might not the key enzyme inducing discoloration of lotus root slices. Therefore, the role of POD in enzymatic browning of different species is different.

In most cultivars, the perimedullary zone of potato tubers was more prone to browning than the pith zone. As expected, PPO and POD activities were higher in the perimedullary tissues than in the pith tissues (*Fig. 2*). Interestingly, the epidermis tissues of potato tubers had the highest PPO and POD activities, which was also verified by Thygesen et al. (1995). In potato tubers, PPO is localized within amyloplasts (Thygesen et al., 1995). The perimedullary tissues of potato tubers have more amyloplasts (Borzenkova and Borovkova, 2003), which could explain why these tissues have a higher PPO activity than pith tissues. However, why the epidermis tissue has the highest PPO and POD activity requires further research. Obviously, the higher PPO and POD activities in the epidermis did not seem to be correlated with the browning potential of potato tubers. Moreover, the epidermis also had the highest total phenol content, which was consistent with the protective function of phenols against fruit bacterial infection (Ende et al., 2014; Jia et al., 2016). Higher levels of PPO and POD activity and phenol content can promote wound healing and decrease rotting (Yang and Bernards, 2006; Golubenko et al., 2007; Kumar et al., 2010; Shao et al., 2010; Lin et al., 2012). In addition, these findings also indicated that the epidermis of potato tubers can be used as a good source of phenols, which are beneficial to people's health (Albishi et al., 2013). Similarly, Albishi et al. (2013) also found that almost 50% of phenolic compounds in potato tubers are located in the tuber epidermis and adjoining tissues and the concentration of these compounds decreases towards the center of the tubers.

We further investigated the changes in PPO and POD activity and total phenol in potato tubers after cutting for different times. In our results, the variation trends of those parameters were similar in BR tissues, which include the pith tissues of Z3 tubers and perimedullary and pith tissues of X2 tubers, and were different between BS and BR tissues. In perimedullary tissues of Z3, the PPO activity gradually declined until 16 h after cutting, and subsequently increased. Due to the consumption of total phenol content after cutting, the initial PPO activity gradually declined and then increased, possibly because latent PPO was converted to its active form when wound stress was applied (Mishra and Gautam, 2016). In BR tissues, the PPO activity exhibited a slight, continuous increase. Unlike PPO, the POD activity did not change significantly during 0-12 h or 0-16 h after cutting. The highest POD activity was reached at 20 h after cutting, which may be the result of gene expression induced by wounding. In the perimedullary tissues of Z3 tubers, the total phenol content gradually declined in early stage after cutting, and subsequently increased due to the production of phenol compounds induced by wounding (Guan et al., 2020). A wound signal originates at the site of injury in lettuce (*Lactuca sativa* L.) leaf tissues and propagates into adjacent tissue where it induces a number of physiological responses including increased PPO, POD, and phenolic metabolism (Choi et al., 2005; Adams and Brown, 2007; Quarta et al., 2013).

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DOI: http://dx.doi.org/10.15666/aeer/1806_81718187

PPO genes are well known to be induced by stresses such as wounding and pathogen infection (Aziz et al., 2019). As the key enzyme of plant enzymatic browning, the induced expression of PPO genes further promotes the occurrence of tissue enzymatic browning. In our study, four genes expressed in potato tubers were up-regulated by wounding. The highest expression levels of StuPPO genes in Z3 tubers were observed 12 h after cutting, while in X2 tubers, the highest expression levels were observed 24 h after cutting. Those results indicate that the expression of StuPPO genes in potato tuber tissues increased more rapidly in the BS cultivars than in the BR cultivars. Consistent with the higher levels of StuPPO gene expression, more browning occurred in a shorter period of time in the BS cultivars than in the BR cultivars. In addition, our results also suggested that *StuPPO1* and *StuPPO2* were the major genes responsible for browning of potato tubers due to higher expression, which is consistent with the result of Chi et al. (2014).

Conclusions

In the present study, the PPO and POD activity were higher in BS cultivar tubers than in BR cultivar tubers, which is consistent with their phenotypes. Compared to pith tissues, the perimedullary tissues had higher PPO and POD activity and total phenol content than pith tissues, and the epidermis had the highest. Furthermore, the PPO and POD activity and expression of StuPPO genes were induced by wounding. The expression of *StuPPO1* and *StuPPO2* were highest in Z3 and X2 tubers. Taken together, our results show that the PPO and POD activity were major contributors in the enzymatic browning of potato tubers. Those results are helpful in understanding the enzymatic browning of fresh-cut potato tubers. In the future, it is necessary to focus on the physiological and molecular mechanisms of the difference in enzyme activity and total phenol content distribution in different parts of potato tuber.

Acknowledgements. We thank Xianzhou Nie of the Potato Research Centre, Agriculture and Agri-Food Canada for constructive comments on an earlier draft of the manuscript. This work was supported by the Guangdong natural science foundation [grant number 2018A030313336], the Science and Technology Planning Project of Guangdong Province [grant number 2017A020208033, 2017B020232002], the Special Fund for Agro-scientific Research in Public Interest of China (201503001), Science Fund opening project of key Laboratory of Crop Germplasm Innovation and Resource Utilization (National Key Laboratory Breeding Base jointly built by provincial and Ministry)(18KFXM03), the Fundamental Research Funds for the Laboratory of Biology and Genetic Improvement of Tuber and Root Crops, Ministry of Agriculture, China (NYBSL201701) and The Guangdong Modern Seed Industry Project (YueCaiNong[2017] #88).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81718187

PERFORMANCE OF DIFFERENT MUNGBEAN (*VIGNA RADIATA* (L.) WILCZEK) GENOTYPES AGAINST *MEGALUROTHRIPS DISTALIS* (THYSANOPTERA: THRIPIDAE) IN THE AGRO-ECOLOGICAL ZONE OF BHAKKAR, PAKISTAN

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(Received 25th Jul 2020; accepted 22nd Oct 2020)

Abstract. The experiment was conducted at the Arid Zone Research Institute (AZRI), Bhakkar, Pakistan during 2018 and 2019 on different mungbean genotypes to find a resistant to thrips and to determine the impact of weather factors on the *Megalurothrips distalis* population. In 2018 twenty-five mungbean genotypes were screened against *M. distalis*. During 2019 three genotypes each of resistant (13TM-04, AZRI-06, NM-16), susceptible (Dera-M, Sawat-I, NM-98) and intermediate reaction (NM-06, 12TM-03, NM-11) towards thrips infestation were chosen for final screening based on per flower thrips population density at 4 days intervals commencing from the first appearance of thrips up to maturity stage of the crop. Maximum HPSI, 15% was recorded in Dera –M while the minimum was recorded on 13TM-04 and NM-16 with 8% of each. Yield and the yield contributing characteristics were also assessed from which NM-16 and 13TM-04 exhibited statistically similar results and produced the highest yields at 935 and 902.8 kg/ha respectively. During both study years and on cumulative basis thrips population fluctuation had positive results and showed a significant correlation with maximum and average temperature. The average number of thrips during 2018, 2019 as well as both years combined showed a non-significant correlation with minimum temperature while negative and highly significant correlation was observed with average relative humidity and rainfall. Maximum temperature was the most important factor which contributed maximum i.e. 51.1% and 55.8% role followed by rainfall which showed 14.8% and 4% role during 2018 and 2019 respectively in the fluctuation of thrips population.
Keywords: *effect, weather factors, thrips population, HPSI (%), mungbean yield*

Introduction

Mungbean, *Vigna radiata* (L.) Wilczek, is an important pulse crop grown in Pakistan. It is able to grow efficaciously in rain fed regions (Anjum et al., 2006). It is a short duration and might face up to unfavorable environmental situations therefore is broadly distributed all around the world. It is the most preferred by the farmer due to high protein content and is easily adopted in the drier and warmer climates (Ratnasekera and Subhashi, 2015; Yaqub et al., 2010).

The yield and quality of mungbean is affected by different biotic (pest and diseases), abiotic (temperature, rainfall, relative humidity etc.) and phenological factors (flower and fruit drop). Among biotic factors insect pests often cause serious threat to mungbean production and increase the cost of production. Insect pests incidence significantly reduces mungbean yield and quality (Malik et al., 1994). Among the insect pests of mungbean thrips had gained major importance. The nature and extent of damage caused by thrips in mungbean varies in distinctive climatic areas because of various agroclimatic conditions. Thrips is the regular, early season and most important

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81898203

insect pest of mungbean which inflict heavy yield losses (Hossain et al., 2004). Flower shedding to the extent of 40-89% has been pronounced in mungbean (Kaul et al., 1976; Sinha, 1977). Flower infestation by thrips results in flower drop, pods deformation, grain quality deterioration and ultimately excessive yield reduction. Yield losses are estimated to be around 65% in mungbean due to thrips attack (Indiati, 2004).

The endurance, growth and reproductive capability of insect pests depends on the climatic factors (Morsello et al., 2014; Rosado et al., 2015). An insect development is influenced by the prolonged duration of low, high or sudden change in temperatures (Iqbal et al., 2010). Weather parameters have significant impact on thrips population (Pedigo, 2004). Different weather factors have positive association for the development and seasonal incidence of thrips (Soni and Dhakad, 2016). Economic loss due to thrips could be reduced by collecting such prediction information (Shuaib, 2004). Such studies are helpful in the establishment of predictive models of thrips attack on mungbean, so that the farmers can determine the correct time to start pest control (Da Silva et al., 2017; Herms, 2004; Rosado et al., 2015)

The varietal development in mungbean focused namely on the selection from germplasm. Several resistant donors for thrips were identified and used to transfer gene(s) for pest resistance. As a result, a number of high yielding genotypes with limited thrips tolerance were developed through interspecific hybridization. There is a need to intensify research for thrips tolerance through integration of desirable alleles from secondary and tertiary gene pool.

Due to the damage significance caused by thrips to the mungbean crop, an attempt was made to sort out the correlation of climate factors with thrips population in mungbean crop under agroclimatic conditions of Bhakkar to predict seasons of abundance. The present study objective was to determine resistant genotype of mungbean against thrips and fluctuation of thrips population in mungbean in relation to weather factors. The present study would be helpful for determining the peak period of thrips attack which in turn may be supportive in developing better thrips management strategy.

Materials and methods

Present research was conducted during the years 2018 and 2019 to find out the resistant genotype of mungbean and to work out the weather role in thrips population fluctuation. Twenty-five genotypes of mungbean were sown at AZRI, Bhakkar, Pakistan on the 10th May, 2018. Plot size was maintained 5 x 2.4 m with 30 cm row to row and 10 cm plant to plant distance. For further investigations three genotypes resistant, susceptible and showing intermediate reaction to thrips infestation were chosen. Thus, in total, nine genotypes were chosen from preliminary screening trial of 2018 based on per flower thrips population density. Sowing of the trial was done on May 10, 2019 in the same experimental area following Randomized Complete Block Design (RCBD) replicated thrice with the same plot size.

During both research years no plant protection measures were applied. Recommended agronomic practices were applied as and when required. From randomly selected five plants thrips population data was taken avoiding border rows from each treatment and three opened flowers of each randomly selected plant were observed at 4 days interval commencing from the first appearance of thrips up to maturity stage of the crop. Thus, from each plot a total of 15 flowers were observed for collection of

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DOI: http://dx.doi.org/10.15666/aeer/1806_81898203

thrips data. The collected flowers were immediately opened on white paper board and thrips numbers were recorded by counting number of thrips (through the use of magnifying lenses). The average thrips population on per flower basis for each genotype was determined by

$$X = \frac{X1+X2+X3\ldots\ldots\ldots\ldots+X14+X15}{15} \quad \text{(Eq.1)}$$

where: X = mean number of thrips flower^{-1} and $X_1 + X_2 + X_3 +$, $\cdots\cdots + X_{14} + X_{15}$ = number of observed flowers.

Mungbean genotypes were classified into three categories based on attack of mean number of thrips per flower:

3 - 3.99 thrips/flower = Resistance; 4 - 4.99 thrips/flower = Intermediate Resistance; 5 - 6 and above thrips/flower = Susceptible.

Host plant susceptibility indices (HPSIs)

The HPSI, based on thrips infestation at different genotypes of mungbean was determined using the following formula:

$$HPSI(\%) = 100 - \frac{B-A}{B} \times 100 \quad \text{(Eq.2)}$$

where A = thrips population in individual mungbean genotype and B = thrips population in all genotypes of mungbean on average per leaf or per flower basis (Ali and Farooq, 2019).

At maturity from five sample plants total number of flowers, flower shedding, total pods and deformed pods were recorded. Then their average number per plant data was calculated. After harvesting of the crop No. of seeds/pod was recorded from the five randomly selected pods. Then from their mean value average number of seed per pod was calculated. Weight of seeds per plant was also calculated from total number of seeds obtained from five randomly selected plants from each plot which were properly sun-dried and their weights were recorded. Then from their mean value weight of seeds per plant was calculated. 100 seed of each selected genotype was separately weighed and data was recorded. After harvesting crop was sun-dried and threshed. Seeds were properly sun-dried and their weights were recorded. Yield per plot was converted in to yield per hectare (Sagar et al., 2017). To find out the significance difference between the different treatments the data on aforementioned parameters were statistically analyzed. By using analysis of variance techniques (Steel and Torrie, 1997) thrips infestation data were analyzed and means were compared by (post-hoc test) using least significant difference test (LSD) at 5% significance level.

Meteorological data related to atmospheric temperature (maximum, minimum and average temperature), average R.H and total rainfall was obtained from the meteorological observatory of AZRI, Bhakkar for the relevant months and were correlated with per flower thrips population of various selected genotypes of mungbean with the objective to determine the impact of different abiotic factors on thrips population. These factors were processed for simple correlation (Steel et al., 1997) between population of thrips and abiotic factors and the factors which showed significant correlation with the thrips population were computed for multiple linear regression analyses of variance with the objective to find the percent role of these

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DOI: http://dx.doi.org/10.15666/aeer/1806_81898203

factors individually as well as in their possible combinations through steps. The data obtained during 2018 and 2019 were transformed to square root transformations and were statistically analyzed. By using Multiple Linear Regression Equation, the combined effect of the abiotic factors on thrips population during 2018 and 2019 were calculated.

Results

Varietal performance of different mungbean genotypes against Megalurothrips distalis during 2018

The results (*Table 1*) showed the mean number of thrips per flower population during 2018 and 2019. It is evident from the results that during 2018 on the basis of mean population of thrips maximum thrips population were observed on Sawat-1 and Dera-M which were at par with each other with 6.03 and 6.00 thrips per flower followed by NM-98 with 5.57 thrips per flower. The minimum 3.07 thrips/flower population was recorded in 13TM-04 which was statistically at par with the genotypes NM-16 and AZRI-06 which showed 3.27 and 3.40 thrips per flower population each during 2018. The genotypes NM-11, 12TM-03 and NM-06 showed 4.77, 4.67 and 4.53 thrips per flower population, respectively which were at par with each other. On the basis of mean number of *M. distalis* population during 2018 all the mungbean genotypes were categorized in descending order as follows: Sawat-1 > Dera-M > NM-98 > TM-1626 > TM-1611 > TM-1609 > Chakwal-06 > Inqilab-M > TM-1607 > TM-1615 > TM-1601 > NM-11 > 12TM-03 > NM-06 > Bahawalpur mung-17 > NCM-13 > Sona-M > NM-92 > TM-1418 > TM-1627 > 13TM-14 > 09TM-11 > AZRI-06 > NM-16 and 13TM-04.

Varietal performance of different genotypes against thrips during 2019

From the results of preliminary screening trial, three highly susceptible genotypes (Dera-M, Sawat-1 and NM-98) three intermediate response genotypes (NM-11, NM-06 and 12TM-03) and three with the lowest thrips population showing resistant response (13TM-04, NM-16 and AZRI-06) were chosen for final screening trial during 2019. The results (*Table 1*) showed that maximum number of thrips 6.27 per flower was observed on Dera-M followed by Sawat-1 and NM-98 with 5.90 and 5.73 thrips per flower respectively which were statistically at par with each other. While minimum 3.50 thrips per flower population was recorded in 13TM-04 followed by NM-16 with 3.53 thrips per flower which was statistically similar with AZRI-06 with 3.87 thrips per flower. The genotype 12TM-03 showed 4.47 thrips per flower which was statistically similar with NM-06 with 4.73 thrips per flower followed by NM-11 which showed 4.87 thrips.

Host plant susceptibility indices (HPSI%)

The results (*Table 2*) revealed the HPSI (%), based on per flower thrips population on different genotypes of mungbean during 2018 and 2019. The genotype Dera-M showed maximum 15% HPSI during 2018 and 2019 on per flower basis followed by Sawat-1 which showed 15% HPSI% during 2018 and 14% during 2019. While NM-98 showed 13% HPSI during both years. These three genotypes were found comparatively susceptible to thrips infestation. Genotypes 12TM-03 showed 11% and 10% HPSI% during 2018 and 2019 respectively while NM-11 showed 11% HPSI each during both years. NM-11 showed 12% during 2018 and 11% HPSI during 2019. The minimum 7%

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HPSI was recorded in 13TM-04 during 2018 while it was 8% during 2019 followed by NM-16 which showed 8% HPSI of each during both years. While AZRI-06 showed 8% during 2018 and 9% HPSI during 2019.

Table 1. *Mean comparison of the data regarding thrips population on various genotypes of Mungbean, at different dates of observations during 2018 and 2019*

Genotypes	Thrips per flower during	
	2018	**2019**
Dera-M	6.00 a *	6.27 a
Sawat-1	6.03 a *	5.90 b
NM-98	5.57 b *	5.73 b
TM-1626	5.43 bc	
TM-1611	5.40 bc	
TM-1609	5.37 bc	
Chakwal-06	5.33 bc	
Inqilab-M	5.13 cd	
TM-1607	4.87 de	
TM-1615	4.87 de	
TM-1601	4.83 def	
NM-11	4.77 efg **	4.87 c
12TM-03	4.67 efgh **	4.47 d
NM-06	4.53 efgh **	4.73 cd
Bhawalpur M-17	4.50 fgh	
NCM-13	4.43 gh	
Sona-M	4.37 h	
NM-92	3.93 i	
TM-1418	3.83 ij	
TM-1627	3.70 ijk	
13TM-14	3.70 ijk	
09TM-11	3.50 jkl	
AZRI-06	3.40 klm ***	3.87 e
NM-16	3.27 lm ***	3.53 ef
13TM-04	3.07 m ***	3.50 f
LSD value @5%	0.36	0.34

Means sharing similar letters are not significantly different by LSD test at P = 0.05
* = Susceptible genotypes
** = Intermediate resistant genotypes
*** = Resistant genotypes

The results (*Table 2*) showed the HPSI (%) on cumulative basis of both study years 2018-19 which showed that maximum HPSI 15% was recorded in Dera-M followed by Sawat-1 and NM-98 with14% and 13% respectively which showed that these three genotypes are more susceptible to thrips infestation. While 12TM-03, NM-06 and NM-11 showed 11% HPSI of each genotype which are found intermediate response. The minimum HPSI was recorded on 13TM-04 and NM-16 with 8% of each followed by AZRI-06 which showed9% HPSI which showed the resistant response to thrips infestation on per flower basis.

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Table 2. *Host plant susceptibility indices (%) based on mean percentage of thrips on different genotypes of mungbean during 2018 and 2019*

Genotypes	Host plant susceptible indices (%) based on per flower during		
	2018	2019	Cumulative of 2018 and 2019
Dera-M	15%	15%	15%
Sawat-I	15%	14%	14%
NM-98	13%	13%	13%
12TM-03	11%	10%	11%
NM-06	11%	11%	11%
NM-11	12%	11%	11%
AZRI-06	8%	9%	9%
NM-16	8%	8%	8%
13TM-04	7%	8%	8%

Response of different mungbean genotypes against thrips attack

Response of different mungbean genotypes against thrips was assessed by comparing the total flowers, flower shedding, total pods and deformed pods per plant basis. The results (*Fig. 1*) showed total number of flower and flower shedding per plant of different mungbean genotypes. It is evident from the present findings that different genotype behaved differently in the case of total number of flowers on per plant basis. Results showed that the highest number of flowers 125.93 per plant were observed in Dera-M which was at par to Sawat-1 and 12TM-03 with 116.60 and 112.80 flowers per plant, respectively. While 13TM-04, NM-98, NM-06, NM-11 and NM-16 behaved statistically at par from each other with 102.53, 100.93, 100.27, 100.13 and 98.13 flowers per plant, respectively. Minimum 85.47 flowers per plant were observed in AZRI-06 which was significantly different with all tested genotypes. The results (*Fig. 1*) also revealed that different genotypes had significant impact on number of flower shedding which may be due to thrips preference. Maximum 99.53 flower per plant shedding was observed in Dera-M which was significantly the highest than other tested genotypes followed by Sawat-1 with 87.67 flower shedding per plant. While minimum flower shedding per plant was recorded in AZRI-06 and 13TM-04 which was not significantly different from each other with 54.73 and 59.87 flowers shedding per plant, respectively. While genotypes NM-06 and NM-11 showed 74.47 and 74.27 flowers shedding per plant, respectively which was statistically at par with each other.

The results (*Fig. 2*) showed the total number of pods and deformed pods on per plant basis of different mungbean genotypes. It is clear from the results that utmost 34.60 pods per plant was observed in 13TM-04 which was statistically similar with NM-16 with 34.80 pods per plant followed by AZRI-06 with 28.73 pods per plant. Minimum 16.07 pods per plant were observed in NM-98 which was statistically at par with Sawat-1 and Dera-M with 18.53 and 18.67 pods per plant. While 12TM-03, NM-11 and NM-06 behaved statistically similar with 22.93, 22.20 and 21.20 total pods per plant, respectively. *Figure 2* also showed that maximum deformed pods 4.93 and 4.33 were recorded in Dera-M and Sawat-1 and were statistically at par with each other. However minimum 2.07 deformed pods per plant were observed in 13TM-04 which was statistically at par with NM-16 and AZRI-06 with 2.33 and 2.60 deformed pods per plant, respectively. The genotypes NM-06, NM-98, 12TM-03 and NM-11 showed 3.27,

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3.07, 2.87 and 2.87 deformed pods per plant, respectively and were statistically at par from each other.

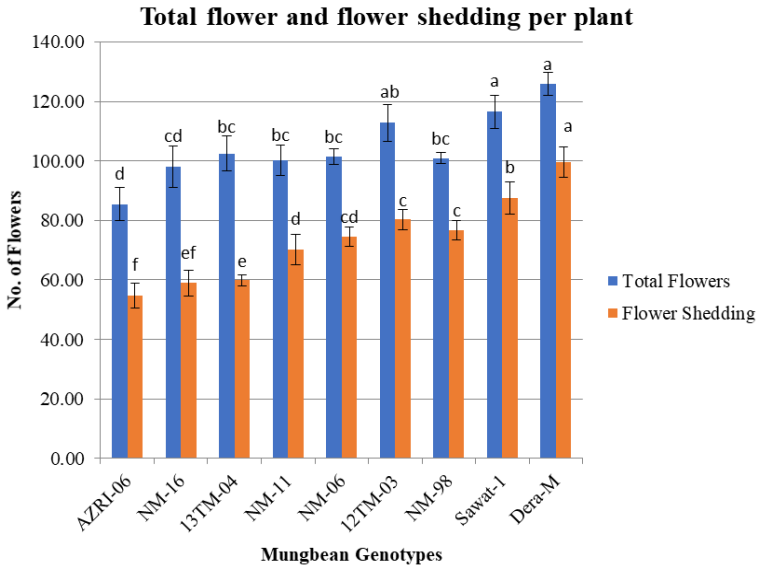

Figure 1. *Response of different mungbean genotypes on number of flowers per plant*

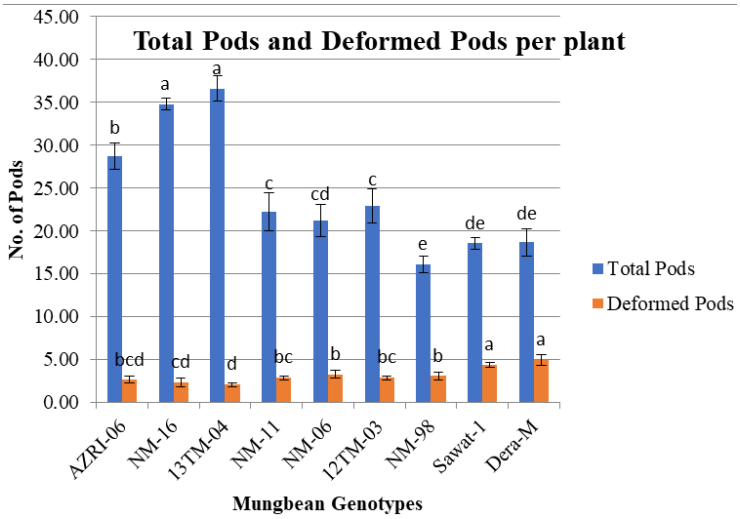

Figure 2. *Response of different mungbean genotypes on number of pods per plant*

Role of different genotypes/varieties on the yield and the yield contributing characters

The results (*Table 3*) showed the yield (kg/ha) and yield contributing characters of different mungbean genotypes/varieties which revealed that 13TM-04 showed maximum number 8.40 seeds per pod which was closely followed by NM-16 with 7.73 seeds per pod and were not significantly different from each other. While minimum 5.80 seeds per pod was observed in Dera-M which was not significantly different from NM-98 and Sawat-1 with 6.60 and 6.67 seeds per pod, respectively. While the genotypes AZRI-06, 12TM-03, NM-11 and NM-06 showed 7.47, 7.47, 7.07 and 6.80 seeds per pod, respectively and were not significantly different from each other.

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The outcome (*Table 3*) also revealed that maximum seed weight 4.77 g per plant was recorded in 13TM-04 which was not significantly different from NM-16 and AZRI-06 which showed 4.22 and 4.31 g seed weight per plant, respectively. Minimum 2.53 g seed weight per plant was recorded in Dera-M which was significantly different from the rest of the other tested genotypes followed by NM 98, Sawat-1 and NM-06 with 3.20, 3.43 and 3.70 g seed weight per plant, respectively, which were statistically at par with each other. The results (*Table 3*) also exposed that no significant difference exists among the, NM-16, 13TM-04, AZRI-06 and NM-11 genotypes of mungbean with 5.57, 5.51, 5.42 and 5.27 g maximum hundred seed weight, respectively. While minimum 3.80 hundred seed weight was recorded in Dera-M which was not significantly different with NM-98 and Sawat-1 with 4.04 and 4.31 g hundred seed weight respectively. The genotypes NM-06, 12TM-03 showed 4.72, 4.49 g hundred seed weight, respectively and did not differ significantly from each other.

The results (*Table 3*) also depicted that the per hectare yield significantly differ in the different selected mungbean genotypes. The highest yield 935 kg/ha was recorded in NM-16 which was at par to 13TM-04 which results 902.8 kg/ha yield and were significantly different from all other selected genotypes of mungbean followed by AZRI-06 and NM-11 with yield 759.4 and 675.0 kg/ha yield, respectively. However minimum yield was recorded in Dera-M followed by NM-98 with 333.3 and 466.7 kg/ha respectively which were significantly different from each other.

Table 3. *Effect of different genotypes/varieties on the yield and the yield contributing characters of mungbean*

Treatments	No. of seed/pod	Seed weight/plant (g)	100 seed weight (g)	Yield/plot (g)	Yield kg/ha
AZRI-06	7.47 bc	4.22 ab	5.42 a	455.7 b	759.5 b
NM-16	7.73 ab	4.31 ab	5.57 a	561.0 a	935.0 a
13TM-04	8.40 a	4.77 a	5.51 a	541.7 a	902.8 a
NM-11	7.07 bc	4.08 bc	5.27 ab	405.0 c	675.0 c
NM-06	6.80 c	3.70 bcd	4.72 bc	369.0 cd	615.0 cd
12TM-03	7.47 bc	3.98 bc	4.49 cd	345.7 d	576.1 d
NM-98	6.60 cd	3.20 d	4.04 de	280.0 e	466.7 e
Sawat-1	6.67 cd	3.43 cd	4.31 cde	332.7 d	554.2 d
Dera-M	5.80 d	2.53 e	3.80 e	200.0 f	333.3 f
LSD $_{0.05}$	0.88	0.66	0.56	42.77	71.28

Means with the similar letter(s) are not significantly different from each other at P = 0.05

Thrips population fluctuation versus climate factors, during 2018 and 2019

The results (*Table 4*) revealed the thrips population fluctuation on per flower basis versus weather conditions. During 2018, the maximum thrips population were observed on the dates, the 26th June, with 5.099 thrips per flower at 43.50 °C, 26.88 °C and 25.19 °C maximum, minimum and average temperature respectively and with 44.125% average R.H with rainfall nil which declined to 4.83 thrips per flower during the 30th June, 2018 and again increased to 5.07 thrips per flower on the date 04th July 2018 at 41.50 °C, 26.75 °C and 34.13 °C maximum, minimum and average temperature respectively and with 58% average relative humidity. While during 2019, the maximum

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5.11 thrips per flower were observed during the 26th, June 2019 which gradually increased to 5.22 thrips per flower during the 30th, Jun2 2019 and a peak population of 5.58 thrips per flower was observed during the 04th, July 2019 at 45.50 °C, 28.25 and 36.88 °C maximum, minimum and average temperature respectively and with 45.50% average R.H with rainfall nil.

Table 4. *Data regarding meteorological observations on various weather factors and thrips population during 2018-19*

Sr.No.	Date	Temperature °C			Average R.H (%)	Average R.F (mm)	Average thrips/flower
		Maximum	Minimum	Average			
1	14.06.18	45.125 (6.755)	29.625 (5.489)	37.375 (6.154)	46.250 (6.837)	0.000 (0.707)	4.562 (2.250)
2	18.06.18	43.500 (6.633)	28.500 (5.385)	36.000 (6.042)	47.500 (6.928)	0.000 (0.707)	4.763 (2.294)
3	22.06.18	41.750 (6.500)	26.750 (5.220)	34.250 (5.895)	49.250 (7.053)	2.500 (1.732)	4.341 (2.200)
4	26.06.18	43.500 (6.633)	26.875 (5.232)	35.188 (5.974)	44.125 (6.680)	0.000 (0.707)	5.099 (2.366)
5	30.06.18	39.750 (6.344)	26.000 (5.148)	32.875 (5.777)	54.500 (7.416)	0.000 (0.707)	4.831 (2.309)
6	04.07.18	41.500 (6.481)	26.750 (5.220)	34.125 (5.884)	58.000 (7.649)	0.000 (0.707)	5.067 (2.359)
7	08.07.18	37.750 (6.185)	25.875 (5.136)	31.813 (5.684)	55.750 (7.500)	17.880 (4.287)	4.028 (2.128)
8	12.07.18	45.250 (6.764)	(29.375) (5.466)	37.313 (6.149)	48.250 (6.982)	0.000 (0.707)	4.999 (2.345)
9	16.07.18	42.500 (6.557)	29.750 (5.500)	36.125 (6.052)	56.125 (7.525)	0.000 (0.707)	4.873 (2.318)
10	20.07.18	38.500 (6.245)	26.000 (5.148)	32.250 (5.723)	68.000 (8.276)	13.500 (3.742)	4.144 (2.155)
11	24.07.18	40.500 (6.403)	28.925 (5.424)	34.713 (5.934)	68.125 (8.284)	0.250 (0.866)	4.503 (2.237)
12	28.07.18	39.000 (6.285)	26.500 (5.196)	32.750 (5.766)	68.750 (8.322)	4.875 (2.318)	4.144 (2.155)
13	01.08.18	39.500 (6.325)	27.125 (5.256)	33.313 (5.815)	60.625 (7.818)	0.000 (0.707)	4.273 (2.185)
1	14.06.19	43.500 (6.633)	28.870 (5.419)	36.185 (6.057)	46.500 (6.856)	0.000 (0.707)	4.867 (2.317)
2	18.06.19	44.380 (6.699)	25.500 (5.099)	34.940 (5.953)	47.630 (6.938)	0.500 (1.000)	4.931 (2.330)
3	22.06.19	38.250 (6.225)	23.000 (4.848)	30.625 (5.579)	54.000 (7.382)	3.750 (2.062)	4.751 (2.291)
4	26.06.19	40.380 (6.394)	26.130 (5.160)	33.255 (5.810)	55.130 (7.459)	1.500 (1.414)	5.111 (2.369)
5	30.06.19	42.500 (6.557)	26.000 (5.148)	34.250 (5.895)	52.880 (7.306)	0.750 (1.118)	5.223 (2.392)
6	04.07.19	45.500 (6.782)	28.250 (5.362)	36.875 (6.114)	45.500 (6.782)	0.000 (0.707)	5.583 (2.466)
7	08.07.19	39.130 (6.295)	25.630 (5.112)	32.380 (5.734)	66.880 (8.209)	8.250 (2.958)	4.763 (2.294)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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8	12.07.19	41.000 (6.442)	28.130 (5.351)	34.565 (5.922)	62.000 (7.906)	0.000 (0.707)	5.215 (2.391)
9	16.07.19	39.250 (6.305)	25.630 (5.112)	32.440 (5.739)	62.000 (7.906)	1.250 (1.323)	4.528 (2.242)
10	20.07.19	39.130 (6.295)	26.750 (5.220)	32.940 (5.783)	65.250 (8.109)	0.550 (1.025)	4.215 (2.171)
11	24.07.19	41.880 (6.510)	29.000 (5.431)	35.440 (5.995)	57.000 (7.583)	0.000 (0.707)	4.647 (2.269)
12	28.07.19	37.950 (6.201)	28.000 (5.339)	32.975 (5.786)	66.630 (8.193)	0.250 (0.866)	4.338 (2.200)
13	01.08.19	37.630 (6.175)	25.100 (5.060)	31.365 (5.645)	68.380 (8.299)	14.500 (3.873)	3.800 (2.074)

Numbers in parenthesis are square root transformed

The data (*Fig. 3*) showed the thrips population and climate factors relationship at various dates of observations on collective basis for both research years 2018 and 2019. The outcome showed that a maximum thrips population (5.32/flower) was observed, on the 04th July, at 43.50 °C, 27.50 °C and 35.50 °C maximum, minimum and average temperature respectively with 51.75% relative humidity and with nil rainfall.

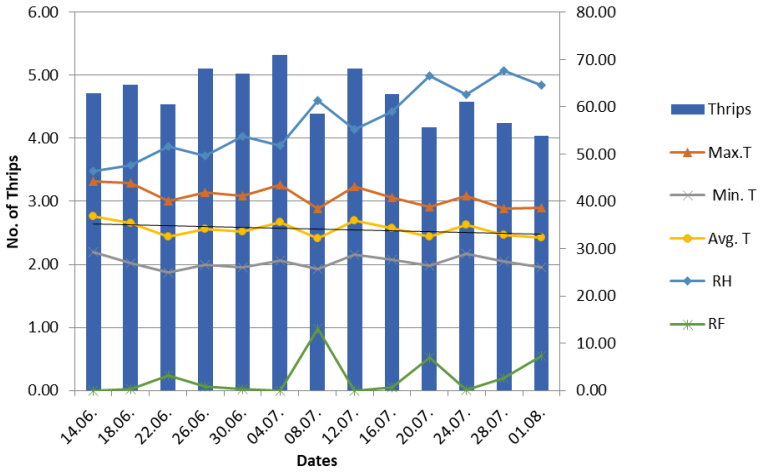

Figure 3. *Thrips population fluctuation versus weather factors during 2018-19 on Cumulative basis*

Weather factors influence on thrips population

The data (*Table 5*) revealed that during both research years 2018-19 maximum temperature and average temperature showed a positive and significant correlation with highly significant correlation with (r) value 0.90 and 0.668 respectively on cumulative basis, whereas average relative humidity and rainfall during both years showed negative and significant correlation with the thrips population with highly significant correlation with (r) value 0.732 and 0.750 respectively on collective basis. Whereas, non significant correlation was found with minimum temperature during both years and on cumulative basis.

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Table 5. *Correlation coefficients (r) between thrips population per flower basis on mungbean, and various weather factors, during 2018 and 2019*

Weather factors	Per flower		
	2018	2019	Cumulative
Max. temperature (°C)	0.715**	0.747**	0.90**
Min. temperature (°C)	0.413 ns	0.250 ns	0.285 ns
Average temperature (°C)	0.644**	0.633*	0.668**
Average relative humidity (%)	-0.555*	-0.715**	-0.732**
Average rain fall (mm)	-0.761**	-0.553*	-0.750**

* = Significant at P ≤ 0.05; ** = Significant at P ≤ 0.01; ns = Non Significant

The weather factors multiple effects on thrips population

The results (*Table 6*) showed the weather factors multiple effects on per flower thrips population, during 2018 and 2019. According to findings during 2018 maximum contribution (51.1%) was observed by maximum temperature in thrips population fluctuations followed by rainfall (14.8%), average temperature (3.0%) and average relative humidity (1.4%). The R^2 value was observed 70.3%, when the effects of all climate factors on the per flower thrips fluctuation on mungbean was analyzed together for the year 2018 and the equation was good fitted. In 2019 maximum contribution was observed by maximum temperature (55.8%) role in thrips per flower fluctuation of population followed by rainfall (4%), average relative humidity (1.2%) and average temperature (1.0%). The R^2 value of all climate factors for the year 2019 was observed 62.0%, on the per flower thrips population fluctuation on mungbean and the equation was not fitted. The regression model regarding the impact of weather factors on thrips population in mungbean varieties revealed that maximum temperature was the most important factor which contributed maximum i.e. 51.1% and 55.8% role followed by rainfall which showed 14.8% and 4% role during 2018 and 2019 respectively in the fluctuation of thrips population. The contribution of other weather factors to the thrips population variation was low (*Table 5*).

Discussion

Results of the present findings showed that out of twenty five tested genotypes against thrips infestation three genotypes namely 13TM-04, NM-16 and AZRI-06 showed resistant response in two years trial which are in confirmatory with Sreekanth et al. (2002) who reported that among thirty eight genotypes, four genotypes of mungbean constantly showed resistance to thrips namely LGG460, 480, 491 and 582 during 2000 and 2001. The results of the present findings are in accordance with Kooner and Malhi (2004) and with Brar et al. (2004) who obtained similar results although they used different mungbean varieties. The present results are in confirmatory with Rani et al. (2008) who found that out of 12 tested mungbean entries against thrips, less attack of thrips was recorded on MGG362 and MGG365 as compared to check MGG 295. The present findings are in agreement with Singh and Singh (2014) who screened 30 genotypes of mungbean against sucking insect pests including flower thrips. Minimum flower thrips infestation was recorded in ML-1628 and Pusa-1171 while maximum thrips infestation was recorded in BPMR-145, HUM-12 and Pusa-0672. In the present

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studies 13TM-04 constantly showed resistance against thrips attack which are in confirmatory with Indiati (2004) who investigated that mungbean cultivar MLG-716 constantly showed the resistance against thrips attack. The resistance of 13TM-04 may be due to antixenosis action.

Table 6. *Multiple linear regression model/s along with (R^2), regarding the impact of weather factors, on the per flower thrips population during 2018-19, on mungbean*

Regression equation	R^2	100 R^2	Percent role	F value	P value
2018					
$Y^{**} = 0.184 + 0.320\ X_1^{**}$	0.511	51.1	51.1	11.47	0.006
$Y^{*} = 0.484 + 0.610\ X_1 - 0.368\ X_2$	0.541	54.1	3.0	5.89	0.020
$Y^{*} = -0.12 + 0.849X_1 - 0.572\ X_2 + 0.0349\ X_3$	0.555	55.5	1.4	3.74	0.054
$Y^{*} = 1.54 + 0.619\ X_1 - 0.562\ X_2 + 0.0117\ X_3 - 0.0376\ X_4$	0.702	70.3	14.8	4.73	0.030
2019					
$Y^{**} = - 0.266 + 0.398\ X_1^{**}$	0.558	55.8	55.8	13.89	0.003
$Y^{*} = - 0.073 + 0.513\ X_1 - 0.159\ X_2$	0.568	56.8	1.0	6.58	0.015
$Y^{*} = 0.89 + 0.335\ X_1 - 0.066\ X_2 - 0.0478\ X_3$	0.580	58.0	1.2	4.15	0.042
$Y = 1.19 + 0.503\ X_1 - 0.333\ X_2 - 0.017\ X_3 - 0.0334\ X_4$	0.620	62.0	4.0	3.26	0.073

* = Significant at $P \leq 0.05$; ** = Significant at $P \leq 0.01$; Y, Thrips population per flower; X_1 = Maximum temperature (°C); X_2 = Average temperature (°C); X_3 = Average relative humidity (%), and X_4 = Rainfall (mm)

Sucking insect pest populations fluctuation is significantly influenced by weather factors (Gogoi and Dutta, 2000; Murugan and Uthamasamy, 2001; Panickar and Patel, 2001). According to our findings maximum temperature has dominant role in thrips population fluctuation and is the most important abiotic factor which has highly significant and positive correlation while minimum temperature has positive and non-significant correlation which is in partial agreement with the Akram et al. (2013) who found that maximum and minimum temperature has strongly positive correlation with thrips population. Our outcomes are in agreement with the findings of Shivanna et al. (2011) who observed that with the rise in maximum temperature thrips population increases. Present results are not in agreement with Zala et al. (2014) who found that thrips has significantly negative association with maximum temperature. According to present findings rainfall and average relative humidity has a strong negative correlation on cumulative basis which confirm the results of the Patel et al. (2013) and Saini et al. (2017), which also affirmed the negative relation of thrips with rainfall. Our results confirmed the findings of Shahid et al. (2012) who observed that thrips population has negative relationship with rainfall and relative humidity. Our results are in agreement with the results of Akram et al. (2013) who found that with the increase in R.H, thrips population decreases because R.H has strong negative correlation. Our findings are not in confirmatory with Saleem et al. (2013) and Janu et al. (2017) who observed that thrips population has positive and noteworthy association with relative humidity and have no considerable relationship with maximum, minimum temperature and rainfall. Thrips population may be affected by rainfall by direct and indirect way (Morsello et al., 2014; Pereira et al., 2007; Semeao et al., 2012). Our findings showed that rainfall have significant and negative correlation during 2018, 2019 and on cumulative basis

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81898203

which is in confirmatory with the finding of Sedaratian et al. (2010) who found that thrips have negative association with rainfall and relative humidity. Rainfall causes mortality of insect due to the mechanical impact of droplets which wash small insects down onto the soil and the indirectly rainfall increases the humidity (Semeao et al., 2012). Similarly, Barbosa et al. (2019) found a negative impact of rainfall on thrips population in multiple regression analysis.

Conclusion

The outcomes of the current findings identified various partially resistant cultivars namely 13TM-04, NM-16 and AZRI-06 against thrips attack. Moreover, to evolve new varieties these genotypes can be used as a source of resistance by the plant breeders for breeding programs. Thrips population in mungbean is influenced by different weather factors in which maximum temperature and rainfall play a significant role. During cropping season the knowledge about thrips dynamics helps in manipulating suitable pest managing strategy. The data generated in the present study will be helpful in the establishment of predictive models of thrips attack on mungbean in relation to weather factors. However, further work is needed to investigate the different sowing times and new varieties which may perform well in the prevalent environmental conditions against thrips. Moreover, role of physio-morphological and chemical plant characters on different mungbean genotypes should be studied.

Acknowledgments. Sincere thanks to the Higher Education Commission, Islamabad for providing the financial grant and Arid Zone Research Institute (AZRI) Bhakkar for providing research facilities.

Conflict of interests. The authors declare that they have no conflict(s) of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_81898203

THE EFFECT OF DIFFERENT PHOSPHORUS DOSES ON AGRONOMIC AND QUALITY CHARACTERISTICS OF CORIANDER (*CORIANDRUM SATIVUM* L.)

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(Received 25th Jul 2020; accepted 22nd Oct 2020)

Abstract. In a two-year study, under the environmental conditions of the plains of Mardin province in Turkey, the effects of different doses of phosphorus (0, 30, 60, 90, 120 kg ha^{-1}) on the yield and quality characteristics of the coriander (*Coriandrum sativum* L.) were examined. Plant characteristics such as plant height, number of branches, number of umbels, thousand seed weight and seed yield were examined, besides quality characteristics such as essential oil rate and composition. The essential oil was isolated with a Clevenger apparatus from mature fruits. Essential oil components were determined with Gas chromatography–mass spectrometry (GC-MS). The results revealed that phosphorus doses affect the plant characteristics of coriander including plant height, number of branches, number of umbels and thousand fruit weight with positive significance. Fruit yield was not affected significantly. Phosphorus doses had a significant positive effect on essential oil rate. Increasing phosphorus doses affected rates of α-pinene, geranyl acetate and camphor positively. However, Linalool and γ-terpinene were not affected by phosphorus doses significantly. The application of 120 kg phosphorus per hectare, which had the highest essential oil rate in both years, can be recommended in coriander farming.
Keywords: *Coriandrum sativum L., essential oil, linalool, phosphorus doses, yield*

Introduction

Coriander (*Coriandrum sativum* L.) is a medicinal and aromatic plant belonging to the Apiaceae family and widely used as a spice (Diederichsen, 1996; Maroufi et al., 2010; Chawla and Thakur, 2013). The fruit is divided into two varieties according to its diameter. The small-fruited var. *microcarpum* is in the diameter range of 1.5-3 mm while the large-fruited varieties var. *vulgare* Alef. var. *sativum* are in the diameter range of 3-5 mm. Each mericarp has 5 wavy ridges. The secretory canal is on the side of the mericarps facing each other and there are two canals (Tanker et al., 1998). In coriander, there is an inverse correlation between fruit size and essential oil rate. As the seed size increases, essential oil rate decreases (Telci et al., 2006a). Large-fruited coriander is generally grown in tropical and subtropical climatic zones and contains low level of essential oil (0.1-0.4%) (Telci et al., 2006b).

Coriander, which is used in many spice compositions and widely used in Indian Curry, is one of the spices with a great reputation all over the world and has a significant place in international trade. According to 2014 data of the Food and Agriculture Organization (FAO), total production amount of Anise, Fennel and Coriander plants is 970.000 tons in the world.

Turkey produced totally 42 tons of coriander in 2016 and earned 221.269 US dollar income from the export (Keykubat, 2016). According to the 2018 data of TURKSTAT, considering the foreign trade numbers in Turkey between 2007 and 2017, import was continuously higher than export on the basis of US dollar expect for 2012 and 2013 and only 29 tons of coriander were obtained in 2018 (Turkish Statistical Institute, 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

The parts of the plant such as fruit, leaf and flower have antimicrobial, antioxidant, antidiabetic, anxiolytic, anticonvulsant, antidepressant, antimutagenic, anti-inflammatory, anti-dyslipidemic, antihypertensive and diuretic effects (Burdock and Carabin, 2009; Sahib et al., 2013).

Coriander fruits contain essential oil and fatty oil. The essential oil rates of coriander fruits vary between 0.03% and 2.6%. The main component of essential oil was reported to be linalool (Gokduman and Telci, 2018; Momin et al., 2012; Izgi et al., 2017; Telci et al., 2006a, b).

Phosphorus is a significant plant nutrient for plant growth. Phosphorus enables potassium uptake by plants and conducts energy transfer in plants, plays a significant role in providing cell division by entering the structure of nucleoproteins. In addition, phosphorus in the soil has great benefits for plant root development. As the root development increases depending on the amount of phosphorus, the contact surface of the root develops in the soil, thus increasing the utilization of the necessary nutrient content of the plants (Marschner, 2011). In agricultural areas of Turkey, lack of available phosphorus and increasingly excessive phosphorus fertilization is still among the most significant problems of plant nutrition and fertilization (Matar et al., 1992).

This study was conducted with an aim to determine the effect of different phosphorus applications on yield and plant characteristics, essential oil rate and their main components in coriander (*Coriandrum sativum* L.), a significant medicinal and spice plant.

Materials and methods

Coriander (*Coriandrum sativum* L.) is a spice plant that belongs to the Apiaceae family and its fruits are used. As the experiment material, Gamze, which is a registered cultivar in Turkey, was used. This study was carried out for two years under the plain conditions (Gollu Village) of Mardin Province in Turkey during the 2014-2015 and 2015-2016 vegetation periods.

The plain conditions of Mardin province where the study was conducted are hot and dry in summers, rainy and warm in winters. When the climate data belonging to past periods and the years when the experiment was conducted were examined, it was seen that the temperature and humidity values were similar in both years; however, when the precipitation values were examined, differences were observed in December, January, February, March and April values and precipitation values were lower especially in the second year (*Table 1*).

Table 1. *Some meteorological values for long years (2004-2016) and 2014-2015-2016 growing periods in Mardin province. (Sources: Turkish State Meteorological Service)*

Months	Rainfall (mm)			Temperature (°C)			Relative humidity (%)		
	Long years	2014-2015	2015-2016	Long years	2014-2015	2015-2016	Long years	2014-2015	2015-2016
November	33.3	76.8	46.0	14	12.7	12.4	51.6	53.1	50.3
December	54.8	100.4	34.8	9.1	5.8	7.3	54.3	72.2	51.7
January	42.8	8.3	73.2	7.1	6.8	5.2	60.3	66.6	75.2
February	47.6	76.0	35.8	8.8	8.2	11.0	60.0	68.7	65.8
March	34.2	89.9	59.9	13.1	10.8	12.0	52.0	60.3	59.0
April	37.7	25.4	9.3	17.5	14.0	17.4	49.3	53.0	41.3
May	17.3	11.1	12.3	23.7	21.2	21.0	37.0	37.3	42.0
June	2.4	0.2	0.5	30.5	26.9	29.1	22.8	29.0	28.2
July	0.4	0.0	0.0	34.1	33.1	32.5	22.0	19.6	22.4

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DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

As seen in *Table 2,* the soil has a clay-loam structure and is poor in terms of organic matter (1.18%). Lime content is very high (36.65%), slightly alkaline (pH = 8.05) and has no salinity problem (0.010%). Phosphorus favorable for intake in soil (31.5 kg ha^{-1}) is low and rich in terms of potassium (104.16 mg kg^{-1}). Soil structure is sufficient in terms of iron (7.9050 mg kg^{-1}), copper (33.000 mg ha^{-1}) and magnesium (292.6 mg kg^{-1}) but insufficient in terms of manganese (3.390 mg kg^{-1}) and zinc (0.8112 mg kg^{-1}) (*Table 2*).

Table 2. *Soil properties of the experimental area*

Analyses (0-30 cm)	Limit values	Analysis results	Analysis method
Phosphorus (P)	< 3 Trace	29.2 mg kg^{-1}	TS ISO 11263
Potassium (K)	> 30 Sufficient	111.44 mg kg^{-1}	TS 8341
Lime (%)	> 25 Excessive calcic	33.39%	TS EN ISO 10693
pH	7.5-8.5 Light alkaline	8.08%	TS ISO 10390
Organic substance (%)	1-2 Little	1.15%	TS8336
Salinity (%)	<2 Salt-free	0.010%	TS ISO 11265
Mangan (Mn)	4-14 Insufficient	5.150 mg kg^{-1}	Tuzuner, 1990
Iron (Fe)	> 4.5 Sufficient	11.121 mg kg^{-1}	Tuzuner, 1990
Copper (Cu)	> 0.2 Sufficient	33.000 mg kg^{-1}	Tuzuner, 1990
Zinc (Zn)	> 8 Excessive	11.314 mg kg^{-1}	Tuzuner, 1990
Calcium (Ca),	1150-3500 Sufficient	1216.6 mg kg^{-1}	Tuzuner, 1990
Magnesium (Mg)	160-480 Sufficient	250.6 mg kg^{-1}	Tuzuner, 1990
Sodium (Na)	--	64.68 mg kg^{-1}	Tuzuner, 1990
Organic carbon	--	0.67%	TS8336
Carbon/nitrogen (C/N)	--	0.55%	Calculation method
Structure	Sand 39.2% Clay 32.7% Silt 28.0%	CL (Clayey loamy)	Tuzuner, 1990

Soil analyses were made in MARTEST Analysis Laboratories Ind.

The experiment was set up according to the Randomized Blocks Experiment Design with three replications. In the phosphorus fertilization, normal superphosphate (NSP) fertilizer was used. The phosphorus fertilizer was applied to the plots by mixing with the soil, with plantation, in 5 different doses (0, 30, 60, 90, 120 kg ha^{-1}). The plot area has a total area of 4.5 m^2 with 5 rows on each plot with 3 m length, 1.5 m width and 30 cm planting distance. In the plantation, 1 m distance between plots and 2 m between blocks were arranged. 10 kg ha^{-1} seed rate was used in the study. Sowings were performed manually on the 15th October 2014 in the first year and on the 16th October 2015 in the second year. After the sowing, weeding was performed three times and irrigation was made as needed by controlling the soil humidity in the summer months. On the 23rd June the harvest was performed manually; calculations were made on the current area by removing 25 cm as edge effect from the row tops and one row on the sides.

Essential oil analysis

Volumetric method was used for the extraction and quantitation of essential oils in matter coriander fruits and for distillation; fruits were ground and subjected to

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DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

distillation for 2.5 h with Clevenger device (Clevenger, 1928). The essential oils obtained were stored in a refrigerator at +4 °C until the analysis.

Essential oil component analysis was performed in GC/MS-QP2020 analysis device. The device conditions were given in *Table 3*.

Table 3. *GC/MS conditions*

System	GC/MS-QP2020
GC capillary column	For essential oil analysis: Rtx-2330 RESTEK (60 m x 0.25 mm x 0.2 μm)
Injection mode	Split
Pressure	For essential oil analysis: 80 kPa
Split rate	For essential oil component analysis: 25
GC oven initial temperature	For essential oil component analysis: Initial 40 °C 2 min holding period 4 °C min^{-1} until 240 °C final temperature 240 °C 3 min holding period
Injection block temperature	For essential oil component analysis: 240 °C
FID temperature	250 °C
Injection volume	1 μl

Data analysis

Agronomic characters were analyzed by using the JMP 5.0.1 statistical program (SAS Institute Inc., 2002), and the differences between means of phosphorus doses were compared using Student's t-test at the 0.05 probability level (Gosset, 1908).

The analysis of essential oil was applied using the IBM SPSS Statistics for Windows (IBM Corp., 2017). The significance of year differences of essential oil between fruit samples was tested by analysis of variance (ANOVA) and represented by critical value from F-test (F) and statistical significance (p). Essential oil means and major components of essential oil with significant variation were compared by using least significant difference (LSD) test. Year difference significances were compared by T-test.

Results and discussion

Agronomic characteristics and yield

Plant height (cm)

Effect of phosphorus doses on mean data of plant height was found statistically significant ($p < 0.05$). Year differences were insignificant. Plant height varied between 36.7 and 51.5 cm in the first year and 39.3 and 55.0 cm in the second year. According to mean data of doses, statistically ($p < 0.05$) the highest value (53.3 cm) was obtained at 90 kg ha^{-1} phosphorus application and the lowest value (38.0 cm) was measured in the control group. It can be stated that the increasing phosphorus doses affected plant height positively (*Table 4*).

In the study of NP fertilization, Khalid (2012) found significant ($p < 0.05$) effect of fertilizer (NP) on plant height of coriander and value between 42.6 and 70.7 cm. He stated that plant height increased by increasing phosphorus doses. Javiya et al. (2017) found significant relation between phosphorus doses and plant height and found values between 51.86 and 60.47 cm. The highest value is obtained at 60 kg ha^{-1} phosphorus application. Farahani et al. (2008) found the effect of phosphorus doses on plant height insignificant.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

Table 4. *Some agronomical characteristics of different phosphorus dose applications in coriander (Coriandrum sativum L.)*

Phosphorus doses (kg ha^{-1})	Plant height (cm)			Number of branches (branches plant^{-1})			Number of umbels (umbels plant^{-1})			1000 fruit weight (gr)			Fruit yield (kg ha^{-1})		
	2015	2016	Mean	2015	2016	Mean	2015	2016	Mean	2015	2016	Mean	2015	2016	Mean
0	36.7	39.3	**38.0c**	4.0	4.9	**4.4bc**	9.5	11.2	**10.3b**	12.8	12.9	**12.9a**	1546	2618	**2082**
30	47.8	51.8	**48.2b**	3.8	5.0	**4.4c**	11.0	12.5	**11.8b**	12.3	12.4	**12.3b**	1957	2511	**2234**
60	50.1	48.6	**51.4ab**	3.5	4.7	**4.1c**	11.6	13.0	**12.3b**	12.9	12.7	**12.8a**	1965	2599	**2282**
90	51.5	52.6	**53.3a**	4.5	6.0	**5.3bc**	14.2	17.2	**15.7a**	12.3	12.2	**12.3b**	203.2	2490	**2261**
120	48.4	55.0	**50.1ab**	5.9	6.8	**6.4a**	15.7	16.2	**16.0a**	12.4	12.3	**12.4b**	1740	2107	**1923**
Mean	**46.9**	**49.5**		**4.3b**	**5.5a**		**12.4b**	**14.0a**		**12.6a**	**12.5b**		**1848b**	**2465a**	

The difference between the mean indicated in the same letter is not significant ($P < 0.05$)

Number of branches (branches plant^{-1})

Regarding number of branches, differences between mean data of doses and differences between mean data of years were statistically significant ($p < 0.05$). It was detected as 4.3 in the first year and 5.5 in the second year. According to mean data of doses, the highest branch number (6.4 branches plant^{-1}) was obtained at 120 kg ha^{-1} phosphorus application) while the lowest branch number (4.1 branches) was obtained at 60 kg ha^{-1} phosphorus application. The results of the study show that the increasing phosphorus doses had a positive significant effect on the number of branches. In the second year, high rainfall values encouraged vegetative growth of the plant, thus increasing the number of branches (*Table 4*).

Regarding the number of branches, Khalid (2012) stated that there was a positive significant relation between phosphorus doses and number of branches. He obtained the highest value (7.8 plant^{-1}) at 56.3 kg ha^{-1} phosphorus application. Javiya et al. (2017) found significant positive effect of phosphorus doses on number of branches and values were between 16.95 and 22.22 branches per plant. They measured the highest number of branches at 60 kg ha^{-1} phosphorus application. In their study of water stress and phosphorus doses effect on coriander, Hani et al. (2015) determined that the number of branches increased significantly by increasing phosphorus doses and values without water stress were between 3.79 and 4.71. They obtained the highest value at 24 kg ha^{-1} phosphorus application. It could be said that phosphorus doses increase number of branches in coriander plant significantly. It can be said that differences of obtained values may be related to the applied cultivation techniques and the effect of ecological and genotypical factors.

Number of umbels (umbels plant^{-1})

The differences between the years and between the doses were found to be significant ($p < 0.05$) in terms of the umbel number. It was detected that there were 12.4 umbels per plant in 2015 while there were 14 umbels in 2016. Considering the mean data of the doses, the highest number of umbels (16 umbels plant^{-1}) was detected in the 120 kg ha^{-1} phosphorus application while the lowest umbel (10.3 umbels) was detected in the control group. In the new studies to be conducted on branch number in plant, 120 kg ha^{-1} and higher phosphorus doses per hectare should be experimented. Furthermore, the differences observed between the years especially in terms of precipitation values affected the high umbel numbers for two years (*Table 4*).

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Hani et al. (2015), Javiya et al. (2017) and Khalid (2012) claimed that number of umbels in coriander plant was significantly affected from different doses. Respectively, Hani et al. (2015), Javiya et al. (2017) and Khalid (2012) obtained values varying between 5.63-6.97; 8.97-12.42 and 13.9-24.7 umbels per plant. The highest number of umbels was measured at 56.3 kg ha^{-1} application in Khalid (2012), 60 kg ha^{-1} in Javiya et al. (2017) and 24 kg ha^{-1} in Hani et al. (2015). Studies on the effect of phosphorus doses on number of umbels prove that increasing doses provide enhancement in number of umbels.

Thousand fruit weight (g)

In terms of the thousand fruit weight, the differences between the doses used in the study were found to be statistically significant ($p < 0.05$) according to both experimental doses and average of the years. The thousand fruit weight was detected as 12.6 g in the first year and 12.5 g in the second year. In the average of the years, the highest value (12.9 g) was obtained from the control group while the lowest value (12.3 g) was obtained from 30 kg ha^{-1} and 90 kg ha^{-1} phosphorus application (*Table 4*).

In their research of row spacing Kizil and Ipek (2004) found values between 12.51 g and 13.90 g with 30 kg ha^{-1} phosphorus application. Javiya et al. (2017) and Jan et al. (2011) found significant effect of phosphorus doses on thousand fruit weight and values between 13.12 g and 13.99 g and 8.59 g and 9.83 g respectively. Javiya et al. (2017) obtained the highest value at 60 kg ha^{-1} phosphorus application and Jan et al. (2011) at 45 kg ha^{-1} phosphorus application. Sannappanavar et al. (2019) and Kan (2007) found effect of phosphorus on thousand seed weight as insignificant and they measured values between 9.45 g and 10.60 g and 9.60 g and 10.14 g respectively. According to results obtained in the study and in other studies, it is revealed that, increasing phosphorus doses has positive effect on thousand fruit weight.

Fruit yield (kg ha^{-1})

In the fruit yield, the difference between the years was significant but not significant between the mean data of the doses. The yield was obtained as 1848 kg ha^{-1} in the first year and 2465 kg ha^{-1} in the second year. In the mean data of doses, the highest value (2282 kg ha^{-1}) was obtained from the 60 kg ha^{-1} phosphorus application while the lowest value (1923 kg ha^{-1}) was obtained from the 120 kg ha^{-1} phosphorus application (*Table 4*). It is thought that the differences between the fruit yield according to the years are related to the higher precipitation values especially in the second year.

According to fruit yield of coriander Hani et al. (2015), Jan et al. (2011), Javiya et al. (2017) and Sannappanavar et al. (2019) found significant effect of phosphorus doses on coriander and they reported positive relation. Respectively they found, values between 2774-3669 kg; 894-1023 kg; 1219-1422 kg and 511-665 kg per hectare. They measured the highest values at the highest phosphorus applications. However, Kan (2007) found the effect of phosphorus doses as insignificant. While literature proves positive effect of phosphorus doses on fruit yield, result in this paper is not significant. Fruit yield shows significant changes depending on variety, climate, genetic and environmental factors, seed time and agricultural practices (Yamanol, 1996; Toncer and Tansi, 1997; Toncer et al., 1998; Gok, 2011).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

Essential oil contents (%)

Difference between mean data of year was not statistically significant for essential oil content (*Table 5*). First year mean value was 0.20% and second year it was 0.21%. Regarding to mean data of dose differences, the highest value (0.23%) was obtained at 120 kg ha^{-1} phosphorus dose and the lowest value was obtained in the control group. Doses and interaction factors were statistically significant ($p < 0.01$) but year factor was not significant. Essential oil increases according to increasing phosphorus doses; While the 1st year increased regularly, the 2nd year became irregular (*Table 6*). If we evaluate according to the year averages; As there is an increase in essential oil rates in parallel with increasing phosphorus doses, 120 kg of phosphorus application may be recommended to hectare.

Table 5. *Variation of mean, standard deviation and F data in coriander essential oil content and components cultivated in two different years*

Components	RT	1st Year	2nd Year	*F* Values
		Mean + SD	Mean + SD	
α-pinene	6.803	7.93 ± 1.64a	6.43 ± 0.70b	10.71**
Camphene	8.115	0.88 ± 0.16a	0.75 ± 0.08b	7.99**
β-pinene	9.502	0.72 ± 0.11a	0.63 ± 0.06b	8.36**
Sabinene	10.003	0.53 ± 0.08a	0.47 ± 0.05b	5.96*
Myrcene	11.554	1.09 ± 0.15a	0.91 ± 0.10b	14.17**
Limonene	12.788	2.16 ± 0.29a	1.86 ± 0.22b	10.79**
γ-terpinene	14.565	7.86 ± 0.93a	7.00 ± 0.54b	9.58**
p-cymene	15.501	4.52 ± 0.59	4.00 ± 0.81	4.07 ns
Terpinolene	15.878	0.40 ± 0.06a	0.34 ± 0.04b	8.27**
Camphor	24.285	4.09 ± 0.71	4.12 ± 0.78	0.02 ns
Linalool	25.254	59.98 ± 6.86	63.00 ± 4.96	1.91 ns
Terpinen-4-ol	26.956	0.33 ± 0.12	0.32 ± 0.14	0.02 ns
α-Terpineol	29.850	0.34 ± 0.09	0.36 ± 0.10	0.39 ns
Isoborneol	30.027	0.10 ± 0.09	0.13 ± 0.09	1.01 ns
Geranyl acetate	31.601	5.05 ± 1.67	5.21 ± 1.32	0.08 ns
L-Citronellol	31.753	0.13 ± 0.08b	0.20 ± 0.06a	5.91*
Geraniol	34.147	2.42 ± 0.76	2.82 ± 0.71	2.25 ns
Dodec-2(E)-enal	34.701	0.12 ± 0.11	0.14 ± 0.10	0.22 ns
Essential oil		0.20 ± 0.03	0.21 ± 0.03	2.49 ns

*p < 0.05; **p < 0.01; ns: not significant

According to the European Pharmacopeia, the coriander fruit should have an essential oil concentration that is higher than 0.03% (Ullah and Honermeier, 2013). Kizil and Ipek (2004) obtained value between 0.28 and 0.32 by application of 60 kg ha^{-1} nitrogen and 30 kg ha^{-1} phosphorus. In their study of cultivar and location, Izgi et al. (2017) measured 0.50% of essential oil in Mardin plain conditions with Gamze cultivar. Regarding different phosphorus doses; Khalid, 2012, 2015; and Hani et al., 2015 claimed that essential oil rate increase with increasing phosphorus doses and they found values as 0.47-0.53; 0.20-0.40 and 0.20-0.60 respectively. However, Kan (2007) did not find the effect of phosphorus doses significant. Results showed that essential oil rate

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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was affected by increasing phosphorus doses. Differences between increase rates and total ratios of essential oil in fruit can be caused from different ecological conditions, different cultivars or various cultural application during cultivation process.

Table 6. *Variation in essential oil content and major components of essential oil of coriander (Coriandrum sativum L.) according to Year (Y) and phosphorus doses (P)*

	Years	Phosphorus doses (kg ha^{-1})					Mean^{Y}
		0	30	60	90	120	
Essential oil	**1st**	0.16	0.19	0.20	0.20	0.23	**0.20a**
	2nd	0.22	0.23	0.17	0.20	0.23	**0.21a**
	Mean^{P}	**0.19b**	**0.21ab**	**0.19b**	**0.20b**	**0.23a**	
Factors		**Y^{NS}**		**P****		**YxP****	
Linalool	**1st**	58.10	58.68	60.40	66.06	56.67	**59.98a**
	2nd	67.66	63.80	59.92	64.10	59.51	**63.00a**
	Mean^{P}	**62.88**	**61.24**	**60.16**	**65.08**	**58.09**	
Factors		**Y^{NS}**		**P^{NS}**		**YxP^{NS}**	
γ-terpinene	**1st**	7.73	7.89	8.24	7.07	8.37	**7.86a**
	2nd	7.61	6.71	6.89	6.84	6.93	**7.00b**
	Mean^{P}	**7.67**	**7.30**	**7.57**	**6.96**	**7.65**	
Factors		**Y****		**P^{NS}**		**YxP^{NS}**	
Geranyl acetate	**1st**	7.49	4.68	5.64	2.82	4.64	**5.05a**
	2nd	3.28	4.56	6.18	5.28	6.75	**5.21a**
	Mean^{P}	**5.39a**	**4.62b**	**5.91a**	**4.05c**	**5.70a**	
Factors		**Y^{NS}**		**P****		**YxP****	
α-pinene	**1st**	5.39	8.31	7.66	9.22	9.08	**7.93a**
	2nd	6.83	7.20	6.48	5.97	5.65	**6.43b**
	Mean^{P}	**6.11c**	**7.76a**	**7.07b**	**7.60ab**	**7.37ab**	
Factors		**Y****		**P****		**YxP****	
Camphor	**1st**	4.94	3.71	4.23	3.31	4.24	**4.09a**
	2nd	3.29	3.91	4.88	3.53	4.99	**4.12a**
	Mean^{P}	**4.12b**	**3.81b**	**4.56a**	**3.42c**	**4.62a**	
Factors		**Y^{NS}**		**P****		**YxP****	

*p < 0.05; **p < 0.01; NS: not significant

Essential oil components

In the study where the effect of different doses of phosphorus in the coriander grown under the plain conditions of Mardin province on essential oil components, 18 components were detected in total. Linalool, γ-terpinene, α-pinene, geranyl acetate and camphor were determined in coriander as the major components (*Table 6*). For linalool, main factors of year, phosphorus doses and interaction were found insignificant statistically (*Table 5* and 6). First and second years were respectively 59.98% and 63%, and year differences were not statistically significant (*Table 5*). Regarding mean data of doses, the highest value was observed at 90 kg ha^{-1} phosphorus application. However, differences between doses were not statistically significant. Regarding γ-terpinene, year factor was statistically significant (p < 0.01). Phosphorus doses and interaction were not

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statistically significant. Statistically ($p < 0.05$) the highest value (7.86%) was obtained in the first year. While the highest mean value (7.67%) of doses was observed at the control group, differences were not significant. Main factor of year was not statistically significant for geranyl acetate. However, factor of doses and interaction were significant ($p < 0.01$). In the second year, higher value (5.21%) was obtained but year differences were not statistically significant. According to mean data of doses, the highest value (5.91%) was obtained at 60 kg ha^{-1} phosphorus application and the lowest value (4.05%) was obtained at 90 kg ha^{-1} phosphorus application. Regarding main factors of α-pinene, year, phosphorus doses and interaction were significant ($p < 0.01$) statistically. For year differences, significantly ($p < 0.05$) higher value (7.93%) was obtained in the first year. According to differences of doses, top value (7.76%) was measured at 30 kg ha^{-1} phosphorus application, the highest value (6.11%) was obtained in the control group. Main factors of phosphorus doses and interaction were found significant ($p < 0.01$) statistically, but main factor of year was not significant. Higher value was measured in the second year as 4.12%, however difference was not significant. Differences between mean data of doses were tested and found significant ($p < 0.05$). Maximum value (4.62%) was observed at 120 kg ha^{-1} phosphorus application, minimum value (3.42%) was obtained at 90 kg ha^{-1} phosphorus doses (*Table 6*).

Rao et al. (1983) reported that the different doses of phosphorus (0, 30, 60 kg ha^{-1}) did not affect the linalool rate varying between 57.9 and 63.3% in coriander. In the study of essential oil content of coriander applying boron fertilization Beyzi and Gunes (2017) determined linalool, camphor and γ-terpinene as the main components by 50 kg ha^{-1} phosphorus and nitrogen application with Gamze cultivar. The highest rates that they detected are between 83.81-91.40%, 2.21-3.67% and 0.88-3.76%, respectively. In their study of cultivar and location, Izgi et al. (2017) determined linalool, neryl acetate, α-pinene and γ-terpinene as the major components of essential oil in coriander by 50 kg ha^{-1} phosphorus and nitrogen application with Gamze cultivar in Mardin plain conditions and they measured values of 82.2%, 3.6%, 0.3% and 4.2%, respectively. According to effect of phosphorus doses, Khalid (2015) obtained 15 components of essential oil and determined linalool (75.5%-75.7%), limonene (6.8%-7.0%) and camphor (3.7-3.9) as major components. He did not find significant effect of doses on major components, but he found significant differences of doses for sabinene and α-pinene. In the study about water regime and phosphorus fertilization effect on essential oil of coriander, Hani et al. (2015) found 19 components and linalool (35.13%-65.58%), nerol (3.52%-22.36%) and α-terpineol (7.17%-16.68%) as major components. They found the effect of phosphorus doses on major components of essential oil in coriander as significant. According to these results, effect of phosphorus doses on essential oil composition of coriander differs to cultivar, climatic conditions, water regime or cultural applications.

Conclusions

Aim of this research was to determine the effect of phosphorus fertilization on coriander to fruit yield and quality. Experiments were carried out in plain conditions of Mardin province in Turkey. The effect of different phosphorus doses applied to the plant on fruit yield was statistically insignificant. The effect of different phosphorus doses applied to the plant on the essential oil ratio of the coriander fruit was statistically significant. The highest essential oil rate (0.23%) was obtained from 120 kg phosphorus

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

application per hectare. Different phosphorus doses applied to the plant had no statistical effect on linalool ratio. Since it contributes to the increase of essential oil ratio; 120 kg of phosphorus application can be recommended per hectare.

Acknowledgments. This work was supported by the Mardin Artuklu University BAP Coordinator, it forms part of the project numbered MAÜ-BAP-16-KMYO-16 and titled "The Effect of Different Nitrogen and Phosphorus Doses in Coriander and Black Cumin on Yield and Quality".

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DOI: http://dx.doi.org/10.15666/aeer/1806_82058216

DIVERSITY PROFILING OF ASSOCIATED BACTERIA FROM THE SOILS OF STRESS TOLERANT PLANTS FROM SEACOAST OF JEDDAH, SAUDI ARABIA

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(Received 28th Jul 2020; accepted 7th Oct 2020)

Abstract. Soils associated with halophytic plants naturally contain a number of ubiquitous microbial communities facing limited nutrients and harsh environmental conditions including salinity and drought. In the present study, metagenomic sequencing of 16S rRNA was used to analyze and classify bacterial communities of the soil associated with halophytic plants *Halopeplis perfoliata* and *Zygophyllum album* collected from various soil samples located in the seacoast of Jeddah province, Saudi Arabia. Analysis of the 16S rRNA sequences at the taxonomic phylum-level revealed that bacterial communities in the soil samples belonged to nineteen phyla, and the most abundant were highlighted for further analysis. Results indicated that the most common phyla were *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Deinococcus-Thermus*, *Gemmatimonadetes*, and an *unclassified phyla*. At the taxonomic genus-level, the most abundant ones were highlighted for further analysis which include *Marinicauda, Altererythrobacter, Maricurvus, Marinobacter, Porticoccus, Salicola, and three unclassified genera* were found belonging to *Proteobacteria. Actinopolyspora,* Geodermatophilus, *Propionibacterium*, *Euzebya* and four unclassified ones were found associated with *Actinobacteria*. *Bacillus, Staphylococcus, Paenibacillus, Lactobacillus, Streptococcus, Symbiobacterium*, and one unclassified genus were found in *Firmicutes*. *Salegentibacter, Haliscomenobacter*, and one unclassified genus of *Bacteroidetes*. *Truepera* was found in *Deinococcus-Thermus* and *Gemmatimonas* was found in *Gemmatimonadetes*. Studying taxonomic, phylogenetic, and functional diversity of soil microbiome will provide a better understanding for novel candidates that can be selected as biological agents to improve agricultural and industrial practices.
Keywords: *microbiome, PGPB, metagenomics, Halopeplis perfoliate, Zygophyllum album*

Introduction

Soil is probably the most complex and dynamic natural ecosystem providing a favorable environment for the growth and production of huge number of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82178231

microorganisms depending on the soil pH, chemical and physical properties (del Carmen Orozco-Mosqueda et al., 2018), and geographic positions (Bui, 2013). These microorganisms play a critical role in regulating plant life cycle and health through biomass decomposition, soil fertility, and cycling of nitrogen, carbon, and other nutrients. Thousands of bacterial, archaeal, and eukaryotic taxa can be found in every gram of soil and this taxonomic diversity is reflected by the diversity of their biological compositions that effect their functions. Moreover, these heterogenous microbial communities can live either as a free-living or symbiotic and their influence can vary from pathogenic to beneficial, or mutualistic (Chaparro et al., 2012; Fierer et al., 2012).

Plant growth-promoting bacteria (PGPB) are a collection of unrelated bacteria that are found in symbiotic relations with plants and have been adapted as a sustainable alternative for crop production. PGPB can be found in the rhizosphere, epiphytes by attaching the surface of plants roots or leaves, or inside the plant tissues as endophytic bacteria (de Zelicourt et al., 2013; Timmusk et al., 2017). Soil salinity can significantly affect the growth of many plants (Waśkiewicz et al., 2013). However, PGPB promote the growth of plants under harsh environmental conditions including draught, high salinity, and temperature (Majeed et al., 2020). Microorganisms live in a relationship with plants are able to stimulate plant growth by directly obtaining nutrients (nitrogen, iron, phosphate) or regulating the hormone levels including auxins and ethylene, and also by indirectly preventing pathogens from attacking plants (Glick, 2012).

Halopeplis perfoliata plants are halophytic and desert plants that are mostly found in association with symbiotic bacteria in order to stand harsh environmental conditions (Etesami and Beattie, 2018). They can withstand and grow in dry seasons and are mainly found in arid and semi-arid regions and wetlands with high salinity (Etesami and Beattie, 2018; Majeed et al., 2020). Moreover, *H. perfoliata* have various ecological and industrial benefits including soap and glass industry (Rasool et al., 2017; Zreik, 1990; Al-Oudat and Qadir, 2011). *Zygophyllum album* is another example of halophytic plants that live in the same community of *H. perfoliate*. It was known as wild desert medicinal plant that was used in folkloric medicine as a diuretic (Mnafgui et al., 2012; Tigrine-Kordjani et al., 2011), local anesthetic (El Ghoul et al., 2011), and for treating various disorders including diabetes mellitus, rheumatism, gout, asthma (Tigrine-Kordjani et al., 2011).

Numerous studies have used the metagenomics approaches in order to understand the soil microbial communities of different ecosystems from different soils collected from cold and hot deserts, forests, grasslands and tundra (Baeshen, 2017). However, very few of them have focused on microbial communities of soils associated with halophytes. It is important to understand how soil microbiome interacts and promotes halophytes to grow and sustain under abiotic stress conditions, and how these halophytes respond to these diverse microbial communities. Such interaction will reveal a huge diversity of microorganisms that are able to foster the growth of diverse crop plants under various biotic and abiotic stresses and can be used as biological agents in numerous industrial and medical applications (Majeed et al., 2020).

The work presented in this study was aimed to discover the microbial diversity found in soils associated with halophytes. We use the metagenomic approaches in order to discover novel promising prokaryotic candidates that can be used as plant growth promoting bacteria and biomolecules of industrial importance.

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Materials and methods

Sample collection

The study area was located in the seacoast of Jeddah (Particularly from the Southern Corniche), Saudi Arabia with latitude: 21.13'08.3" N and longitude: 39.10'29.7" E and altitude: 3 m above sea level. The climate in the Jeddah region is classified as hot, arid, and sandy with a lower amount of rainfall and an average temperature in January on the day ranges between 26-33 °C. Despite all these characteristics, the halophytic plant *Halopeplis perfoliata* and the *Zygophyllum album* as a member of *H. perfoliata* communities grow significantly in this region.

Sampling was carried out on the 9th of January 2019 at noon and the temperature was 35 °C. A total of four soil samples associated with two halophytic desert plants namely *Halopeplis perfoliata* and *Zygophyllum album* were collected. Two samples from the rhizosphere of each plant with a depth of 10 cm beneath the first layer and two samples from the crust soil samples associated with *Halopeplis perfoliata* and *Zygophyllum album.* in addition, one plant free soil sample was collected from a nearby area that has no plant growth and was used as a control. An amount of 10 g soil was collected from each sample and were immediately kept in dry ice and store in -80 °C until further analysis. Samples codes were as the following; control sample (L3. S3 Control), *Halopeplis perfoliata* rhizosphere sample (L1.S1.R), *Halopeplis perfoliata* crust sample (L1.S1.C), *Zygophyllum album* rhizosphere sample (R3), and *Zygophyllum album* crust sample (C3).

Identifications of theses salt tolerance plants of the study was carried by our team member, Professor Nabih A. Baeshen as compared to the collection of the preserved specimens in the herbarium of the Department of Biology, Faculty of Science, King Abdulaziz University (Batanouny and Baeshin, 1982, 1983; Sejiny et al., 1980; Zaki et al., 1980).

Genomic DNA isolation, PCR amplification, and 16S rRNA gene sequencing

Soil samples were shipped to Macrogen Inc. Company (Seoul, South Korea) and genomic DNA was extracted from the soil samples. DNA purity and quantification were evaluated using the Picogreen (Invitrogen, cat. #P7589) fluorescence-based quantification method.

Bacterial V3-V4 16S rRNA gene regions fragments was amplified by PCR with the universal primers (Bakt_341F: CCTACGGNGGCWGCAG) and (Bakt_805R: GACTACHVGGGTATCTAATCC). The PCR amplification program was performed using an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 40 s, and extension at 72 °C for 1.30 s with a final elongation at 72 °C for 10 min (Rawat and Joshi, 2019).

Purified amplicons were used for library construction and deep sequencing on an Illumina SBS technology to recover 300 bp pair-end reads of the V3 and V4 regions.

16S dataset processing and statistical analysis

Raw sequencing data was analyzed using Quantitative Insights into Microbial Ecology (QIIME) software (http://qiime.org), which is a bioinformatics open-source tool used in performing microbiome analysis from raw DNA sequencing data that is generated on the Illumina or other sequencing programs. Furthermore, QIIME offers

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quality pretreatment of raw reads, OTU picking, taxonomic assignment, and phylogenetic reconstruction, and diversity analyses and graphical displays (Caporaso et al., 2010).

V3-V4 16S rRNA sequence reads were filtered and trimmed using the CD-HIT-OTU software (http://weizhongli-lab.org/cd-hit-otu/). The FLASH software (http://ccb.jhu.edu/software/FLASH/) was used for merging paired-end reads from next-generation sequencing experiments to eliminate the low-quality sequences. Operational taxonomic units (OTUs) were used to linked and classified unique sequence set with a cutoff of 97% identity. The Ribosomal Database Project (RDP) Classifier was used for taxonomic composition.

Alpha diversity was assessed by Chao1, which was estimated based on the report that came from Macrogen (Chao and Bunge, 2002). Shannon and Simpson indices that were estimated by Mothur software package (http://www.mothur.org) to analyze the complexity of species. Drawing rarefaction curve was based on calculating OTU numbers of the extracted tags and detecting the maximum depth allowable to retain all samples. Beta diversity was detected by calculating the weighted and unweighted UniFrac distances and plotted through principal coordinate analysis (PCoA). UniFrac uses the system evolution information to compare bacterial communities' species between samples. The highest abundance of each genus was selected and genus level phylogenetic tree was drawn by Interactive Tree of Life (ITOL) (https://itol.embl.de).

Results

16S rRNA statistical analysis

In the present study, metagenomic approach was used a powerful tool to investigate the microbial community structure and diversity of five different soil samples associated with *Halopeplis perfoliata* and *Zygophyllum album*. Illumina SBS was used in analyzing the soil different samples based on the 16S rRNA.

The percentage of the read quality of the five soil different samples is shown in *Figure A1*. Total number of sequences reads and the results of the assembly for the five samples were carried out from FLASH software and is shown in *Figure A2* and *Table 1,* respectively. Data showed a total of clean sequences reads 546,713 with the highest value of 126,158 found in *H. perfoliata* crust sample and the lowest 88,446 value found in the control sample.

Table 1. *Result of assembly of the five soil samples*

FLASH Software					
Sample name	**Total bases**	**Read count**	**GC (%)**	**Q20 (%)**	**Q30 (%)**
L3S3.Control	39,889,093	88,446	57.84	98.11	93.32
L1S1R	46,175,526	102,358	56.67	98.19	93.67
L3S1C	57,623,348	126,158	55.03	98.24	93.91
R3	55,897,213	124,341	57.98	98.1	93.18
C3	47,494,924	105,410	57.52	98.34	94.08
Total number	247,080,104	546,713			

Total bases: The total number of bases in reads identified. Read count: The total number of sequence reads. GC (%): the GC percentage in sequence reads. Q20 (%): the percentage of bases in which the phred score is above 20. Q30 (%): The percentage of bases in which the phred score is above 30

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Operational taxonomic unit (OTU) analysis

CD-HIT-OTU program and rDnaTools were used to filter the sequences from any contamination. Results of clustering of the five soil samples which were assigned to the OTU is shown in (*Fig. 1*). The highest value of number of OTUs was 333 belongs to *H. perfoliata* crust sample while the lowest was 108 belongs to the control sample.

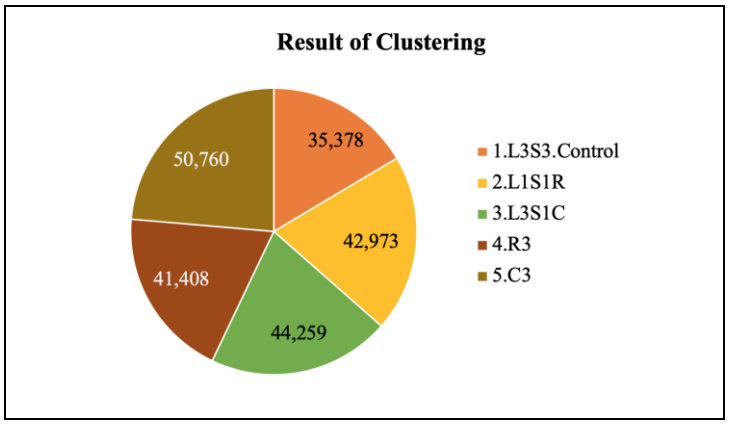

Figure 1. *Result of clustering. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

Community richness and diversity

Alpha diversity was applied to study the complexity of species through several indices; A. Chao1 value describes richness estimates for an OTU definition. B. Shannon value describes the species diversity of the community that affected by both species' richness, and species evenness. C. Inverse Simpson value which represents the probability that two randomly selected individuals in the habitat will belong to the same species. *Table 2* shows the results of the OTUs and the alpha diversity metrics (Chao1, Shannon, Simpson) on each sample. The number of OTUs on each sample is shown in *Figure 2*. Different curves based on observed Shannon value and Inversed Simpson value is shown in *Figure 3* and alfa rarefaction curve observed is shown in *Figure 4*.

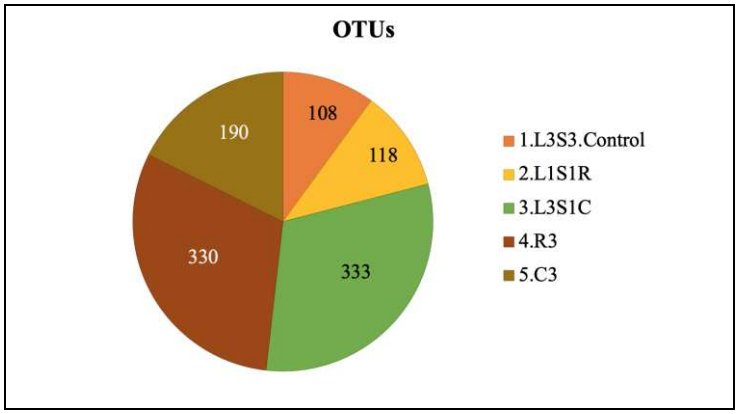

Figure 2. *The number of OTUs generated for each sample. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

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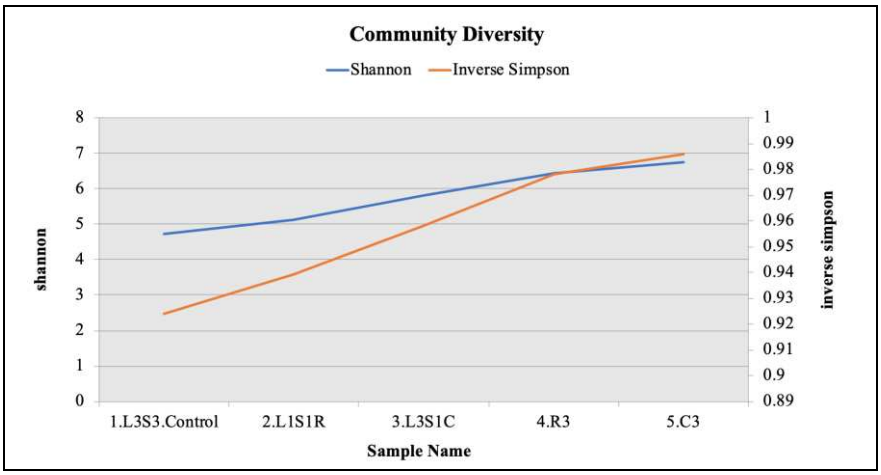

Figure 3. *Different curve based on observed Shannon value and inversed Simpson value. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

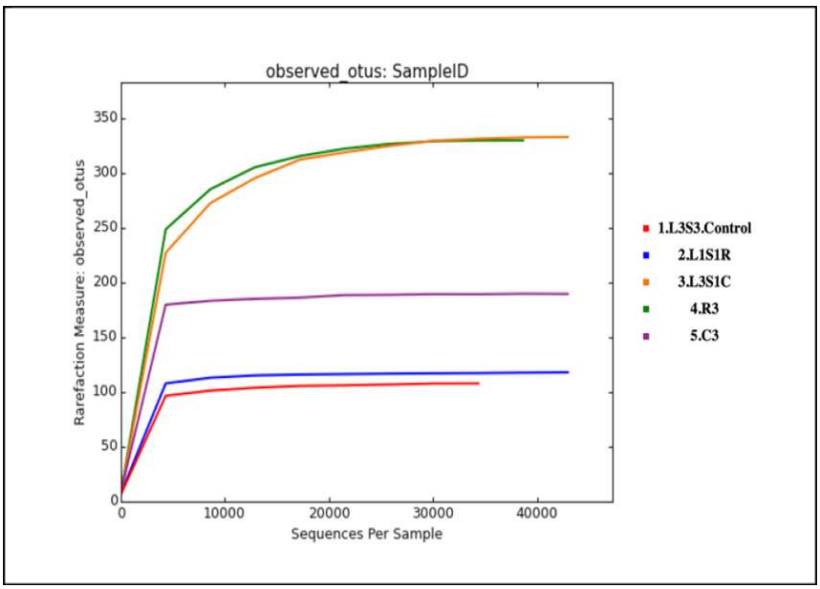

Figure 4. *Alfa rarefaction curve observed based on observed species (OTUs) value. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

Principal coordinate analysis (PCoA) was used in order to show the diversity and differences of OTU composition in the different soil samples. PCoA based on OTU abundance of different samples and the unweighted UniFrac that refers to unique species is shown in *Figure 5*. The red triangle indicates to the control sample. The blue square indicates to the rhizosphere of *Halopeplis perfoliata*. The orange triangle indicates to the crust of *Halopeplis perfoliata*. The green circle indicates to the rhizosphere of *Zygophyllum album*. The Purple triangle indicates to the crust of *Zygophyllum album*. Similarity is high between samples when they are closely located.

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Table 2. *Community richness and diversity*

Community richness and diversity				
Sample name	**OTUs**	**Chao1**	**Shannon**	**Inverse Simpson**
L3S3.Control	108	111	4.739486514	0.923819556
L1S1R	118	118	5.135939679	0.939106837
L3S1C	333	343	5.799493094	0.958314098
R3	330	330	6.429642998	0.978116633
C3	190	190	6.747126231	0.985840649

Chao1: Species richness estimators estimating the total number of species present in a community by using the frequency of occurrence of rarer OTUs. If a sample contains many singletons or doubletons, it is likely that more undetected OTUs exist, and the Chao 1 index will estimate greater species richness than it would for a sample without rare OTUs
Shannon: A quantitative measure that reflects the number of different types (species) present within a dataset. It also simultaneously takes into account of how evenly the basic entities (individuals) are distributed among those types
Inversed Simpson: An indication of how evenly the species are distributed and measures the degree of concentration when individuals are classified into species

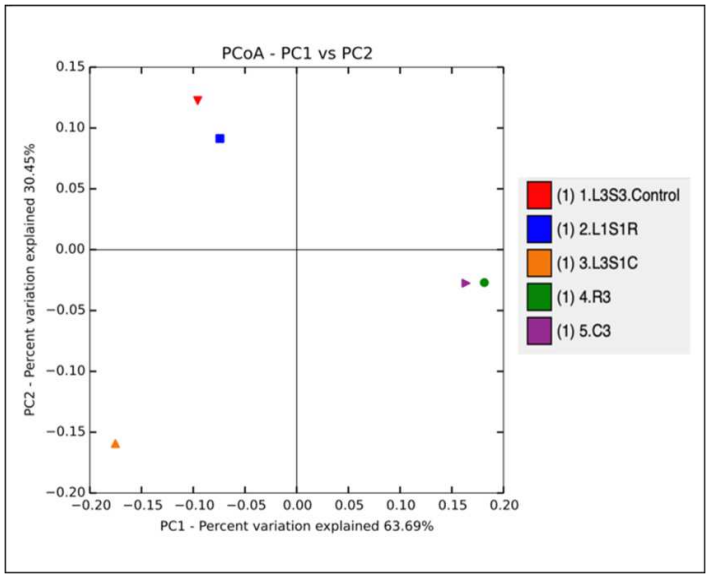

Figure 5. *Beta diversity analysis. Unweighted PCoA of UniFrac distances. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

Taxonomic classification at the phyla and genera levels

Phylogenetic tree based on 16S rRNA showing the diversity and taxonomy of bacteria isolated from the five different soil samples at both the phyla and genera levels are shown in (*Fig. 6*). A phylogenetic tree is a diverging schema presenting the inferred evolutionary relationships among diverse biological taxa constructed upon similarities and differences in their physical or genetic features. The shorter the length of the branch, the closed evolution distance between taxa. Moreover, phylogenetic tree can explain the species evolution relationship in addition to the taxa composition and abundance analysis.

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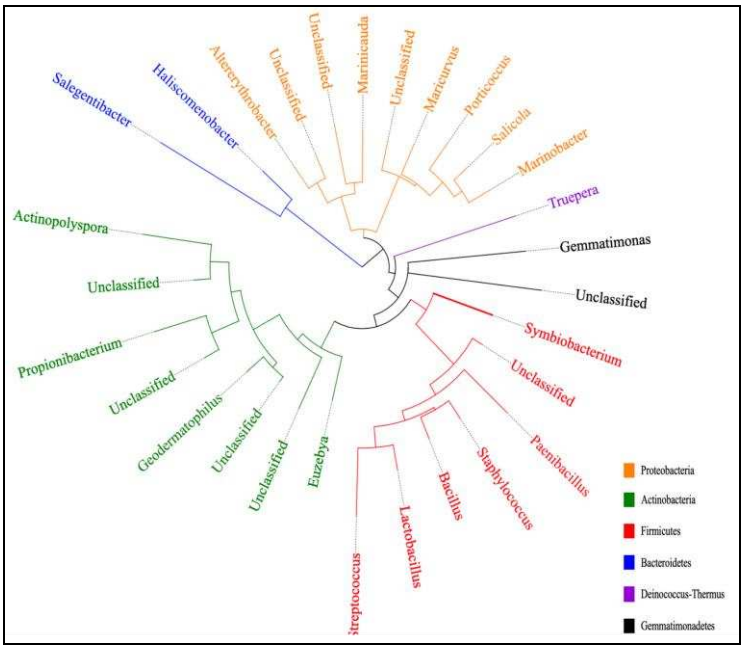

Figure 6. *Un-rooted phylogenetic tree based on 16S rRNA gene sequence representing the diversity of bacteria isolated from the various soil samples at booth the phylum and genus level*

Results of the bacterial communities in the five soil samples at phylum-level taxonomic distribution showed that they are belongs to nineteen phyla, and the most abundant of them were highlighted for further analysis. The results indicated seven most common phyla which are *Proteobacteria* (199 genera), *Actinobacteria* (47 genera), *Firmicutes* (39 genera), *Bacteroidetes* (33 genera), *Deinococcus-Thermus* (one genus), *Gemmatimonadetes* (one genus), and *unclassified* phyla (one genus).

Analysis of the bacterial communalities at the phylum classification showed that *Proteobacteria* and *Bacteroidetes* were the most found in the sample collected from the crust of the *H. perfoliata* (3.L3.S1.C) (*Fig. 7A, B*). *Actinobacteria* and *Firmicutes* were significantly showed in the samples collected from both the rhizosphere and the crust of the *Zygophyllum album* (4.R.3 and 5.C.3) (*Fig. 7C, D*). *Gemmatimonadetes* were found in the control sample and the rhizosphere of the *H. perfoliata* sample (*Fig. 7E*). *Deinococcus-Thermus* was found in the control sample followed by the rhizosphere of the *H. perfoliata* sample (*Fig. 7F*). However, the *unclassified phyla* were found in all of soil samples with a significant abundance in the control sample and the rhizosphere of the *H. perfoliata* sample (*Fig. 7G*).

The previously mentioned highly abundant seven bacteria found at the phylum-level include large numbers of genera, estimated at 321 genera. At the genus level, *Actinopolyspora, Geodermatophilus, Propionibacterium, Euzebya,* and four unclassified were the most abundant found in the *Actinobacteria*. *Salegentibacter*, *Haliscomenobacter*, and one unclassified genus were found in *Bacteroidetes*. Whereas *Sphaerobacter* and one genus unclassified were found in *Chloroflexi*. *Truepera* was found in *Deinococcus-Thermus,* and *Gemmatimonas* was found in *Gemmatimonadetes*. *Marinicauda*, *Altererythrobacter, Maricurvus, Marinobacter, Porticoccus, Salicola,* and three unclassified were found in *Proteobacteria*. *Bacillus, Staphylococcus, Paenibacillus, Lactobacillus, Streptococcus, Symbiobacterium*, and one unclassified genus were found in *Firmicutes* (*Fig. 8*).

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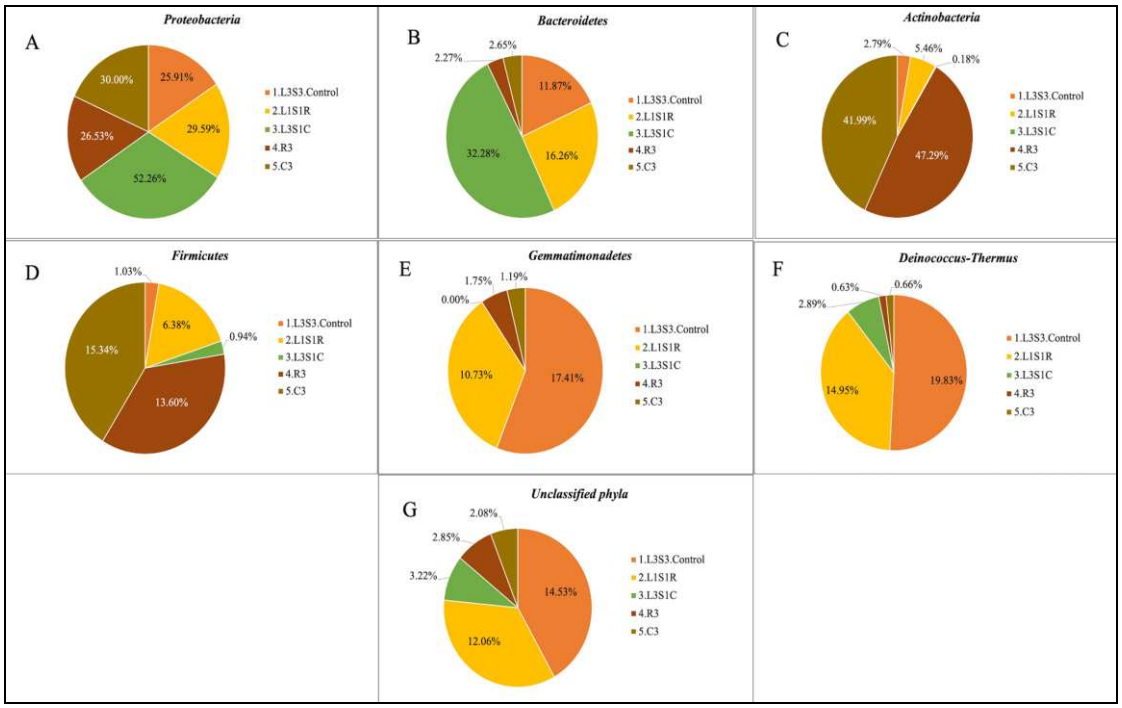

Figure 7. *bacterial communities at the phylum classification among the samples. (A) The amount of Proteobacteria among the samples. (B) The amount of Bacteroidetes among the samples. (C) The amount of Actinobacteria among the samples. (D) The amount of Firmicutes among the samples. (E) The amount of Gemmatimonadetes among the samples. (F) The amount of Deinococcus-Thermus among the samples. (G) The amount of the unclassified phyla among the samples. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

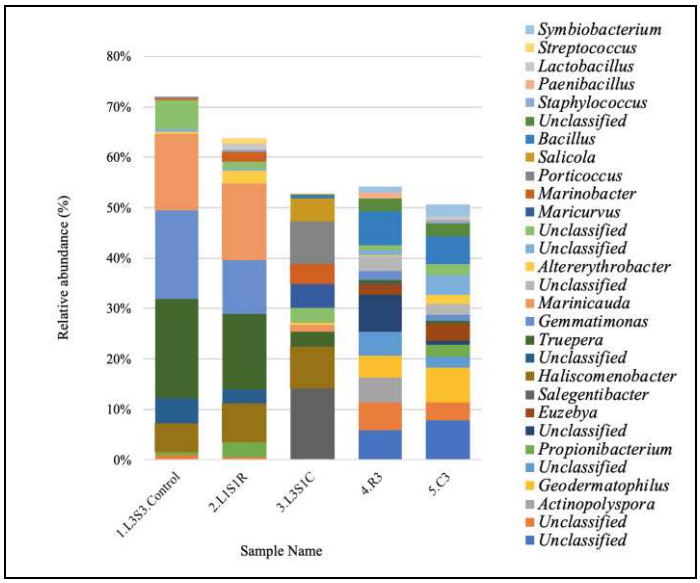

Figure 8. *The relative most abundance in the taxonomic composition distribution in samples of Genus -level. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

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Discussion

Over the past decade, the interest in studying the bacterial diversity in soil salinity and the hot desert has increased due to global climate change with arid regions believed to be more vulnerable (Osman et al., 2016). The purpose of the presented study was to discover the bacterial diversity that inhabit salty soil associated with halophytic plants, which located in the Southern Corniche of Jeddah, Saudi Arabia, in order to pinpoint beneficial strains that promote crops to survive with different environmental stress and have several industrials outcomes. Bacterial cultivation-based isolation methods, which are commonly used, are not very effective due to the limitation of several factors including media compositions, nutritional and environmental requirements of soil microbial communities (Fierer et al., 2012). Our microbiome analysis involves sampling collection, processing, NGS sequencing, and bioinformatics analysis to provide composition of those microbiota populations associated with halophytes. Five various soil samples were collected from different locations of the same area which included; the rhizosphere and crust of both the *H. perfoliata* and the Z. *album* plants. The fifth sample was collected from an area that has no plant and was considered as a control. Amplification of the v1-v3 bacterial 16S rRNA genes regions by PCR detected bacterial biodiversity in these extreme conditions. Therefore, 546,713 high-quality sequences were obtained and classified at the phylum and genus levels, and the differences between bacterial combinations were studied in the five soil samples. The richness and diversity of the bacteria were examined in each sample, and a slight change was found among the samples (from 108 to 333 OTUs) in the five samples from the same region.

Sequencing results showed that the taxonomic distribution of the bacterial communities at the phylum-level indicated nineteen phyla. More importantly, numerous studies have shown that these bacteria have many environmental and ecological benefits including plant growth-promoting bacteria (PGPB) (Gupta et al., 2015). The most dominant phyla among the five different soil samples were *Proteobacteria*. *Proteobacteria* have been used as a biological treatment for various toxic complexes and as naturally bioactive products (Bodenhausen et al., 2013; Mukhtar et al., 2018). Moreover, they are well known to be sensitive to climate change and have a influence on the soil biosphere due to their involvement in the global carbon, nitrogen and sulfur cycles (Zhao et al., 2018). *Actinobacteria* were found frequently in the rhizosphere and the crust of the *Z. album* samples. *Actinobacteria* are commonly used as a source of active antimicrobial biomaterials (Elbendary et al., 2018). They are also essential in restating the uncontrolled biomaterials through the decomposition of plants and dead animals. Moreover, they have a significant function in the production of antibiotics and providing the high resistance of the UV radiation and dehydration (Barka et al., 2016; Zhao et al., 2018). *Bacteroidetes* were shown to be a sensitive biological indicator for agricultural soil use and they have a potential association between antibacterial and antifungal performance (Eida et al., 2018; Wolińska et al., 2017). *Firmicutes* were found to be more abundance in both of the *Z. album* samples. They have been used as a prevailing species among all the marine enzyme-producing microscopic organisms (Divya et al., 2010). They are able to produce salt stress compounds to overcome salt causing osmotic pressures (Meena et al., 2017). *Gemmatimonadetes* were found to be more in the control and the rhizosphere of the *H. perfoliata* sample. They are able to live under aerobic and anaerobic respiration, and this indicates that they can adapt low soil

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DOI: http://dx.doi.org/10.15666/aeer/1806_82178231

moisture (DeBruyn et al., 2011). They also play a major role in the biochemical transformations (Kadam and Chuan, 2016; Zhang et al., 2003). *Deinococcus-Thermus* were found to be significant in the control sample in addition to both of the *H. perfoliata* sample and they were known to be highly resistant to harsh environmental stresses and radiation (Theodorakopoulos et al., 2013). Unclassified bacteria at the phylum level were found with 2.08% in the crust of the *Z. album sample* and 14.53% in the control sample. The emergence of the unclassified bacteria at the phylum-level may be due to the lack of a reference sequence in the database and theses bacteria might include a potential candidate that are still not identified.

At the genus-level, several studies have shown the benefits of the bacteria in agricultural, environmental, medical or industrial applications. For example, *Altererythrobacter,* one of the most abundant genera were found in the soil samples, which fall under the *Proteobacteria*. Many physiological studies have notified that *Altererythrobacter* strains possess degrading activity against rebellious organic petroleum-derived hydrocarbons (Maeda et al., 2018). *Propionibacterium* (of *Actinobacteria* Phylum) one of the genera that were found in the soil sample and thy are widely used in many applications including the production of vitamin B12 and probiotic and cheese industries (Kiatpapan and Murooka, 2002). *Bacillus* were found significantly and they are well known as a source of antibiotics and probiotics. There are a few types of *Bacillus* that hurtful to human *Bacillus anthracis* which cause *anthrax* and *Bacillus cereus* that are responsible for the food poisoning. Moreover, some strains have a greater capacity not only in the soil but also in their ability to produce compounds that can be used for several applications (Ahmad et al., 2018). *Bacillus subtilis* and *Bacillus halotolerans* were shown to produce Polyhydroxyalkanoates (PHAs) that were found to be used as an alternative for petrochemical-derived plastic (Valappil et al., 2007). PHAs were found to be ecofriendly, biodegradable, biocompatible and microbial thermoplastic (El-Hamshary et al., 2018; Zaki, 2018).

Conclusion

This study shows diversity of the microbial communities that present in the soil associated with stress tolerance halophytic plants located in the Southern Corniche of Jeddah, Saudi Arabia. The Kingdom of Saudi Arabia is characterized by extraordinary environments beginning from the brutal desert of the Arabian Peninsula, passing through the salty areas and saline marshes and ending with the Red Sea which is known by its exceptional decent variety. Microbial communities inhibited this ecosystem should be investigated. Our finding showed that rhizospheric microbes can be studied as biomarkers of plant growth rate as well as its power to survive under harsh environmental conditions. This will give a better understanding for novel candidates that can be used as biological agents to improve agricultural and industrial practices. In addition to the identification of the soil bacterial communities through high-throughput molecular tools for the characterization of the taxonomic and phylogenetic, future detailed representation and comparative functional and biochemical studies for the diversity of the soil microbiome is needed to highlight different metabolic pathways. Moreover, future correlation between the taxonomic composition and the functional characteristics of the soil microbiome, will help in the discovery of novel promising candidates to improve the fate of humankind and its resources.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82178231

Acknowledgements. This work was funded by the Deanship of Scientific Research (DSR), University of Jeddah, Jeddah, under grant No. (UJ-01-18-ICP). The authors, therefore, acknowledge with thanks DSR technical and financial support.

Conflict of interests. The authors declare that they have no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82178231

APPENDIX

Figure A1. *The percentage of read quality of the five soil different samples. Q20(%): The percentage of bases in which the phred score is above 20. Q30(%): The percentage of bases in which the phred score is above 30. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

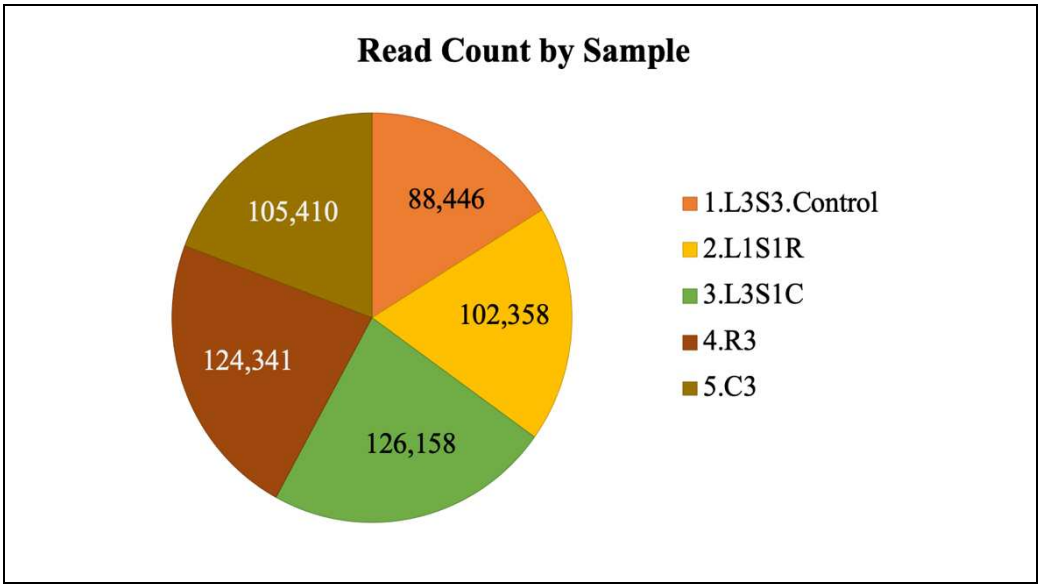

Figure A2. *The total number of sequences reads among the five soil samples. (L3. S3 Control): Control sample; (L1.S1.R): H. perfoliata rhizosphere sample; (L1.S1.C): H. perfoliata crust sample; (R3) Z. album rhizosphere sample; (C3) Zygophyllum album crust sample*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82178231

EFFECTS OF DEGRADABLE MULCHING FILM ON SOIL TEMPERATURE, SEED GERMINATION AND SEEDLING GROWTH OF DIRECT-SEEDED RICE (*ORYZA SATIVA* L.)

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(Received 28th Jul 2020; accepted 6th Oct 2020)

Abstract. The effect of degradable mulching film on dry direct- seeded rice remains largely unknown. Then the aim of this research is to investigate the effects of degradable mulching film on dry direct-seeded rice. A field investigation of four treatments (CK: non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)) was conducted to evaluate the effects of degradable mulching film on the rice seed germination, seedling growth, soil temperature, and grain yield of dry direct-seeded rice. The results showed that compared to CK, mulching film treatments increased soil temperature, especially at night time, improved seed germination rate, plant height, leaf area of seedlings, and grain yield. MF1 showed good degradation performances and had the highest soil temperature at the night time of 13.65 ℃ - 14.08 ℃, grain yield at 7.938t ha^{-1}, and seedling growth with shoot dry mass at 46.73 mg $plant^{-1}$ and root dry mass at 31.34 mg $plant^{-1}$. The germination rate significantly increased by 6.99%-755.60% at MF1 as compared to CK. Overall, mulching films resulted in high yield due to the increasing soil temperature, seedling germination, and improving seedling growth, amongst MF1 performance the best.
Keywords: *hill-drop drilling, grain yield, degradation progress, leaf area, root to shoot ratio*

Introduction

Mulching film in the field provided a suitable microclimate for crop growth (Diaz-Perez et al., 2009; Namaghi et al., 2018; Zhang and Miles, 2020), enhanced the disaster resistance ability of crops and ultimately increased crop production (Bu et al., 2013; O'Loughlin et al., 2017; Deschamps et al., 2019). The use of plastic film mulching allows for an early seeding date and a shortened germination time and increases the germination rate and the emergence rate, and achieves water-saving effects (Li et al., 2013; Biswas et al., 2015; Cosic et al., 2017). Especially, dry direct-seeded rice with mulching film could improve water utilisation rate, inhibit weeds growth, and improve soil temperature after sowing, which were conducive to high and stable yield (Towa et al., 2013; Fawibe et al., 2020). Traditional mulching film is polyethylene (PE), its application has caused environmental pollution and affected the food safety (Chae et al., 2018; Boots et al., 2019;

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82338249

Hu et al., 2020). Therefore, the use of degradable mulching film is essential for the development of future agriculture.

Previous studies have reported the application of biodegradable mulching films (Brodhagen et al., 2015; Moreno et al., 2017; Cozzolino et al., 2020). For example, the effects of biodegradable films on the production of peanut (Sun et al., 2018). The effect of mulching films on the biomass and soil organic matter mineralization and the yield of tomato has been evaluated (Moreno et al., 2008). A previous study has reported that biodegradable polymers improved root growth conditions and fruit quality of tomato (Sekara et al., 2019). The biodegradable paper mulching increased the yield of tomato and improved the fruit quality due to reduced nitrate but increased vitamin C (Zhang et al., 2019a). It has been reported that degradable mulching films affect the corn production and that degradable mulching films have comparable temperature conservation and water retention effects in response to common mulching films (Yang et al., 2010). Besides, previous report has indicated that the corn yield with biodegradable mulching film was similar to that with common mulching film and the biodegradable mulching film was sufficiently degraded after crop harvest (Hu, 2015). Research has shown that there were no significant differences in yields of *Brassica chinensis* L. among the degradable films, but the yield under degradable films was increased by 80% as compared to that with the non-mulching film and by 50% compared to that of common mulching film, and the natural protein, soluble sugar, and other components in *B. chinensis* L. were adequately improved (Shi et al., 2012). The effect of biodegradable film mulching on the production test of winter oilseed rape has been investigated and results showed that no significant difference in the average yield and water use efficiency in degradable film mulching were detected, whilst degradable film mulching showed higher seed quality than PE (Gu et al., 2017). Moreover, Bilck et al. (2010) have reported the effects of biodegradable mulching film on the production and quality of strawberry. Further study about the degradable film mulching on crop growth and development is still needed.

The effect of the application of degradable mulching film on the growth and yield of many crops such as tomato, corn, strawberry has been studied. Few studies have been conducted to evaluate the effect of degradable mulching film on the rice grown and yield. In this study, a field experiment was conducted to evaluate the degradable mulching film on the rice seed germination, seedling growth, soil temperature, and grain yield of direct-seeded rice. The results of this study may provide a basis and reference for the application of degradable mulching film in the rice field.

Materials and methods

Description of study field

The experimental site is located at Wudaohezi Village, Haolibao Town, Jalaid Banner, Hinggan League, Inner Mongolia Autonomous Region, P.R. China (46°35'43"N, 123°04'36"E; 174 m in altitude). It has a temperate continental monsoon climate. There is a large temperature difference between day and night. The average annual temperature is 4.4 °C; the average annual precipitation is 430 mm, with the precipitation is primarily concentrated from June to August. The average frost-free period is 130 d. The site has a meadow soil type with deep and thick layers, and upon tillage, it is finely broken with a flat surface. The mass fraction of organic matter in the plough layer was 22.36 g/kg, and the pH of the topsoil was 5.89.

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DOI: http://dx.doi.org/10.15666/aeer/1806_82338249

Mulching film performance

In this study, three types of mulching film, MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China), MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China), and MF3: a common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China), were used. The performance measurements of each mulching film were repeated three times. The transmittance is an important index of optical performance that determined the amount of solar energy absorbed by the soil. In this study, transmittance/fog tester is used (Brand: Shanghai Shenguang Instrument & Meter Co., LTD.; Model Number: WGT-S; Division value: 0.01%). The light transmittance was in trend of MF1>MF3>MF2. Microcomputer controlled electronic universal material testing machine (Brand: Shanghai Hengyi Precision Instrument Co., LTD.; Model No.: Hy-0580; Maximum load: 500 N; Precision grade: 0.5 magnitudes) was used to detect the film tensile strength, elongation, and elastic modulus. The tensile strength and modulus of elasticity in MF2 and MF3 are better than that of MF1. The elongation of MF1 and MF3 was superior to that of MF2. (as shown in *Table 1*).

Table 1. *The properties of Mulching film*

Mulch	Transmittance (%)	Tensile Strength (Mpa)	Elongation (%)	Elastic Modulus (Mpa)
MF1	6.48A	9.51B	278.45B	148.80B
MF2	0.58C	10.51AB	93.64 C	297.39A
MF3	4.50B	11.90A	469.28A	263.27A

Different uppercase letters followed by the same column among the treatments means significant differences ($p<0.01$) according to LSD. MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)

Experiment design

Three mulching film treatments (MF1, MF2, and MF3) were carried in this experiment, and the non-mulching treatment was taken as control (CK). The three types of mulching film are black, with 0.01 mm in thick, and 1550 mm in wide. The planting pattern of "one film, two drip irrigation belts, and eight rows" (as shown in *Fig. 1*) was adopted, and all the field work was completed by a multi-functional machine that integrated film laying, seed sowing, and soil covering.

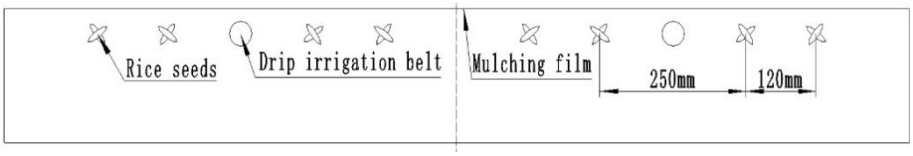

Figure 1. *Planting pattern diagram*

In the experiment, the length of each treatment was 50 m, and each treatment was repeated 3 times, and the total plot area was 1250 m^2 (50×25 m). The distance between treatments was 600 mm which was increased that the external influences and interactions between the treatments were reduced. During planting, wide and narrow row spacing was

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DOI: http://dx.doi.org/10.15666/aeer/1806_82338249

used in an alternating manner, with the narrow row spacing being 120 mm and the wide row spacing being 250 mm. One drip irrigation belt was set in the middle of the wide row, which was shared by four rows.

The same water and fertilizer management were used in the four treatments, which was consistent with local field management. The pump station was used to supply water and fertilizer in equal amounts through drip irrigation belts, and the time of water and fertilizer application was recorded. On April 10, 2019, base fertilizer was applied, and the fertilizer was mixed fertilizer (N:P:k=12:18:15) of 150 $kgha^{-1}$ and biological fungus fertilizer of 150 kg ha^{-1}. Topdressing twice during the growth cycle: June 5 (urea: 37.5 kg ha^{-1}, liquid fertilizer (high nitrogen type): 22.5 kg ha^{-1}) and June 20 (liquid fertilizer (high potassium type): 22.5 kg ha^{-1}). According to the growth stage of rice and weather conditions to determine the time and amount of water supply. In the mulching treatments, manual weeding was used, while the combination of mechanical and herbicide weeding was applied in the non-mulching treatment.

The tested rice variety was Suijing 18, which covers the largest local cultivation area. It has a growth period of 135 d. The technique of seeding in dry soil and drip irrigation for emergence was adopted. The seeds were sown on April 29, 2019, and 15±3 seeds were sown per hill with a hill spacing of 120 mm; and the plants were harvested on September 25-28, 2019. The degradation progress of mulching film was observed until the next sowing season (April 15, 2020).

Sampling and measurement

Soil temperature

The soil temperature at different soil depths for each treatment was measured by a set of sensors (Manufacturer: Sonbest Company of Shanghai; Model Number: KM3002B; Configuration: three probes), which were installed in the soil at 5, 10, and 15 cm below the ground surface. The system collected and storaged data automaticly at intervals of 30 min.

Germination rate

The germination rate is an important basis for guaranteeing that a crop has full germination with a single sowing, and it can reflect the effects of different treatments on germination. After the first water supply, the rice seeds would readily have the appropriate germination conditions. After 5 d of water treatment, the germination condition was closely monitored. For each treatment, 10 hills of plants were selected to determine the germination rate, which was recorded once a day until the germination rate of the all treatments was stable; the germination rate for each treatment was rendered as the final germinate rate.

$$\text{Germination rate (\%)} = \text{number of buds / number of seeds} \times 100\% \quad \text{(Eq.1)}$$

Plant height and leaf area

The plant height and leaf area are the most commonly used indicators for measuring the growth of rice seedlings at the seedling stage. At the three-leaf stage, 10 rice plants with uniform growth were selected for each treatment; their plant heights were measured using a steel ruler, and the leaf area of each plant was calculated using an LA-S series

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plant image analyser (Manufacturer: Wseen Ltd., Hangzhou, China; Model Number: LA-S Series).

Biomass

The root and shoot biomass of rice plants were measured at the three-leaf stage. for each treatment, 10 rice seedlings with uniform growth were selected and excavated as a whole. They were washed and rinsed with water, air-dried, and then cut with scissors at the top of the root system. The plants were separately placed in a drying container, which was in turn placed in an electric thermostatic drying oven (Manufacturer: Shanghai Heheng Instrument & Equipment Co., Ltd.; Model Number: DHG-9050A) at 105 °C for fixation (30 min); next, the temperature was adjusted to 80 °C to dry the materials to a constant weight. The samples were weighed using a high-precision electronic balance (Model: Hengji Electronic Analytical Weighing Scale FA1204; Precision level: level 1; Range: 120 g; Division value: 0.1 mg).

The degradation progress of mulching film

Mulching film cannot degrade prematurely to affect the growth of rice seedlings, meanwhile, the degradation cycle cannot be too long to affect the planting of the next crop season. In this study, the degradation process was recorded and measured by visual assessment and picture comparison. The induction period, cracking period, major cracking period, fragmentation period, residue film entrainment at harvest, and the degradation of residue film under conventional tillage were recorded.

Grain yield

The grain yield was randomly harvested from three points (1 m^2) for each treatment. After artificial threshing, electronic scale is used to weigh the rice grains, and then the average value of the three points is calculated, which is the grain yield under this treatment.

Statistical analysis

Data processing (the analysis of variance (ANOVA) and correlation coefficients) were performed using Microsoft Excel and Design Expert software. The differences amongst means separated by using the least significant difference (LSD) test at 5% significance level.

Results

Soil temperature

MF1 showed higher in the soil temperature at the 5 cm depth as compared to CK. A higher in the soil temperature at the 5 cm depth from 7:00-22:00 was detected at MF2 when compared to CK. MF3 showed lower in the soil temperature at the 5 cm depth from 7:00-16:00 but higher in the soil temperature at the 5 cm depth from 16:00-7:00. Compared with CK, MF1 and MF2 showed higher in the soil temperature at the 10 and 15 cm depth. MF3 showed lower in the soil temperature from 7:00-16:00 and 8:00-17:00 at 10 and 15 cm depth than CK, respectively. Compared with CK, higher in the soil

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temperature from 16:00-7:00 and 17:00-8:00 at 10 and 15 cm depth was detected for MF3 (*Figure 2*).

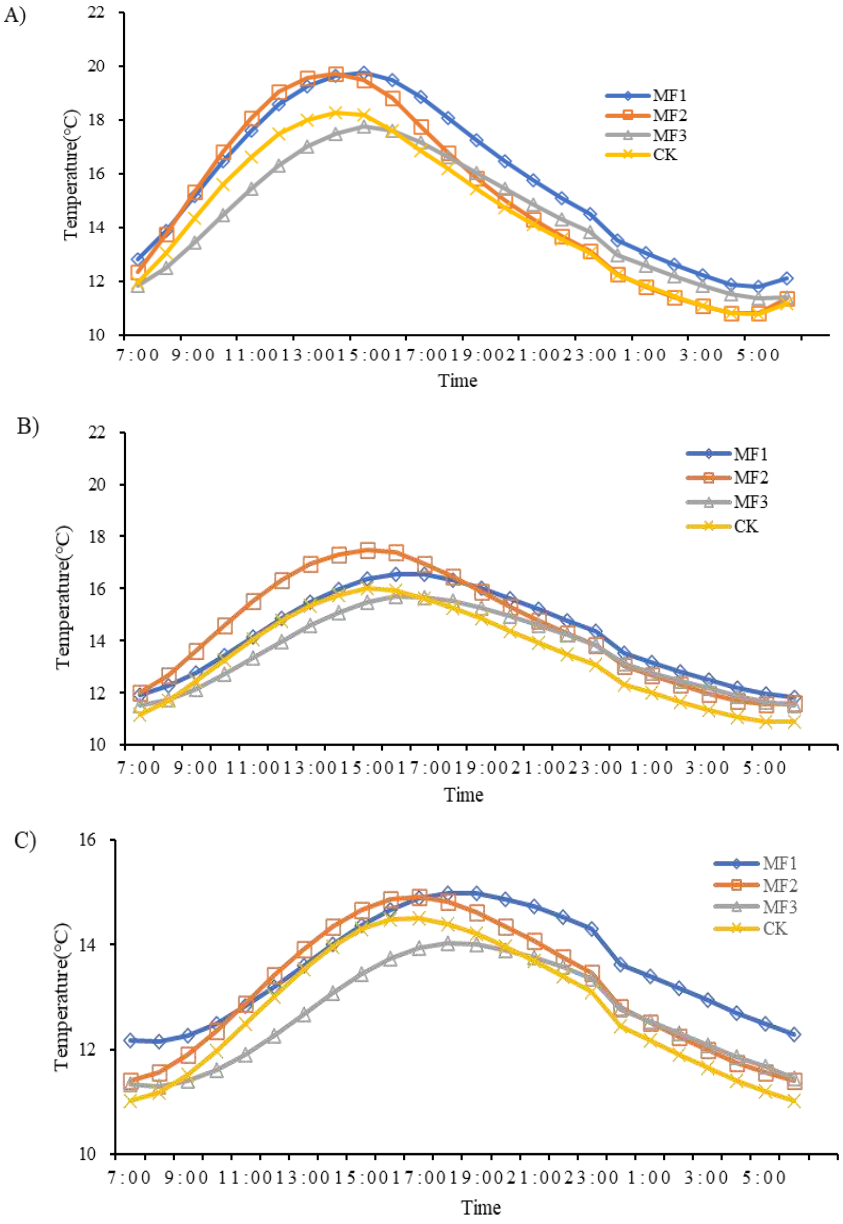

Figure 2. *Changes of the daily soil temperature in soil depth of 5 cm (A), 10 cm (B), and 15 cm (C). CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

From 8:00-18:00, compared with CK, MF1, and MF2 significantly increased the average soil temperature at 5cm depth by 7.63% and 7.42%, respectively. The average soil temperature at 10 cm depth significantly increased by 9.58% at MF2 as compared to CK. No significant difference was detected in the average soil temperature at 15 cm depth among the treatments (*Table 2*).

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Table 2. *The average soil temperature under different treatments*

Treatment	Temperature (℃) during 8:00-18:00			Temperature (℃) during 18:00-8:00		
	5 cm	10 cm	15 cm	5 cm	10 cm	15 cm
MF1	17.862a	14.84b	13.451a	14.077a	13.732a	13.653a
MF2	17.826a	15.882a	13.482a	12.894bc	13.387a	12.911b
MF3	15.912b	14.036c	12.534b	13.34b	13.249a	12.755b
CK	16.596b	14.493bc	13.096ab	12.738c	12.595b	12.54b

Different lowercase letters followed by the same column among the treatments means significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)

From 18:00-8:00, the average temperature in the soil at 5 cm depth significantly increased by 10.51% and 4.73% at MF1 and MF3 as compared to CK, respectively. Compared with CK, MF1, MF2, and MF3 significantly increased the average temperature in the soil at 10 cm depth by 9.03%, 6.29%, and 5.19%, respectively. The average temperature in the soil at 15 cm depth significantly increased by 8.86% at MF1 as compared with CK (*Table 2*).

Germination rate

Compared with CK, significantly increased germination rate by 213.02% was detected at MF1 on 10 May. On 12 May, the germination rate significantly increased by 755.60%, 450.71%, and 621.54% at MF1, MF2, and MF3, respectively as compared to CK. Significantly increased in the germination rate at MF1, MF2, and MF3 were detected on 13 May (increased by 331.26%- 378.49%), 14 May (increased by 20.97%-22.13%), 15 May (up to 8.63%), and 16 May (up to 6.99%) (*Figure 3*).

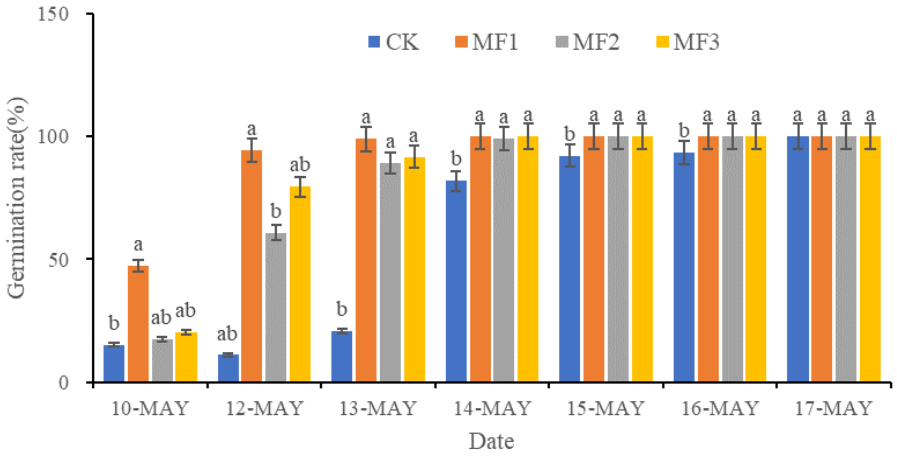

Figure 3. *The germination rate under different treatment. Different lowercase letters above bars indicated that there were significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

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Seedling growth

The plant heights significantly increased by 46.88%, 57.29%, and 41.67% at MF1, MF2, and MF3 as compared to CK, respectively (*Figure 4*). The leaf areas significantly increased by 26.12%, 25.64%, and 30.64% at MF1, MF2, and MF3 as compared to CK, respectively (*Figure 5*). MF1 had the highest dry mass in the shoot (46.73 mg plant^{-1}) and root (31.34 mg plant^{-1}), whilst no significant difference in shoot biomass, root biomass, and root to shoot ratio was detected (*Figures 6-8*).

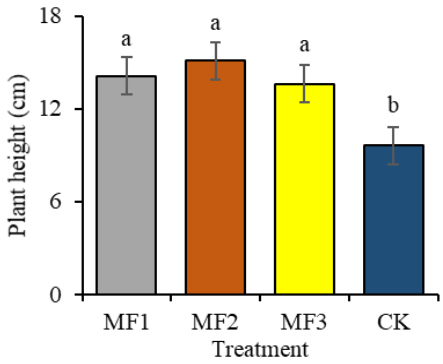

Figure 4. *The plant height of seedling under different treatments. Different lowercase letters above bars indicated that there were significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

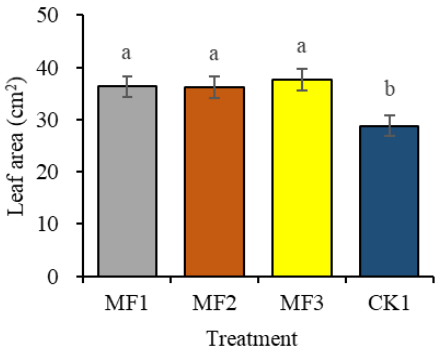

Figure 5. *The leaf area of seedling under different treatments. Different lowercase letters above bars indicated that there were significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

Grain yield

The highest grain yield was detected at MF1 (7.938 t ha^{-1}). The grain yield was increased by 11.83%, 4.82%, and 5.73% at MF1, MF2, and MF3 as compared to CK, respectively (*Figure 9*).

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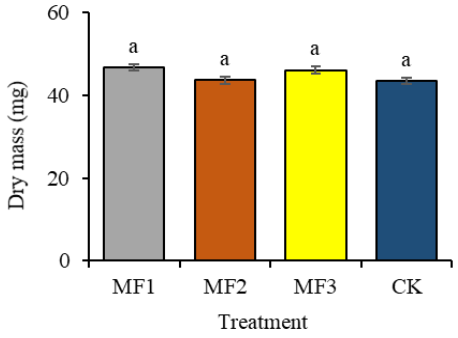

Figure 6. *The aboveground dry mass of seedling under different treatments. Different lowercase letters above bars indicated that there were significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

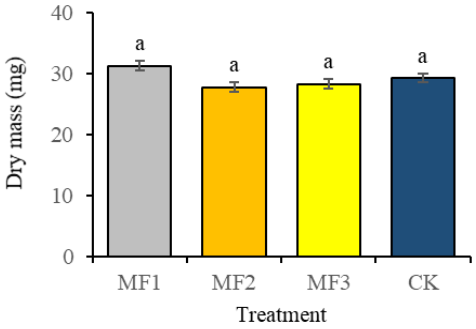

Figure 7. *The root dry mass of seedling under different treatments. Different lowercase letters above bars indicated that there were significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

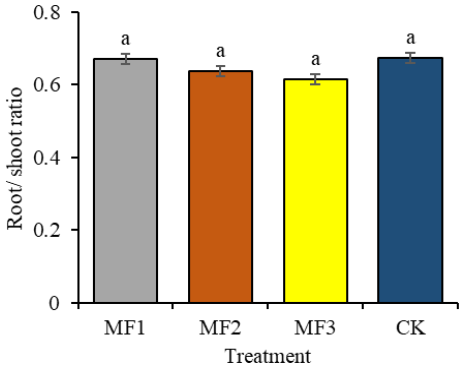

Figure 8. *The root/shoot ratio of seedling under different treatments. Different lowercase letters above bars indicated that there were significant differences ($p<0.05$) according to LSD. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

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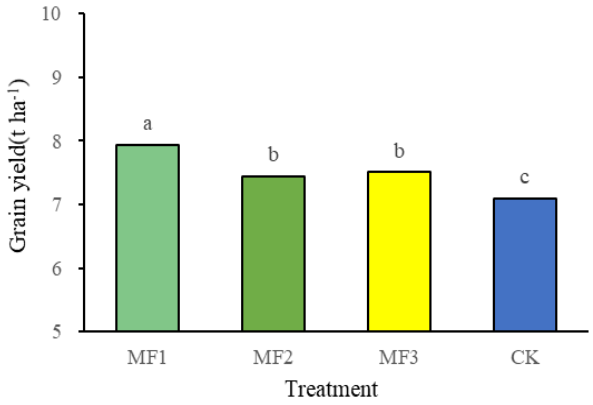

Figure 9. *The grain yield under different treatments. CK: Non-mulching; MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: Common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)*

The degradation progress of mulching film

The induction period for MF1 and MF2 was 19-June and 28-June, respectively. The cracking period for MF1 and MF2 was 28-June and 7-July, respectively. A similar major cracking period and fragmentation period were detected for MF1 and MF2. Both MF1 and MF2 showed good degradation performances. MF3 showed no degradation (*Table 3*).

Table 3. *The degradation progress of mulching film in field*

Period	MF1	MF2	MF3
Induction period	June 19	June 28	-
Cracking period	June 28	July 7	-
Major cracking period	September 7	September 7	-
Fragmentation period	September 17	September 17	-
Residue film entrainment at harvest	No entrainment	No entrainment	No entrainment
Degradation of residue film under conventional tillage	Good degradation	Good degradation	No degradation

MF1: a degradable film (Shanghai Hongrui Biotech, Shanghai, China); MF2: a degradable film (Xifeng Plastic Corp. Ltd., Baishan, China); MF3: common agricultural mulching film (Jialiming New Material Corp Ltd., Hinggan League, China)

Correlation analysis

The germination rate on 10 May was significantly positively related to the mean temperature at 5 cm depth in the soil from 18:00-8:00 and the mean temperature at 15 cm depth in the soil from 18:00-8:00. The plant height is highly related to the germination rate on 14-May, 15-May, and 16-May. The leaf area is highly related to the germination rate on 13-May 14-May, 15-May, and 16-May (*Table 4*).

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Table 4. *Correlation analysis between the investigated parameters*

Index	V001	V002	V003	V004	V005	V006	V007	V008	V009	V010	V011	V012	V013	V014	V015	V016
V002	0.7059															
V003	0.5390	**0.9598***														
V004	0.4664	0.9414	**0.9962****													
V005	0.4410	0.9254	**0.9935****	**0.9987****												
V006	0.4410	0.9254	**0.9935****	**0.9987****	**1.0000****											
V007	0.7859	0.8488	0.6709	0.6388	0.6000	0.6000										
V008	0.8700	0.2894	0.0574	-0.0247	-0.0560	-0.0560	0.5767									
V009	0.3809	-0.3781	-0.5129	-0.5846	-0.5906	-0.5906	-0.1456	0.7240								
V010	0.3622	0.8250	0.9476	**0.9526***	**0.9661***	**0.9661***	0.4019	-0.1390	-0.5282							
V011	0.3862	0.9230	**0.9770***	**0.9903****	**0.9869***	**0.9869***	0.6441	-0.1009	-0.6788	0.9195						
V012	0.5101	0.2720	0.3444	0.2911	0.3147	0.3147	-0.0407	0.3462	0.4302	0.4867	0.1581					
V013	-0.0204	0.0254	0.2358	0.2275	0.2715	0.2715	-0.4502	-0.2249	0.0842	0.5091	0.1320	0.8321				
V014	0.3836	0.0255	0.0957	0.0412	0.0677	0.0677	-0.2346	0.3369	0.5854	0.2649	-0.0941	**0.9674***	0.8273			
V015	**0.9551***	0.8403	0.6701	0.6139	0.5824	0.5824	0.9309	0.7564	0.1237	0.4489	0.5681	0.3041	-0.1870	0.1303		
V016	0.7559	0.9407	0.9406	0.9084	0.9044	0.9044	0.6890	0.3372	-0.1919	0.8837	0.8473	0.5810	0.3298	0.3594	0.8000	
V017	**0.9628***	0.7655	0.6667	0.5994	0.5858	0.5858	0.6962	0.7381	0.2907	0.5603	0.5046	0.6817	0.2284	0.5342	0.9043	0.8732

* and ** significant at 0.05 and 0.01 level, respectively.

V001: germination rate on 10-May; V002: germination rate on 12-May; V003: germination rate on 13-May; V004: germination rate on 14-May; V005: germination rate on 15-May; V006: germination rate on 16-May; V007: aboveground dry mass; V008: root dry mass; V009: root/shoot ratio; V010: plant height; V011: leaf area; V012: mean temperature at 5 cm depth in soil during 8:00-18:00; V013: mean temperature at 10 cm depth in soil during 8:00-18:00; V014: mean temperature at 15 cm depth in soil during 8:00-18:00; V015: mean temperature at 5 cm depth in soil during 18:00-8:00; V016: mean temperature at 10 cm depth in soil during 18:00-8:00; V017: mean temperature at 15 cm depth in soil during 18:00-8:00

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Discussion

The effect of degradable and traditional mulching films on soil temperature has been widely documented. Generally, the degradable mulching film and PE mulching film have similar effects of increasing soil temperature at crop early growth stages. For example, Shen et al. (2012, 2019) reported that in the early period of maize growth, the degradable films had good warming effects on soil, which were similar with the plastic film. The degradable films fulfilled successfully all the functions of the plastic film, thus they were recommended as viable option to the plastic film due to their good degradability. Zhang et al. (2020) reported that biodegradable plastic film mulching increased the soil temperature at soil depths of 5 cm, 15 cm, and 25 cm, over the maize's entire growth period, by 3.1 °C-3.2 °C in 2017 and 1.2 °C-2.1 °C in 2018 compared with the non-mulched treatment. This study shows that biodegradable plastic film could be used as a substitute for common plastic film (Zhang et al., 2020). But, previous study reported that the soil temperature under degradable mulch was higher than that of PE mulch (Subrahmaniyan et al., 2008), which was maybe related to the transmittance of mulch. The amount of heat exchange and radiation at night was related to the mulch materials, as the description of previous studies (Sekara, 2019). Therefore, in the present study showed that MF1 degradable film and the PE plastic film had good warming effects on soil in the early period of rice growth, but the increased temperature performance of degradable mulching films at the 5 cm, 10 cm, and 15 cm soil depths outperformed that of PE mulching film and the non-mulching treatment (*Table 2, Figure 2)*. Both degradable mulching film and PE mulching film had the effect of raising soil temperature, especially the thermal insulation effect at night promoted the germination of rice seeds and affected the growth of seedlings. Previous study reported that degradable mulch could significantly improve the temperature of soil tillage layer and promote plant growth and development. The grain yields for degradable films No.2, No.1, and No.3 which had different degradation cycles were significantly improved, and with no significant difference between degradable films and the plastic film (Shen et al., 2019). Previous study found that the night temperature of biodegradable mulch was higher than that of ordinary mulch, promoting the growth of crops (Zhang et al., 2016). Yin et al. (2012) described that biodegradable film mulching could significantly improve the temperature of soil tillage layer, with better thermal insulation performance, and was conducive to the growth and development of crops in the early stage. Alamro et al. (2019) reported that the current results showed that all mulching plot had significantly higher soil temperature than bare soil. The improvement of tomato plant growth was noticed mainly in leaf area, fresh and dry weight of shoot under biodegradable mulch treatment. Based on the results, the using of biodegradable mulch could perform similar and/or better than polyethylene mulch in term of tomato growth and yield, which could be adopted as a sustainable alternative to polyethylene mulch. Lopez-Tolentino et al. (2017) found that different plastic mulches impact positively on the yield of cucumber crop. The benefit in yield by the different plastic mulches in the conditions of this study was due to their soil warming ability that results in improved soil temperature, leaf area, and plant dry weight (Lopez-Tolentino et al., 2017). In the present study, degradable mulching film showed better warming effect, which was due to its higher light transmittance. Meanwhile, MF1 had the highest soil temperature at the night time of 13.65 ℃ - 14.08 ℃ (*Table 2, Figure 2*), which effectively extended the growth time of crops, and promoted rice germination and plant growth. The results of the present study were consistent with those of previous studies.

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MF1 degradable mulching film could be used as an alternative to PE mulching film due to the its similar properties in temperature.

As is known to all, temperature is an important factor affecting the germination of seeds. Mulching films increases the soil temperature and creates good conditions for seed germination. In this study, compared with CK, mulching film treatments increased soil temperature, especially at night time, which promoted the germination of seeds. Adamczewska-Sowinska and Sowinski (2020) reported that low soil and air temperatures hinder the germination of sweet maize seeds and their early growth. Similarly, previous study showed that degradable mulch has the same effect as PE plastic film, which promotes the germination and emergence of seeds and speeds up the growth process (Zhang, 2010). All of the researches were the same as the present study, in the present research both degradable mulching films and PE mulching film promoted the germination progress of rice seeds, and MF1 was 4 d earlier than that under the non-mulching treatment, and these effects may be due to the insulation and water retention of mulching films.

In the present study, the plant height, and leaf area of rice in the different mulching film treatments were higher than those in the bare soil, with no significant differences detected among the three mulching films. In this study, the positive effect of MF1 degradable mulching film on rice plant height, and leaf area in this region was comparable to that of the PE plastic film (*Figures 4-8*). These results are similar to previous studies, which have described that no significant differences were recorded in leaf area of seedling between degradable mulching films and common mulching film, whereas leaf area was lowest under the not mulched control. Moreover, degradable black mulching film N8 and common mulching film had the highest fruit yield, 77.8 t/ha on average, the not mulched control had the worst performance, 68.8 t/ha (Sekara et al., 2019). Zhang et al. (2019b) reported that the average plant height had no significant difference across all mulched treatments, but was greater than no mulch control. Furtherly, total fruit yield was greater across mulched treatments than the bare ground control. Zhang (2010) found that degradable mulch increased plant height and dry matter accumulation of crops, with no significant difference from normal mulching film. And the analysis of yield characteristic indicated that covered by normal mulching film and degradable film had great significance with bare land, but the influence of liquid film was not significant. Deng et al. (2019) found that both the biodegradable mulch film and the polyethylene plastic film significantly increased various physiological parameters, such as crop height, stalk diameter, and leaf area. And all the mulch film treatments significantly increased the yield of maize and cotton, but there was no significant difference among the three mulch film treatments. In this research, these mulching film treatments increased rice grain yield by 4.82-11.83% as compared to CK, due to mulching film treatments to promote the growth and health of plants, it was associated with the increase in rice grain yield. So, the present study was in agreement with previous studies.

The degradability of degradable mulching film is an important factor to be adopted. A lot of studies have been carried out on the degradation of degradable mulching film. Sintim et al. (2020) studied the degradation of different biodegradable plastic film in compost and soil. The results showed that the degradation performance of degradable plastic film was mainly affected by its physical and chemical properties. Meanwhile, the degradation rate of degradable plastic film in compost was faster than that in soil, the degradation rate in summer was greater than that in winter, and the degradation rate in rainy season was better than that in dry season. Yin et al. (2019) carried out a study on

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the effects of different degradation rates of biodegradable mulching film on soil environment and maize growth. The study showed that the degradation rate of biodegradable mulching film was affected by climate and crop species. For example, high temperature increased the degradation rate of biodegradable mulching film. Previous study reported that under controlled conditions, the aerobic biodegradation of Agrobiofilm®1 had an increase of 55.8% when comparing the continuous and the batch system, for 72 days of assay (Costa et al., 2014). In a maize experiment, Zhang et al. (2020) described that at the end of the growth period the degradation rates of biodegradable film1, 2, and 3 were 35.2%, 19.7%, and 18.1% in 2018, respectively. In the current study, the two kinds of degradable mulching film showed good degradation performance (*Table 3*). There was no bulk mulching film residue during tillage and preparation, which had no effect on the tillage and the next sowing season. Meanwhile, the degradation cycles could meet the requirements of rice growth. The present results consistent with the ones achieved.

Overall, mulching film treatments affected the soil temperature, lead to changes in the germination rate, plant height and leaf area of seedlings, and ultimately affected the grain yield. MF1 showed good degradation performances and the highest soil temperature at night time, grain yield, germination rate, and seedling growth.

Conclusions

Mulching film treatments increased soil temperature at night time, germination rate, plant height and leaf area of seedlings, grain yield. MF1 showed good degradation performances and the highest soil temperature at night time, grain yield, germination rate, and seedling growth. So MF1 degradable mulching film could be considered as an alternative for the common agricultural mulching film.

Degradable mulching film has a positive effect on the growth and yield of rice. In the future, the trial area of degradable mulching film in rice cultivation should be expanded to further verify the feasibility of this technology and the adaptability to different soil conditions. Meanwhile, the influence of degradable mulching film on soil should be studied.

Acknowledgements. The work was supported by the Special Fund for Construction of Modern Agricultural Industry Technology System of China (CARS-01-41), and Special Projects in Key Area of 'Artificial Intelligence' of General Colleges and Universities in Guangdong Province (2019KZDZX1003).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82338249

COVID-19 INFECTION IN INFLAMMATORY BOWEL DISEASE PATIENTS TREATED WITH TNF-A ANTAGONISTS: A POSSIBLE CRITICTAL ENROLLMENT OF GUT MICROBIOTA AND VITAMIN D LEVEL – A REVIEW

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(Received 1st Aug 2020; accepted 22nd Oct 2020)

Abstract. The ongoing pandemic of coronavirus disease 2019 (COVID-19) is emerging as a public health crisis worldwide. Patients with COVID-19 range from being asymptomatic to suffering from severe pneumonia, acute respiratory distress syndrome (ARDS), and multiple organ failure (MOF). Gastrointestinal (GI) symptoms have been reported in a number of patients with COVID-19, suggesting that GI microbiota may play a role in the pathogeneses of the disease. Theoretically, patients with confirmed inflammatory bowel disease (IBD) treated with immune-based therapies may be at a higher risk of manifesting a severe form of COVID-19, owing to immune system impairment. This hypothesis has evoked the concerns of patients and treating physicians throughout the pandemic. However, surprisingly, the findings of a number of studies show that immunosuppressive therapies, such as anti-TNF agents, could reduce the severity of symptoms associated with Covid-19. Dysbiosis of gut microbiota, characteristic of IBD patients, can be positively changed after using anti-TNF agents. Vitamin D has been revealed to have a profound effect on reducing the viral infections, aside from its role in modulating the gut microbiome. In this review, we discuss possible susceptibility of IBD patients to SARS-CoV-2 infection, the impact of immunosuppressive therapies on the course of SARS-CoV-2 infection in patients with IBD, and the potential protective role of gut microbiota against COVID-19 in patients with IBD in the presence of normal vitamin D levels.
Keywords: *Crohn's disease, ulcerative colitis, SARS-CoV-2, IBD, biologics, microbiome, VDR*

Abbreviations: IBD: inflammatory bowel disease, COVID-19: corona virus disease that is a novel virus detected in 2019, CD: Crohn's disease, UC: ulcerative colitis, SCFAs: short chain fatty acids, VDR: vitamin D receptors, MOF: multi organ failure, ARDS: acute respiratory distress syndrome, MODS: multiple organ dysfunction syndrome, GI diseases: gastrointestinal diseases, ICU: intensive care unit, HCoV: human corona viruses, IFX: infliximab, ADA: adalimumab, Anti-TNF: anti-tumor necrosis factor, ARBs: angiotensin receptor blockers

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82518265

Background

The challenge of controlling this infectious disease stems from the rapid spread of the causative virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), across all nations and across all borders. The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection was first reported in December 2019 in Wuhan, central Hubei Province, China and has subsequently spread worldwide (Wang et al., 2020). As of mid-June 2020, approximately 10 million cases and 500 000 deaths in 200 countries have been reported (https://www.worldometers.info/coronavirus/). Ten percent of patients have required hospitalization or admission to an intensive care unit (ICU) (Danese et al., 2020; Huang et al., 2020). This has resulted in a dramatic challenge for governments and services, putting significant pressure on healthcare providers owing to the potential for exceeding the capacity of healthcare systems around the world, including IBD centers

Notably, the gastrointestinal (GI) tract can be a transmission route for SARS-CoV-2, as some GI warning signs, such as nausea, diarrhea, and abdominal pain have been recorded in about 20-50% of COVID-19 patients (Lin et al., 2020; Zang et al., 2020). These symptoms likely appear before the development of respiratory symptoms, indicating that the GI tract may be a target organ for the virus. Therefore, patients suffering from intestinal disorders, particularly IBD, could potentially be vulnerable to COVID-19 infection in the current epidemic.

During the course of the disease, a large number of patients with IBD receive biologics treatments such as α-TNF antagonists, which have double-edged sword effects. These medications contain molecules that can cause inflammation, increasing the risk of infections such as respiratory illnesses as a result of immune system impairment (Dipasquale and Romano, 2018). This can be complicated with dynamic bidirectional interactions with the host microbiome. Although recent studies have suggested that most immune system responses are driven by the host genome's stimulations, about 10% of immune system responses were reported to be stimulated by a direct interaction with the host microbiome (Gilbert et al., 2018; Schirmer et al., 2016). The GI microbiota is documented to have an effect on the quality of the host immune system, which participates, in turn, in modulating gut microbiota composition. Prior studies have approved the major influence of nutrient substances and diet on GI microbiota composition and localization, and their link with immunological pathways (Gao et al., 2018; Thorburn et al., 2014). For example, vitamin D was found to be linked to an individual's GI microbial diversity and to healthy interactions between GI microbiota and intestinal immunity, including short chain fatty acid (SCFA) butyrate (Celiberto et al., 2018).

Supportive evidence has led to speculation about the role of GI microbiota and vitamin D in COVID-19 patient outcomes. It has been observed that individuals at higher risk of evolving severe COVID-19 are those aged >60 years and with underlying chronic diseases, such as hypertension, diabetes, cardiovascular illness, chronic respiratory illness, and cancer (Alifano et al., 2020)—all of which are known to coincide with GI microbiota dysbiosis. Also, elderly people have been shown to suffer more often from severe deficiency in vitamin D (Kweder and Eidi, 2018).

In both humans and mice, it has been found that the expression of the angiotensin I-converting enzyme 2 (ACE2), to which SARS-Cov-2 binds when entering the cell, is considerably higher in the small intestine than in other organs, including the lungs (Zang et al., 2020). Thus, it may be important to investigate the degree of susceptibility

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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of IBD patients to COVID-19 infection. Other clinically relevant questions include whether the use of immune-based therapies has a negative effect on prognosis, or otherwise can help in fighting COVID-19 complications on one hand, or whether gut microbiota composition and vitamin D level have a protective role against COVID-19 infection on the other hand.

SARS-CoV-2 and COVID-19 infection across the globe

Severe acute respiratory syndrome coronavirus 2 belongs to the *Coronaviridae* (*β-coronavirus*) family, which share the characteristics of being single-stranded, positive sense (+ ssRNA) viruses and have an enveloped glycoprotein surface that appears like a crown under microscope. This family includes the largest identified RNA viruses, which extend between 26 to 32 kb in length (Lai and Cavanagh, 1997; Weiss and Navas-Martin, 2005). For unknown reasons, coronaviruses, like MERS and SARS, have the ability of pass through species barriers causing diseases in humans, which range from being asymptomatic to more severe signs and symptoms. These viruses, termed human coronaviruses (HCoVs), were first discovered in 1960, and shown to be transferred to mammals from various other organisms (Al-Ani et al., 2020). Other novel viruses of the *Coronaviridae* family may arise in the future, because these viruses interact together in nature. This makes genetic recombination among their genetic materials simple and therefore likely (Su et al., 2016). As the virus rapidly evolves, we need to investigate whether or not it continues to be as virulent.

To date, there are seven identified HCoVs. The latest HCoV is proposed to be originated from bats and transferred to other mammals before affecting humans (Cascella et al., 2020). Examples of mammals that have been infected by HCoVs that have crossed the species barrier are the Himalayan palm civet, which was found to have a form of SARS-CoV (~79% genomic similarity to SARS-Cov-2), and the dromedary camel, which was infected with MERS-CoV (~50% genomic similarity to SARS-Cov-2) (Cao and Li, 2020). However, the dynamics of SARS-Cov-2 are still unclear; it has been speculated that the virus might be transmitted to humans from other animals (*Fig. 1*) (Su et al., 2016). Routine viral transmission between hosts has not been well documented for HCoVs, however, it seems that they are much more transmissible among humans (Zhou et al., 2020). Among human individuals, transmission can occur either by direct contact or through the air, or possibly indirectly through touching inanimate surfaces contaminated with the virus. It is unclear how long the virus can survive on or in non-biological materials, but approximately nine days is estimated to be the survival time for most of these viruses; by contrast, influenza virus can survive on surfaces for many months (Conti et al., 2020).

On 28 February, 2020, the World Health Organization (WHO) raised the threat level of the SARS-Cov-2 epidemic to “very high”, followed by an announcement on 11 March that the COVID-19 pandemic is a very high risk to health owing to its rapid spread internationally (Cascella et al., 2020). The WHO’s chief executive, Tedros Ghebreyesus, has criticized nations that made no adequate tests for their populations, or had limited testing (Al-Muharraqi, 2020). A number of countries, owing to capacity problems regarding COVID-19 test availability, initially restricted testing to individuals that suffer from severe acute respiratory syndromes (Al-Muharraqi, 2020). The problem of asymptomatic patients, the high infectivity of SARS-Cov-2, a lack of an effective

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DOI: http://dx.doi.org/10.15666/aeer/1806_82518265

vaccine or antiviral, and late quarantine decisions in many countries contributed to the sharp increase in the number of affected individuals worldwide.

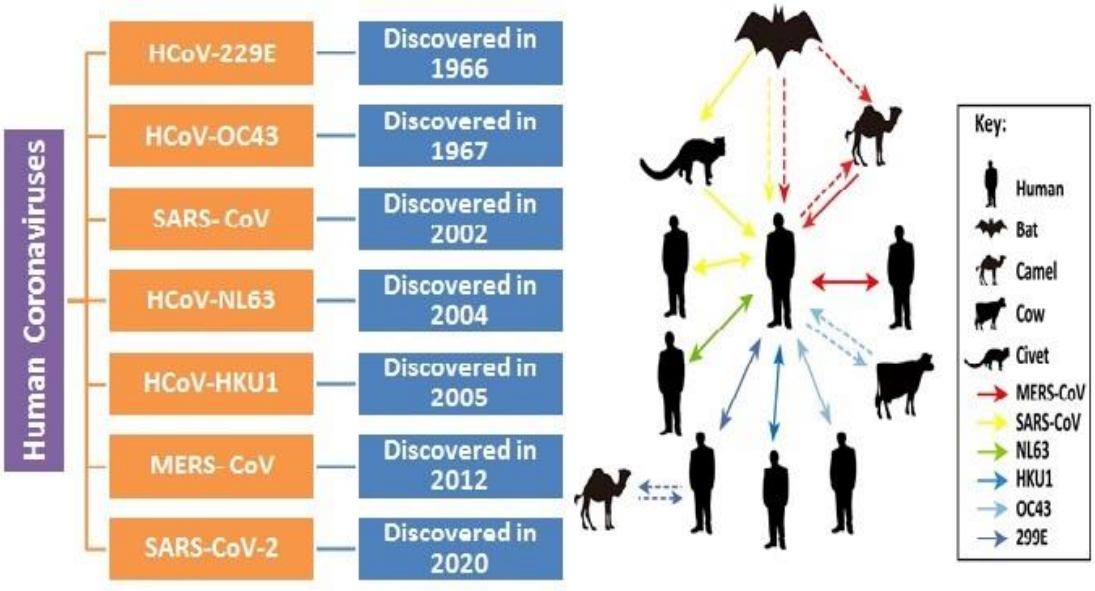

Figure 1. *The seven reported human coronavirus (HCoVs) and intra- and inter-species transmission of human coronaviruses. Figure was taken after Su et al., 2016*

Recent reports have indicated that elderly individuals, in particular those treated with immune-suppressives or biologic therapies or have a history of coronary artery disease, are more prone to a severe form of the disease and a higher mortality rate compared to other age groups, such as children and adolescents (Monteleone and Ardizzone, 2020). These findings have shown an almost null death rate in children between 0 and 10 years, and 0.2% death rate in children between 10 and 19 years (Huang et al., 2020; Zhu et al., 2020). Also, SARS-Cov-2 is believed to affect males more than females, possibly owing to the large number of immune genes located on the X chromosome (Conti et al., 2020; Ghosh and Klein, 2017).

Invasion of the host cells by SARS-CoV-2 and COVID-19 symptoms

Similar to SARS-CoV-1, SARS-CoV-2 can enter human cells via the renin-angiotensin system (Guo et al., 2020; Rico-Mesa et al., 2020). This system comprises of two main enzymes that control peptide balance in the angiotensin family. These two enzymes are angiotensin I-converting enzyme 2 (ACE2), expressed in the respiratory epithelium, intestines, and macrophages (Lu et al., 2020), and transmembrane serine protease 2 (TMPRSs2). Both enzymes are important for viral infectivity (Hoffmann et al., 2020). The successful isolation of a sub-genomic messenger RNA (sgRNA) of the live virus from throat swabs indicates that SARS-CoV-2 has the ability to actively replicate in upper respiratory tissue cells (Lu et al., 2020; Wölfel et al., 2020). However, similar to SARS-CoV-1, CoV-SARS 2 firstly replicates in the lower respiratory tissues. Following this, organs with high expression of ACE2 (such as heart, kidney,

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gastrointestinal tract, and vast distal vasculature) start to be aggressively attacked by the virus, specifically in the later stage of the disease course—generally the second week following the onset of COVID-19 (Cao and Li, 2020). Other significant complications induced by SARS-CoV-2 have been recognized, such as direct viral damage and consequences of immune-mediated injury. Of particular note, sever acute cases of COVID-19 often have two critically distinctive features, which are progressive increase of inflammation and the unusual trend of hyper-coagulation (Cao and Li, 2020).

Clinically, severity of SARS-CoV-2 can range from asymptomatic to very severe pneumonia, leading to acute respiratory distress syndrome or multi-organ failure (MOF). Many patients develop dyspnea and hypoxemia, leading to mild respiratory syndromes that require O_2 therapy. Others, however, rapidly develop acute respiratory distress syndromes (ARDSs), resulting on occasion in septic shock, metabolic acidosis, coagulation dysfunction with disseminated intravascular coagulation (DIC), and multiple organ dysfunction syndrome (MODS) (Singhal, 2020). However, it is broadly acknowledged that at present there is no indication about which individuals are highly susceptible from being seriously negatively affected from COVID-19. Broadly, SARS-CoV-2 could affect any individual, causing acute respiratory symptoms that can result in death. Nearly one in four infected individuals require intensive healthcare and approximately 4.3% of infected patients eventually die (Wang et al., 2020). Thus, COVID-19 is a serious and complex disease that is multi-faceted, involving all the systems and organs of the human body.

Regarding IBD patients, the expression of ACE2 was reported to increase in inflamed intestines (Garg et al., 2020). Proteomic investigations showed higher expression of ACE2 in Crohn's disease (CD) compared to Ulcerative colitis (UC) (Ning et al., 2019). The invasion of SARS-CoV-2 in a cell is mediated by a specific type of fusion, using a 'spike' protein (Ibrahim et al., 2020). This protein is activated via a proteolytic cleavage, triggered by the host cell using trypsin-like proteases: enzymes that are demonstrated to be upregulated in IBD patients (Jablaoui et al., 2020). This evidence suggests that the increased probability of inflamed intestinal tissues in IBD patients may be an optimal environment for SARS-CoV-2 activity. Nevertheless, no research has yet examined the frequency of COVID-19 occurrence in IBD patients.

Immune-based medication in IBD patients with COVID-19: is there a risk of increased occurrence and severity?

There is no clear evidence that patients confirmed with IBD are more susceptible to developing severe COVID-19 symptoms. Nevertheless, there are indications in previous studies that IBD patients that take immune-suppressive drugs are at high risk of having infectious diseases, possibly since the intracellular signaling cascades needed for the host to resist pathogens are inhibited (Beaugerie et al., 2020; Holmer and Singh, 2019; Singh et al., 2020). Earlier reports indicate that infections of upper and lower respiratory tract increased when using anti-TNF therapies, besides other several opportunistic pulmonary infections (Lichtenstein et al., 2012; Long et al., 2013; Shah et al., 2017). A study conducted on IBD patients to assess the increasing risk of pneumonia infection indicated that the use of biologics resulted in an increased risk (OR 1.28, 95% CI 1.08-1.52), and corticosteroids showed a significant increase (OR 3.62, 95% CI 3.30-3.98) (Singh et al., 2020). However, the study did not highlight if the medications were combined or not.

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A recent systemic review with a meta-analysis of clinical trial data, which included 4135 patients that underwent anti-TNF treatment (Ford and Peyrin-Biroulet, 2013), concluded that individuals are at risk of doubling bacterial and viral infectivity (such as tuberculosis, herpes simplex infection, oral or esophageal candidiasis, herpes zoster virus, cytomegalovirus, and Epstein-Barr virus) with anti-TNF treatment (OR 2.05, 95% CI 1.10 –3.85) compared to a placebo. Consequently, the monotherapy of anti-TNF therapy seems to be safer than in combination with other immunosuppressive agents. Importantly, a significant increased risk of developing infectious disease is shown when combining anti-TNF treatments with corticosteroids (Singh et al., 2020; Ye et al., 2020). This is because the early induction of corticosteroids can eliminate the initiation of the host immune system defense, possibly leading to adverse consequences.

In contrast to the above findings, recent research has examined the role of anti-TNF therapy as a treatment for COVID-19. Many pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, TNF, and interferon γ (Gong et al., 2020; Huang et al., 2020), have been reported to be upregulated in the blood of patients confirmed with COVID-19 (Monteleone and Ardizzone, 2020). Patients hospitalized under intensive care showed high concentrations of a number of cytokines. Furthermore, some other cytokines and chemokines are deregulated in the plasma of affected cells, causing what is generally termed a 'cytokine storm'. TNF and IL6 are well noted in acute respiratory conditions to potentially lead to lunge injury (Kovalchuk et al., 2020). In animal studies, TNF cytokines have been shown to contribute to the development of lung injury and weakening of T cell response in mice infected with SARS-CoV. The loss of TNF receptors and the neutralization of the activity of TNFs have resulted in protection against the morbidity and mortality associated with SARS-CoV (Channappanavar et al., 2016; McDermott et al., 2016). Accordingly, many potential therapeutic agents were nominated for controlling inflammation in COVID-19, of which, anti-TNF antagonists (infliximab IFX or adalimumab ADA, which have been approved, and widely available) are superior, because they show potential in being effective, particularly for acute inflammation cases (Feldmann et al., 2020). Clinically, in many inflammatory diseases and conditions, the blockage of TNF cytokines is effective, despite the existence of other pro-inflammatory cytokines (Feldmann et al., 2020). Therefore, the effectiveness of anti-TNF antibodies in treating COVID-19 warrants further consideration, especially when we know that TNFs are not detected in the serum of SARS-affected patients at later stages (Ye et al., 2020). Currently, α-TNF antagonists have not been used to treat patients with COVID-19, but it requires further exploration.

GI microbiota composition and vitamin D level in IBD patients with COVID-19 treated with TNF antagonists

Since the use of biologics to treat IBD can result in impairment of the immune system (Dipasquale and Romano, 2018), which can lead to an increased possibility of infection, it is important to investigate the probability of infection of IBD patients with COVID-19 that have been treated with TNF antagonists. Reducing the gut microbiota diversity, known as dysbiosis, has been well documented in IBD patients (Sartor, 2006), increasing the number of pro-inflammatory cytokines, such as *Escherichia coli.* (Celiberto et al., 2018). Onset and progression of IBD has been shown to be associated with insufficient serum vitamin D levels (30-75 nmol/L). Almost 68% of IBD patients suffer from vitamin D insufficiency, and more than 50% of those suffer from vitamin D deficiency (<30

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DOI: http://dx.doi.org/10.15666/aeer/1806_82518265

nmol/L) (Celiberto et al., 2018; White, 2018). Vitamin D has been shown to be of considerable importance in immune modulation, with a potential to improve IBD and COVID-19 patients (Celiberto et al., 2018; Grant et al., 2020). It can modulate pro-inflammatory Th1 and Th17 cells, which express TNFα, interferon-γ and IL-17, similar to those that appear in COVID-19 cytokines storms (Wu and Yang, 2020).

CoV-SARS-2 was documented to use ACE-2 receptors for cellular entry (Zhou et al., 2020), and, as noted above, the receptors can be highly expressed in the small intestine over other organs, including the lungs (Leung et al., 2003; Zang et al., 2020). In addition to this, the feces-oral route has also been suggested (Zang et al., 2020), with the higher mortality rate of the elderly affected with COVID-19 (Liu et al., 2020), and those elderly individuals previously documented to have less diverse GI microbiota composition (Claesson et al., 2011, 2012; Yatsunenko et al., 2012) and often suffer from deficient vitamin D levels (Kweder and Eidi, 2018). Recent literature showed that COVID-19 has an association with increased pro-inflammatory cytokines (Wang et al., 2020), whereas the immune-modulatory properties owing to vitamin D can downregulate the pro-inflammatory cytokines (Arboleda et al., 2019; Greiller and Martineau, 2015; Panarese et al., 2019; Zdrenghea et al., 2017; Zhang et al., 2012). Furthermore, vitamin D has shown to have the ability of preventing lung injury via blocking these observations, with the evidence that vitamin D-VDR signaling prevents lung injury by blocking effects on the renin-angiotensin pathway (Kong et al., 2013).

In a cohort study conducted in the US, approximately 61% of COVID-19 patients manifest GI symptoms (Redd et al., 2020). Also, the RNA of CoV-SARS-2 was shown to be detected in patient stools (Cheung et al., 2020; Holshue et al., 2020; Wu et al., 2020; Xiao et al., 2020; Zhang et al., 2020). Another study found that vitamin D signaling has a critical role in healthy association between the GI tract microbiota and intestinal immunity, including the SCFAs in particularly butyrate, which increases the vitamin D receptors (VDRs) in vitro. Also, butyrate can directly bind to the VDR, resulting in down-regulation of TNF-α production (Schwab et al., 2007). Altogether, this data led suggests a critical role for gut microbiota and vitamin D in fighting against COVID-19 infections and limiting the severity of the disease.

There are complex interaction networks among GI microbiota composition and the host immune system, which are needed for a physiological microbiota-host balance, which have a profound effect on health and disease (Lagkouvardos et al., 2017). Dysbiosis in the gut microbiota composition has been linked to many disorders; one of these is IBD (De Musis et al., 2020; Khan et al., 2019; Lavelle and Sokol, 2020). Pathogen-induced microbiota can diminish the chance of beneficial bacteria colonization, leading to inflammation development. Similar to GI microbiota, research has shown that the lungs have distinct microorganisms (Bingula et al., 2017; Zhang et al., 2020). Interestingly, pulmonary health has been shown to be affected by gut microbiota composition, through a vital 'cross-talk' between the gut and lungs, known as the 'gut-lung axis' (Keely et al., 2012). The latter is thought to be a bidirectional relationship, as the effects of endotoxins and metabolites of gut microbiota, on one hand, and lung inflammation, on the other hand, is mutual (Dumas et al., 2018). Several studies have illustrated the association between respiratory infections with GI microbiota alterations (Groves et al., 2020). Interestingly, this attests to the effect of SARS-Cov2 infection on gut microbiota composition and the converse, since patients confirmed with COVID-19 have been found to suffer from ARDS, particularly elderly and immune-compromised individuals (Lake, 2020).

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DOI: http://dx.doi.org/10.15666/aeer/1806_82518265

The relationship between lung infections including COVID-19 and potential role of healthy gut microbiome were speculated in recent studies that have explored the faces-oral route. A study conducted in Hong Kong covered 59 patients with confirmed COVID-19 (n = 59), where 15 individuals (25.4%) had GI symptoms and 9 individuals (15.3%) had tested positive for SARS-CoV-2 in their stool RNA. The results are more pronounced if the patient was suffering from diarrhea (38.5% and 8.7% in patients with and without diarrhea, respectively) ($P = 0.02$) (Cheung et al., 2020). They searched for supplementary meta-analysis data published in various databases concerning patients with GI symptoms and the presence of SARS-CoV-2 RNA in their stools. Approximately 17.6% of patients were shown to have GI symptoms, and SARS-CoV-2 was detected in the genetic material of stool samples of about 48.1% of patients, after being found negative in their previous respiratory tests (Cheung et al., 2020). Another study conducted in February 2020 targeted 73 hospitalized patients confirmed with COVID-19 aged between 10 months and 78 years old to test the GI infection and presence of SARS-CoV-2. The analysis of the patients stool RNA showed that 39 (54.42%) of tested samples were positive for SARS-CoV-2. The positivity of the test lasts from 1-12 days. However, 17 patients (23.29%) that had tested negative for respiratory infection continued to show positive results in their stools (Xiao et al., 2020). This enhances the possibility of fecal-oral transmission, where the virus targets GI tract, corroborating the findings that viral RNA causing COVID-19 has been detected in feces of patients, despite testing negative for respiratory infection (Effenberger et al., 2020; Zang et al., 2020). The detected virus was described to be non-infective (Zang et al., 2020), which drives our speculation that this impaired virus may have potential for use in the production of an effective vaccine against the infecting version of the virus.

The use of biologics, such as anti-TNF treatments in IBD patients with less diverse gut microbiota alterations, was shown to improve the microbiota profile in responders (Alshehri et al., 2020). This was found in a study carried out on Chinese pediatric patients with Crohn's disease (CD), which were treated using IFX. The microbial dynamic changes were assessed during treatment and investigated for the influence of the anti-TNF agent on the microbiota composition. The use of IFX was shown to alter gut microbiome structure and metabolic function, diminishing CD-correlated GI microbial dysbiosis, accompanied with a significant increase in the number of beneficial bacteria (Li et al., 2017). This may indicate that using an anti-TNF agent can be of great help for improving the GI microbiota profile, as well as a defense against COVID-19, which come along with the importance of and normalizing the vitamin D level.

Further recommendations

1. It seems that there is no documented evidence on the predisposition of IBD patients that undergo biologics treatment, such as anti-TNF agents, to COVID-19. Conversely, anti-TNF agents may be an effective therapy against SARS-CoV-2.
2. Physicians should update (at least temporarily) their viral screening lists for IBD patients to include testing for SARS-CoV-2, besides the other tests, such as hepatitis B, hepatitis C, HIV, varicella zoster, and tuberculosis, before starting biologics or other immune-based therapies. This is because of the confirmation on the susceptibility of IBD patients to infectious illnesses and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82518265

the detection of the SARS-CoV2 virus in stool samples of COVID-19 patients, especially those with diarrhea.

3. The intersection between COVID-19 infection, IBD course, and effective usage of immune-based therapies is yet to be fully explored. However, physicians could carefully make individual risk assessments of patients before initiating immune-suppressive drugs therapies with IBD patients to potentially avoid complications, especially if they are combining drugs. Physicians should avoid combination therapies, in particular, using anti-TNF agents with corticosteroids.
4. Improving the GI microbiota profile and vitamin D levels deserve further consideration for improving the fight against COVID-19. Furthermore, additional studies on infection caused by SARS-Cov2 are required to address the intervention of intestinal and lung commensal microorganisms (*Fig. 2*).

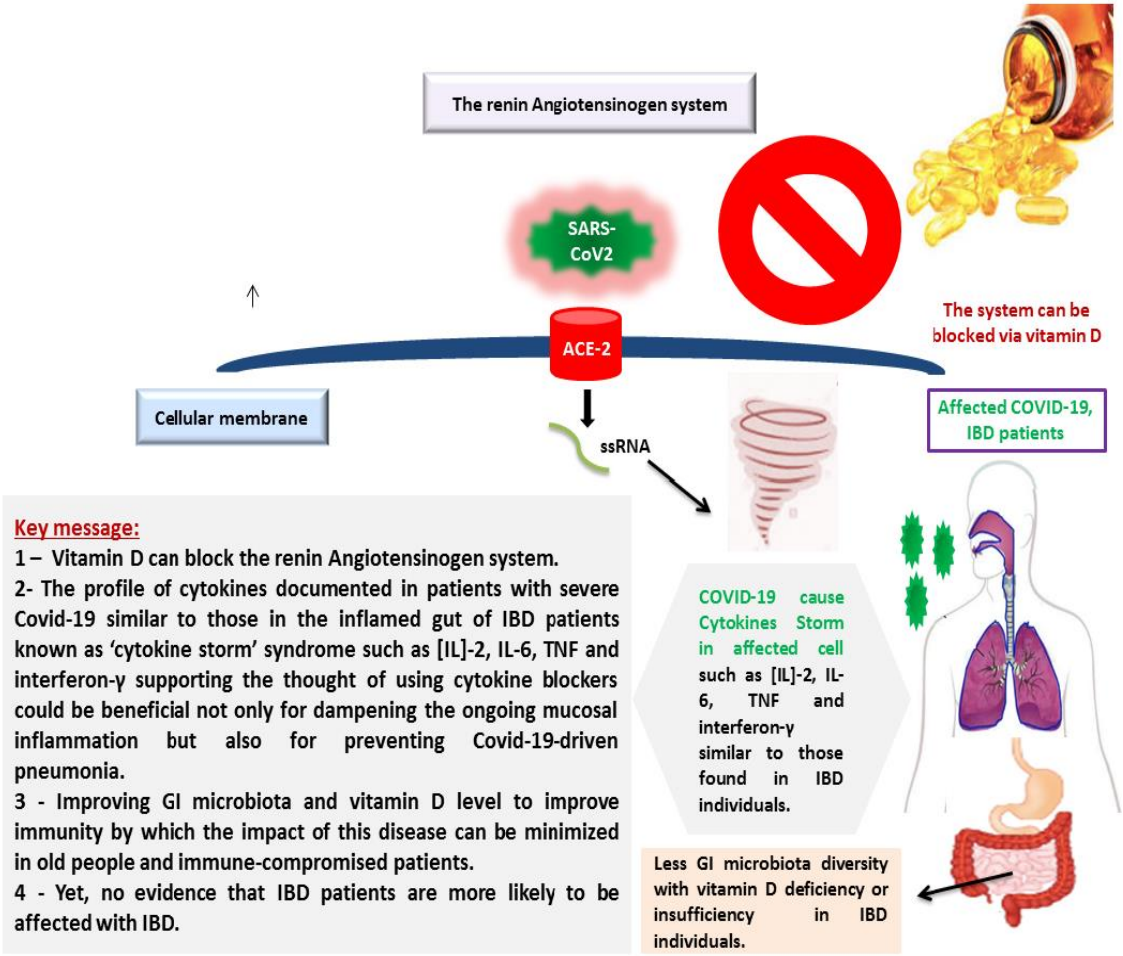

Figure 2. *Summary and key messages from this review*

Conclusions

It has been reported that using biologics and immunomodulators can impair the patient's immune system; immunotherapies used to treat IBD may increase susceptibility to COVID-19 infection. However, infection with COVID-19 causes a

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cytokines storm that can result in hyper-induction in various pro-inflammatory cytokines of the immune system, which may be modulated using immunomodulators such as α-TNF antagonists and immune-nutrition, such as vitamin D. Since the gut microbiota and vitamin D are acknowledged to have a critical role in immunity, and using TNF therapy has been reported to improve GI microbiota profile in IBD patients, treating IBD patients with COVID-19 using low-risk immune-based approaches could be a sensible way forward.

Acknowledgments. We thank the Inflammatory Bowel Disease Research Group and Microbiome Research group at King Abdulaziz University for discussion and constructive criticisms on drafts of this article.

Conflict of interests. The authors declare no conflict of interests.

Funding. No funding was received.

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DOI: http://dx.doi.org/10.15666/aeer/1806_82518265

CHANGES IN SOIL ORGANIC CARBON AND ITS FRACTIONS AFTER 13 YEARS OF CONTINUOUS STRAW RETURN IN A SOYBEAN-MAIZE CROPPING SYSTEM

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(Received 6th Aug 2020; accepted 22nd Oct 2020)

Abstract. Straw return to the soil is proposed as an effective practice to increase soil organic carbon (SOC) storage in croplands. Based on a 13-year field experiment with soybean-maize cropping system, we studied the changes of total SOC and SOC fractions under no fertilizer (NF), mineral fertilizers (NPK) and mineral fertilizers with straw return (NPKS). Compared with the initial soil, SOC storage in the bulk soil significantly increased by 7.19% in the NPKS treatment, with an annual increase of 0.32 Mg ha^{-1}; while the SOC storage significantly decreased by 3.47% in the NF treatment, and no significant change was recorded in NPK treatment after 13 years. The NPKS treatment significantly increased the storage of free light fraction carbon (fLFC), occluded light fraction carbon (oLFC), heavy fraction (HFC), humic acid carbon (HAC) and fulvic acid carbon (FAC) by 44.4, 31.8, 5.47 10.5 and 3.92%, respectively. The HAC contributed the highest percentage (47.0%) of carbon to the improvement of SOC after 13 years of straw return. Therefore, straw return was conducive to the accumulation of labile fractions (fLFC and oLFC) which were in favor of soil fertility. Simultaneously, the increased HAC after straw return are beneficial to carbon sequestration.
Keywords: *labile fraction carbons, Mollisol, soil density fractionation, long term fertilization, soil organic carbon storage*

Introduction

Soil organic carbon (SOC) is an important pool in the global carbon (C) cycle, and has the function of mitigating climate change (Stockmann et al., 2013). Elevated levels of SOC have been positively related to crop productivity by enhancing soil fertility and plant nutrient supply (Lal, 2010; Singh et al., 2020). Therefore, management strategies that increase the net SOC of agricultural soils are considered soil health improvement strategies and promoted worldwide (Oliveira et al., 2019). However, this requires implementing agricultural practices adapted to local conditions that will increase the net soil C input, with outputs remaining stable or increasing, thus maximizing the soil C storage. In recent times, the impact of agricultural management on SOC storage has attracted much attention (Poeplau and Don, 2015; Chenu et al., 2019).

Crop straw return to the soil has been regarded as an environmentally friendly approach for straw utilization due to its positive effect on SOC storage in croplands (Liu et al., 2014), and widely adopted in diverse cropping systems. A number of studies have demonstrated that the duration of straw return to the soil has linearly increased SOC word wide in agroecosystem (Zhang et al., 2017; Jian et al., 2020). However, some studies have

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also reported a slight or no increase in SOC storage in response to crop straw return (Niu et al., 2011; Guo et al., 2015; Poeplau and Don, 2015). These differences might occur due to the duration of experiment. Jian et al. (2020) have reported that SOC could be significantly increased under the treatment experiencing straw return of more than 10 years, which depends on climate conditions, soil types, and agronomic practices. A meta-analysis has demonstrated that SOC could reach saturation under continuous straw return after 12 years (Liu et al., 2014). Therefore, medium- to long-term experiments are necessary to explore the dynamics of SOC under continuous straw return in agroecosystem.

SOC is heterogeneous and composed of several functional pools with different stability, which results in their turnover rates ranging from a few months to hundreds of years (von Lützow et al., 2007). To estimate the changes in SOC, it is crucial to quantify and understand the sensitivity of the different functional SOC pools to agricultural practices, for example straw return (Poeplau and Don, 2013). In view of SOC stabilization mechanisms, SOC can be separated to the following fractions: (1) unprotected fractions; (2) physically protected fractions by soil aggregates; (3) chemically or biochemically protected (Six et al., 2002; Yang et al., 2018). Various physical and chemical fractionation methods have been developed to separate SOC fractions with distinct degradability and turnover times. Density fractionation method highlights the observation that the physical location of SOC within the soil matrix is a key factor determining its turnover (Llorente et al., 2010). The obtained fractions are free light fraction (FLF), occluded light fraction within aggregates (OLF) and heavy fraction (HF). The FLF and OLF representing labile SOC pools with a rapid turnover time are both sensitive indicator of agronomic practices (Golchin et al., 1994; Tamn et al., 2005; Llorente et al., 2010). In contrast, HF is considered as stable pool, with turnover times ranging from decades to centuries. Generally, crop straw return has a positive effect on soil light fraction C (Nayak et al., 2012; Chen et al., 2019). The HF could be further chemically fractionated into three humic fractions (humic acid, fulvic acid and humin). Humic substances are operationally defined by a standardized extraction procedure, but previous studies have demonstrated that they are a heterogeneous pool of substances with distinct turnover rate (von Lützow et al., 2007). Crop straw is one of the major sources of humic substances in cropland soil (Guimarães et al., 2013). Several studies have addressed the effect of straw return on the size and composition of different humic fractions (Zhang et al., 2017, 2019a; Mi et al., 2019). Zhang et al. (2017) observed that continuous maize straw return increased humic and fulvic acid C concentrations in Mollisol. However, Mi et al. (2019) found that application of rice straw increased the concentrations of humic acid C only. Despite the widely researched response of different SOC fractions to straw return, the relative contributions of various functional SOC fractions to SOC change related to long-term straw return are still unclear.

Mollisols are one of the most important soil resources for food production and climate change mitigation due to their high organic C (Liu et al., 2012; Sanford et al., 2012). However, the SOC content of Mollisols has decreased rapidly over the past decades in the Northeast China, which is caused by long-term intensive cultivation and lower crop residue return (Li et al., 2016; Xu et al., 2020). In recent times, returning crop straw into soil is encouraged and widely applied in Northeast China aimed at improving soil fertility instead of burning the straw in the field (Wang et al., 2018). Previous studies have reported, that straw return could improve C sequestration by physically protecting soil macroaggregates and the occluded microaggregates (Guan et al., 2019), and also increase

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DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

the soil recalcitrant C content (Zhang et al., 2019b). However, those studies were mainly conducted in continuous maize cropping systems in the south of the Mollisol region in Northeast China. Soybean-maize rotation is the primary cropping system in the north of the Mollisol region of China. Yet, a detailed examination of the changes in SOC and its fractions after straw return has not been considered in this region. The objectives of this study were to 1) identify the changes in SOC in the bulk soil and various fractions (light fractions, heavy fraction and humic fractions) after 13 years of continuing straw return under soybean-maize rotation, 2) assess the relative contributions of different SOC fractions to SOC change in relation to continue straw return. We hypothesize that continuous straw return would increase SOC storage both in the bulk soil and its fractions. Alternatively, the rate of increase of different SOC fractions caused by straw return would be different.

Materials and Methods

Site description

The field experiment was located at the National Field Observation and Research Station of Hailun Agroecosystems, Chinese Academy of Sciences (47°27′ N, 126°55′ E), in the central region of Mollisol in northeast China (*Fig. 1*). The region has a typical temperate continental monsoon climate with an average annual temperature of 1.5 °C. The lowest mean monthly temperature is −23 °C in January and the highest monthly mean temperature is 21 °C in July. The mean annual precipitation of the region is 550 mm, with more than 80% occurred from May to September. The frost-free period of the region is about 120 days. The soil of the study area is classified as Mollisol according to the USDA Soil Taxonomy System (Soil Survey Staff, 2010), which developed from sedimentary materials of loamy loess. The study site is a flat plain and had been under native prairie before the land was reclaimed for cropping about 120 years ago (Song et al., 2007). Before 1993, the cropping system was inter-annual rotation between wheat (*Triticum aestivum* L.) and soybean (*Glycine max* (L.) Merrill.), and between wheat, maize (*Zea mays* L.) and soybean rotation in 1993-2003.

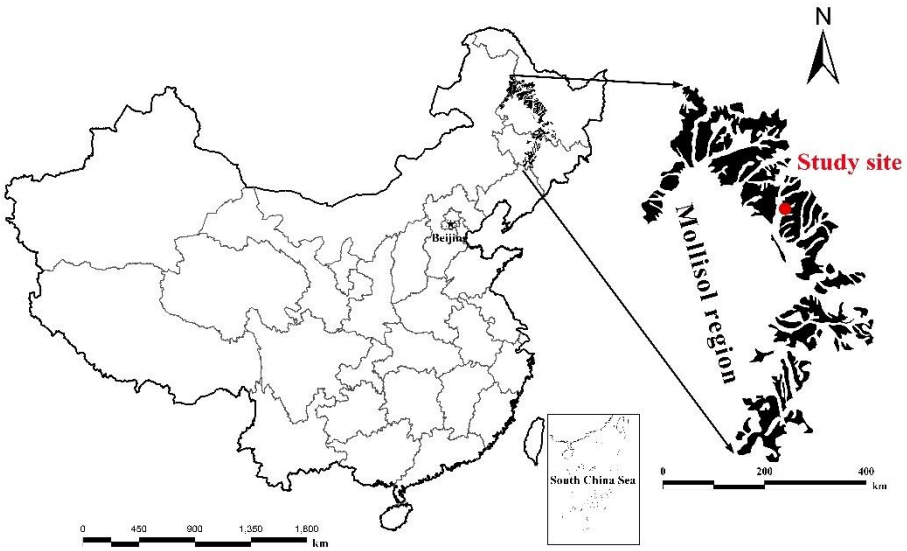

Figure 1. *Geographical distribution of study site in northeast China*

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Experimental design

The long-term experiment was established in 2004. The cropping system was inter-annual rotation between soybean and maize. Before 2004, all crop residues were removed from the field after harvest. Three treatments were set up in 2004, including no fertilizer (NF), mineral fertilizers (NPK) and mineral fertilizers with straw return (NPKS). The area of each treatment was 1800 m^2 (width 30 m × length 60 m). Our experimental design was pseudo-replicated for NF, NPK and NPKS treatments, from which six composite soil samples were collected from each treatment. Our specific interest was assessing the SOC and SOC fractions in site-specific soil that had been treated for 13 years, representing the long-term agronomic outcomes. We presumed that any significant difference among those plots could be attributed to the effects of long-term treatment based on the random selection and low spatial variability of the soil characteristics. In the NF and NPK treatments, all aboveground straw was removed after harvest. In the NPKS treatment, maize or soybean straw was chopped to 3-4 cm in length after harvest, spread evenly in the treatment plot. All maize or soybean straws were plowed into the topsoil (0-20 cm depth) by rotary tillage. In the NPK and NPKS treatments, the mineral fertilizers were applied at rates of 64 kg N ha^{-1}, 70 kg P_2O_5 ha^{-1} and 20 kg K_2O ha^{-1} for soybean, and 138 kg N ha^{-1}, 70 kg P_2O_5 ha^{-1} and 20 kg K_2O ha^{-1} for maize. For the soybean, the mineral fertilizers were applied once as basal fertilizer at sowing. For maize, P and K fertilizers, and one third (33%) of the N fertilizer were applied as basal fertilizer at sowing, and the remainder two thirds (67%) was applied as topdressing at jointing stage. Mineral fertilizers were applied as urea (46% N), ammonium hydrogen phosphate (18% N; 46% P_2O_5) and potassium sulphate (51% K_2O). In all treatments, soils were subjected to conventional tillage, and were ridged by rotary tillage to a depth of 20 cm after harvest in autumn.

Plant-derived C input estimates

Every year, maize and soybean grain were manually harvest from five randomly selected sub-plots (6 m^2) of each treatment plot to estimate grain yield at crop maturity. Organic matter derived from crop straw, roots, stubble and rhizodeposition in this study, was estimated by the average ratios of crop yields to the above residues from 2004 to 2017. The ratios were estimated by harvesting 10 individual plants of soybean and maize in September every year. Each plant was separated into grain, straw, stubble and root (0-20 cm soil depth), and oven-dried at 60 °C to constant weight and weighed to estimate the dry matter mass. The C concentration of each tissues was determined using a CN elemental analyzer (EA3000, Euro Vector，Italy). The average ratios of grain to straw, grain to stubble and grain to root were 1:0.73, 1:0.05 and 1:0.14 for soybean, respectively, and were 1:1.2, 1:0.13 and 1:0.26 for maize, respectively during experimental years. The C input derived from rhizodeposition was assumed to be equal to root biomass C (Bolinder et al., 1999).

Soil sampling

Soil samples were collected from the NF, NPK and NPKS treatments after harvest in each experimental year. Thirty randomized soil cores (depth 0-20 cm and diameter 5 cm) were collected in each plot and every five soil cores were mixed as six composite soil samples. After removing visible plant fragments and roots, the fresh soil samples were sieved to pass through a 10-mm sieve by gently breaking soil clods along the natural failure surfaces of the soil, air-dried, and then stored in glass bottles. A portion of the air-

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dried soils was ground to pass through a 2-mm sieve for analyses of SOC and its fractions. During soil sampling, five randomized point were selected at each sampling plot for the determination of soil bulk density. At each point, four soil cores (100 cm^3) were sampled, and soil bulk density was measured after drying the soil cores at 105 °C for 48 h.

SOC fractions analysis

Soil density fractionation

Density fractionation of SOC was carried out following Llorente et al. (2010). 10 g air-dried soil sample (<2 mm) was placed in a 100 mL centrifuge tube with 50 mL sodium iodide (NaI) solution (d=1.8 g cm^{-3}). The tube was gently turned upside down 5 times by hand. After centrifugation for 30 minutes (min) at 4000 revolutions per minute (rpm), the supernatant was passed through a 0.45 μm membrane filter into a millipore vacuum unit. The separation method was repeated three times. The soil particles on the membrane were collected, washed with deionized water and considered as the free light fraction (fLF, d<1.8 g cm^{-3}). The residue remaining in the tube was then added with 50 mL NaI. The tube was placed in an ice bath and sonicated at 300 J ml^{-1} for 15 min with a probe-type ultrasonic disintegrator. The floating material was the occluded light fraction (oLF, d<1.8 g cm^{-3}) protected by soil aggregates, then recovered by centrifugation, filtered and washed in the same way as the fLF. The leftover soil in the centrifuge tube was washed with distilled water until the water became clear and used as the heavy fraction (HF, d>1.8 g cm^{-3}). All fractions were dried at 50 °C, weighed, ground in a mortar, and analyzed for C.

Humic substance extraction

The extraction of humic substance was preformed according to the method described by Stevenson (1994). Briefly, 50 mL 0.1 mol L^{-1} NaOH and 0.1 mol L^{-1} $Na_4P_2O_7$ solution (50:50, v/v) was added into the heavy fraction, and the tubes were then placed in a water bath at 70 °C for 1 h. The supernatant solution was centrifuged at 3500 rpm for 15 min and collected as the humic extractable substances (HE). The HE was acidified to pH 1.0 with 0.5 mol L^{-1} H_2SO_4, the precipitated fraction was acid-insoluble humic acid (HA), and the solution was fulvic acid (FA), the two fractions were separated by centrifugation at 3500 rpm for 15 min. The HA was re-dissolved by 0.05 mol L^{-1} NaOH. The residue soil was humin (HM) fraction. The C contents of HE (HEC) and HA (HAC) was determined by the $K_2Cr_2O_7$ oxidation method. Carbon content of FA (FAC) and HM (HMC) were calculated using the following formulas:

$$\mathrm{FAC} = \mathrm{HEC} - \mathrm{HAC} \quad \text{(Eq.1)}$$

$$\mathrm{HMC} = \mathrm{SOC} - \mathrm{fLFC} - \mathrm{oLFC} - \mathrm{HEC} \quad \text{(Eq.2)}$$

where, fLFC and oLFC are the C contents of fLF and oLF.

Total C contents in bulk soil and light fractions were determined using the CHN elemental analyzer (EA3000, Euro Vector，Italy).

Statistical analysis

Statistical analyses were performed by SPSS V19.0. One-way ANOVA with Tukey test was conducted to analyze the differences of SOC and SOC fractions among

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treatments at 5% level of significance. Homogeneity of variance and normality assumption were tested using Levene's Test. The date differences between 2004 and 2017 were compared with paired t-test. Regression analysis was performed to determine the relationships between SOC content and experimental year.

Results

Plant-derived C input

During the 13-year experimental period, the cumulative C input in the NF, NPK and NPKS treatments were 10.92, 15.12 and 45.96 Mg ha^{-1}, respectively (*Table 1*). In the NPKS treatment, the cumulative C input was 321% and 204% higher than NF and NPK, respectively, and 66.2% was derived from straw return, and more than 80% of cumulative C input were derived from root and rhizodeposition in the NF and NPK.

Table 1. *Plant-derived C input in different treatments in 2004-2017 (Mg ha^{-1}). NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return*

Treatment	Straw C [a]		Root C		Stubble C		Rhizodeposition C [b]		Cumulative C input
	Soybean	Maize	Soybean	Maize	Soybean	Maize	Soybean	Maize	
NF	0	0	0.88	3.52	0.31	1.81	0.88	3.52	10.92
NPK	0	0	1.03	5.05	0.37	2.59	1.03	5.05	15.12
NPKS	5.99	24.42	1.09	5.17	0.39	2.65	1.08	5.17	45.96

[a] Soybean straw including the pod husk. [b] Carbon input from rhizodeposition was assumed to be equal to root biomass C (Bolinder et al., 1999)

SOC storage in bulk soil

Different agronomic practices had a significant impact on both SOC storage. After 13 years of different fertilizer treatments, significant differences in the storage of SOC were observed among treatments, shown as NPKS > NPK > NF (*Fig. 2*). Compared with the initial soil, the SOC storage significantly increased by 7.19% in the NPKS treatment ($P < 0.05$), with an annual average increase of 0.32 Mg ha^{-1}. In the NF treatment, SOC storage significantly decreased by 3.47%, and without any significant change in the NPK treatment over the past 13 years. The changes of SOC in the NPKS treatment, NPK and NF treatments were 4.10, 0.11 and -2.02 Mg ha^{-1}, respectively (*Fig. 2*).

Temporal changes of SOC content

Compared with the initial soil, SOC content in the NPKS treatment significantly increased ($P < 0.001$), and no significant changes ($P > 0.05$) in the NF and NPK treatments over the 13 years (*Fig. 3*). The SOC content in the NPKS treatment was significantly higher ($P < 0.05$) than those in the NF and NPK treatments in 2008, four years after the experiment was established (2008). Subsequently, the significant differences among three treatments were recorded in the thirteenth year of the experiment with the highest SOC content in the NPKS treatment (28.37 g kg^{-1} soil), followed by the NPK treatment (26.35 g kg^{-1} soil), and the lowest in the NF treatment (25.16 g kg^{-1} soil). Compared with the initial soil, SOC content increased by 19.5% for NPKS, but decreased

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by 6.64% for NF. It is worth noting that there were relatively high standard deviation values in the NPK treatment in 2013 and 2014, this might be caused by the variation of sample plots.

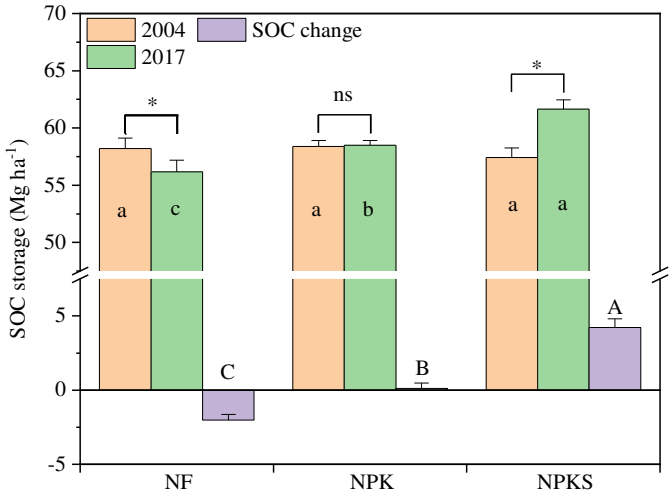

Figure 2. *Soil organic carbon (SOC) storage change in bulk soils (0-20 cm) after 13 years of different treatments. NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return. Different lowercase letters indicate significant differences (P < 0.05) in SOC storage among treatments in the same year. Different uppercase letters indicate significant differences (P < 0.05) in SOC storage change among treatments. *Indicate significant differences (P < 0.01) between 2004 and 2017. Bars represent the standard deviations (n = 6)*

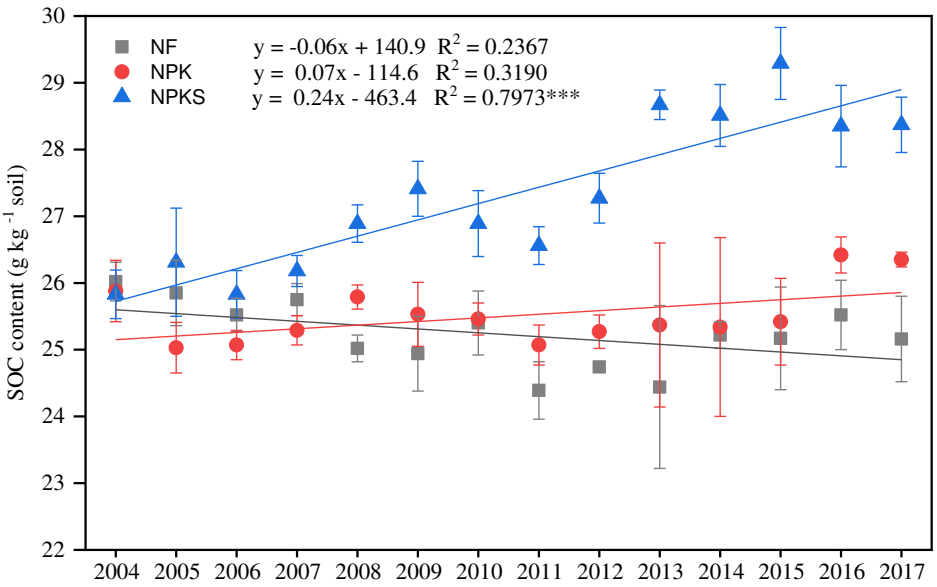

Figure 3. *Changes in soil organic carbon (SOC) content (0-20 cm) in different treatments during the period 2004-2017. NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return. ***Indicate significance level at P< 0.001. Bars represent the standard deviations (n = 6)*

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SOC storage in density and humic fractions

Different agronomic practices have impacted the storage of SOC fractions in this study (*Fig. 4*). Compared with the initial SOC, treatment with continuous straw return with mineral fertilization significantly increased ($P < 0.05$) the storage of fLFC, oLFC, HFC, HAC and FAC by 44.4%, 31.8%, 5.47%, 10.5% and 3.92%, respectively (*Fig. 4a, b, c, d, e*). However, all of the above mentioned SOC fractions significantly decreased by 17.7%, 11.2%, 2.76%, 7.21% and 3.41% for fLFC, oLFC, HFC, HAC and FAC, respectively, under NF treatment. In the NPK treatment, there were no significant differences in all the SOC fractions between the soil samples taken in 2004 and 2017. After 13 years of experiment, the significant differences ($P < 0.05$) in the storage of fLFC, oLFC, HFC and HAC were recorded among the three treatments in the following order of NPKS > NPK > NF (*Fig. 4a, b, c, d*). However, there was no significant difference in the storage of HMC among three treatments (*Fig. 4f*).

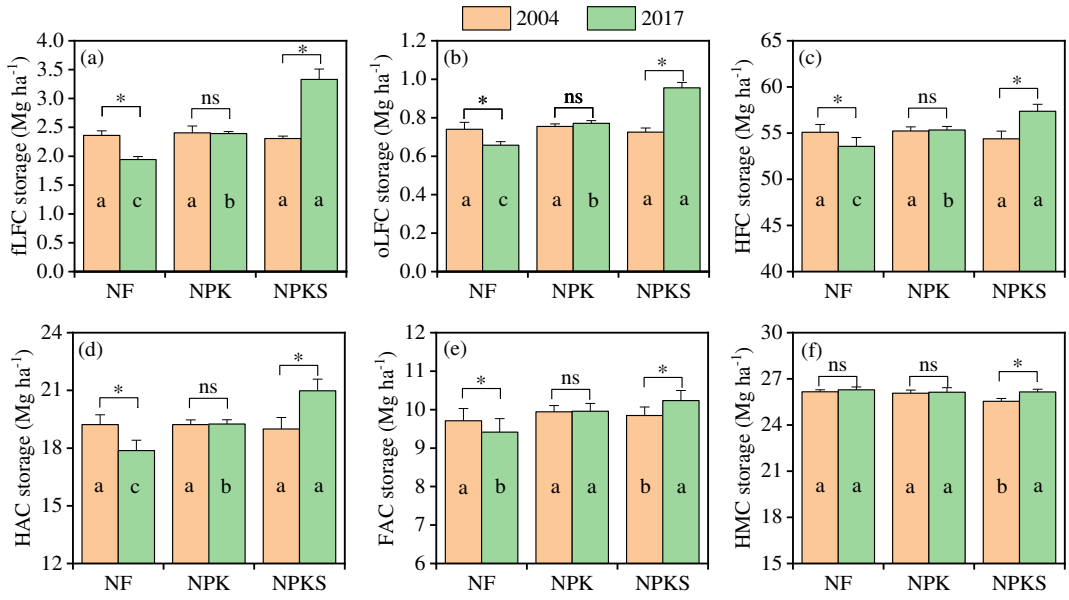

Figure 4. *Carbon storage of soil organic carbon (SOC) fractions (0-20 cm) in different treatments in 2004 and 2017. NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return. fLFC, free light fraction carbon; oLFC, occluded light fraction carbon; HFC, heavy fraction carbon; HAC, humic acid carbon; FAC, fulvic acid carbon; HMC, humin carbon. Different lowercase letters indicate significant differences (P < 0.05) among treatments in the same year. * indicate significant differences (P < 0.01) between 2004 and 2017. Bars represent the standard deviations (n = 6)*

HMC, HAC, FAC, fLFC and oLFC storage accounted for 44.4%-46.8%, 33.1%-34.0%, 16.6%-17.2%, 3.46%-5.40% and 1.17%-1.55% of total SOC, respectively (*Fig. 5*). The NPKS treatment significantly increased ($P < 0.05$) the proportion of fLFC, oLFC and HAC by 34.5%, 22.7% and 2.88%, respectively, but decreased the proportion of HMC by 4.66%. The proportions of fLFC, oLFC and HAC under NF treatment were significantly decreased. Meanwhile, the different agronomic practices had a significant influence on the HA/FA ratio of humus, the largest value was recorded in the NPKS

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treatment with 2.05, followed by NPK treatment with 1.93 and NF treatment with 1.90, NPKS of which were higher than the initial soil with 1.93 (*Table 2*).

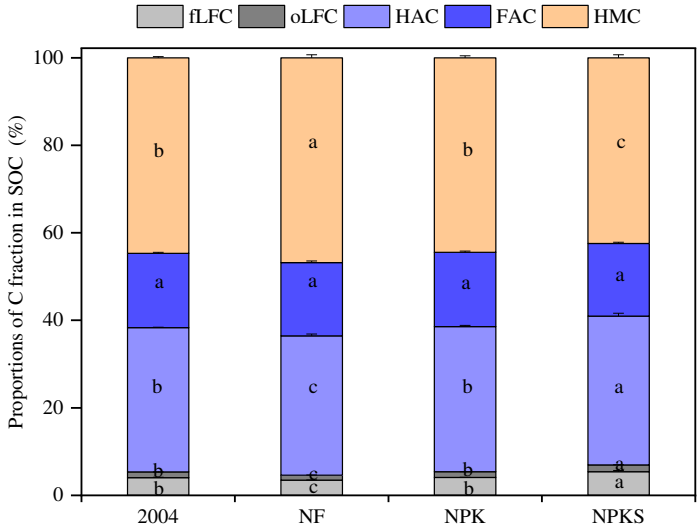

Figure 5. *Proportion of carbon fractions in bulk soil organic carbon (SOC) (0-20 cm) in 2004 and 2017.The proportions of carbon fractions in 2004 are the mean value of the three treatments. NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return. fLFC, free light fraction carbon; oLFC, occluded light fraction carbon; HFC, heavy fraction carbon; HAC, humic acid carbon; FAC, fulvic acid carbon; HMC, humin carbon. Different lowercase letters indicate significant differences (P < 0.05) among treatments. Bars represent the standard deviations (n = 6)*

Table 2. *The HA/FA ratio of humus in different treatments in 2004 and 2017*

Treatment	2004	2007
NF	1.98 ± 0.03 Aa	1.90 ± 0.04 Bb
NPK	1.93 ± 0.03 Ab	1.93 ± 0.04 Ab
NPKS	1.93 ± 0.03 Bb	2.05 ± 0.02 Aa

NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments in the same year. Different uppercase letters indicate significant differences ($P < 0.05$) between 2004 and 2017. The results are shown as the mean ± SD (n=6)

Contribution of C fractions on the improvement of SOC after straw return

The increased amount of SOC fractions under NPKS treatment shown as the order of HAC > fLFC > HMC > FAC > oLFC (*Table 3*), and HAC accounting for 47.0% of SOC change was significantly higher than the other SOC fractions. Although the light fraction C accounted for only about 5% of SOC (*Fig. 5*), its contribution to the improvement of SOC was as higher as 29.8%, including the increase of 24.3% from fLFC and 5.5% from oLFC, respectively (*Table 3*). However, the contribution of HMC to the improvement of SOC in the NPKS treatment was only 14.3%, even though the HM accounted for more than 40% of SOC.

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DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

Table 3. *The change amount and the percentage contribution of different organic carbon fraction in SOC (0-20 cm). NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return*

C fraction	Change of C fractions (Mg ha^{-1})			Proportion to SOC change (%)		
	NF	NPK	NPKS	NF	NPK	NPKS
SOC	-2.02	0.11	4.23	-	-	-
fLF	-0.42	0.00	1.02	21.1	-29.9	24.3
oLF	-0.08	0.02	0.23	4.2	-11.3	5.5
HA	-1.35	0.12	1.99	66.4	45.7	47.0
FA	-0.29	0.06	0.39	15.1	-63.8	9.0
HM	0.13	0.19	0.60	-6.9	159.3	14.3

SOC, soil organic carbon; NF, No fertilizer; NPK, mineral fertilizers; NPKS, mineral fertilizers with straw return; fLFC, free light fraction carbon; oLFC, occluded light fraction carbon; HFC, heavy fraction carbon; HAC, humic acid carbon; FAC, fulvic acid carbon; HMC, humin carbon

Discussion

Change in bulk SOC in response to continuous straw return

Our results clearly support the hypothesis that straw return to the soil has a positive impact on SOC storage and sequestration. SOC storage increased by 7.19% after 13 years of straw return to soil, with an annual increase of 0.32 Mg ha^{-1} in this study (*Fig. 2*). This increase in SOC was within the range presented by Zhang et al. (2010), who found that the rate of SOC sequestration ranged from 0.07 to 1.46 Mg ha^{-1} year^{-1} under different upland cropping systems across northern China. Similarly, Wang et al. (2018) also reported a SOC sequestration rate of 0.28 Mg ha^{-1} yr^{-1} in a 21-year straw return experiment in the same pedoclimatic region as this study. Generally, a significantly linear relationship occurs between straw C input and SOC sequestration rate when the soils do not reach the C-saturated point (Kong et al., 2005; Duval et al., 2016; Jiang et al., 2017). The average C input was 3.5 Mg ha^{-1} yr^{-1} under NPKS treatment in this study (*Table 1*), which was 318% and 206% higher than the NF and NPK treatments. Consequently, the highest storage of SOC was found under NPKS treatment after 13 years (*Fig. 2*).

A meta-analysis has demonstrated that the effects of straw return on SOC content were not evident in short-term field experiments (1-3 years), but significantly increased in medium-term experiments (3-15 years). SOC contents showed a significantly increasing trend with the duration increase of straw return (Liu et al., 2014), which was consistent as our result. Although a significantly linear relationship between SOC content and experimental years of continuous straw return was found (*Fig. 3*), there was no significant change of SOC content in the first 3 years of straw return, which could be attributed to the relatively lower input of straw return under the soybean-maize rotation system. Different with our results, Liu et al. (2019) reported that the maize monoculture had continuously increased SOC content during the 8-year field experiment with straw return. The response of SOC to organic matter input depends on the initial SOC content (Chenu et al., 2019). When the initial SOC content is high, it is difficult to measure the change of SOC within a shorter period of time (Campbell et al., 1991). Berhane et al. (2020) has also described that soils with lower initial SOC have a higher potential to store and sequester SOC than soils with higher initial SOC following combined application of

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DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

chemical fertilizer and straw. At the same study site, You et al. (2017) studied SOC changes during the early stages of the development of Mollisol with excessively low SOC (4.79 g kg^{-1} soil), and demonstrated that the return of maize and soybean straw induced a steady increase in SOC storage in the early years of the experiment with an annual SOC sequestration of 0.80 Mg C ha^{-1}, which was relatively higher than that in this study (0.32 Mg ha^{-1}) (*Fig. 3*). In addition, the effect of straw return on SOC accumulation was affected by straw return approaches and climate (Liu et al., 2014; Han et al., 2020; Jian et al., 2020). In general, crushing and incorporating straw into soil increased SOC more than mulching straw on soil surface (Han et al., 2020). Straw incorporation by tillage increases the contact between soil and straw, and thus promotes more straw C was sequestrated by soil. The decomposition rate of crop residue in this study area is substantially lower than other areas due to the low temperature in northeast of China (Xu et al., 2017). Therefore, incorporating straw in to the plough layer is an effective approach to accelerate the decomposition of straw in northeast of China (Han et al., 2020).

It has been widely accepted that SOC content could not be growing constantly with C input because of the potential upper limit of SOC concentration called the SOC saturation point, where there is an equilibrium between the C inputs and outputs (Stewart et al., 2007). Prior research has reported that due to the high base status of SOC, Mollisols can appear C saturation after many years of organic material input (Chung et al., 2010). In this study, a significantly linear relationship was found between SOC content and experimental years under the treatment with straw return (*Fig. 3*), which is an indication that the tested Mollisol has not reached an upper threshold of C sequestration over the experimental period. This could be explained by the high clay content of Mollisols (>40%) that could enhance the capacity of soil to store more C (Ding et al., 2012). However, we cannot conclude from this study that SOC saturation will occur with increasing straw-C inputs. Therefore, further long-term research is needed in this region to establish that conclusion of SOC saturation.

The above-ground biomass under the NF and NPK treatments was removed from filed and more than 80% of C input was derived from root (root biomass and rhizodeposition) in this study. Moreover, the root-derived C input is relatively lower under the NF treatment. Consequently, the cumulative C input was significantly lower in NF as compared with the NPK and NPKS treatments (*Table 1*), which contributed lower SOC storage under NF treatment (*Fig. 2*). Similar results have been reported in different climate and soil conditions (Lee et al., 2009; Ding et al., 2014; Yang et al., 2018). Applying mineral fertilizer alone for 13 years did not change SOC concentration and storage (*Figs. 2, 3*), indicating the SOC was in equilibrium and the input of C could compensate for the decomposition of SOC. The result from a 35-year field trial demonstrated that SOC would maintain the balance under the treatment with annual C input of 1.4 Mg ha^{-1} in northeast China (Hao et al., 2016), which is very close to the value under NPK treatment of our study. However, some studies showed that application of mineral fertilizer alone decreased SOC in Northeast China (Yan et al., 2007; Wang et al., 2013; Li et al., 2016). This reduction in SOC from the application of mineral fertilizer alone, could be attributed, in part, to the serious soil erosion in this area from slopes in fields due to hills in the landscape (Liu et al., 2010). The slope at this study site, is less than 2° and the effects of soil erosion can be ignored. In this case, incorporating crop stubble and root biomass into soils consequently can compensate for the loss from the SOC mineralization. Including legumes in crop rotations has been introduced as a sustainable alternative to nitrogen fertilizer-based systems, due to the increased N

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

availability to following crops, particularly when residues are added to the soil (Oliveira et al., 2019). In addition, legume crops have a positive effect on soil biology, promote the stabilization of soil aggregates, which protect native SOC from microbial decomposition, leading to increased soil C storage (Franke et al., 2018). Hence, rotation system including soybean is an effective practice to maintain the level of SOC in Mollisols.

Effect of straw return on SOC density fractions

Light fractions of SOC, mainly consisted of fresh plant-derived materials, represented labile SOC pools with rapid turnover rates (Golchin et al., 1994; Tamn et al., 2005; Llorente et al., 2010). In general, the light fractions of SOC is closely associated with soil nutrient cycling and soil fertility owing its impacts on soil food webs (Chen et al., 2017). The combined application of straw return and mineral fertilizer significantly increased not only the storage of fLFC and oLFC but also both proportion in SOC (*Figs. 4, 5*), which could be attributed to large straw inputs provided abundant source for the formation of light fractions under NPKS treatment. Moreover, the increase rate in light fraction C (44.4% for fLFC, and 31.8% for oLFC) was much higher than that in bulk SOC (7.19%), indicating that the light fraction C was more sensitive to straw return than bulk SOC. Previous studies have also reported that straw return significantly increased the light fraction C compared to no straw return (Nayak et al., 2012; Chen et al., 2017; Guan et al., 2019; Yan et al., 2020). After plant residue was incorporated into soil, part of residue directly existed in the soil as unprotected fLF, and part was protected by aggregates, which then slowly decomposed and utilized by soil microorganisms (Six et al., 2002). Although the light fraction C accounted for only 5% of SOC (*Fig. 5*), its contribution to the improvement of SOC was as high as 29.8% after 13 years of experiment with straw return to soil, including the increase of 24.3% from fLFC and 5.5% from oLFC, respectively (*Table 2*). Similar result was reported by Yan et al. (2020), who found that more than 34% of the gain in SOC storage was stored in light fractions. Thus, light fraction C may be of greater importance in defining SOC turnover. Previous studies have indicated that the decrease of root biomass caused by the changes of soil management were the main factor that induced the decrease of light fraction C (Angst et al., 2018). In addition, higher amounts of root residue and exudates could promote the formation of soil aggregate, and thereby increase the oLFC that was physically protected within aggregates (Yamashita et al., 2006). Root-derived C in no fertilizer treatment was obviously lower than that in the fertilizer treatment (*Table 1*), which resulted in a significant decline of light fraction C in NF treatment compared with the initial soil (*Fig. 4a, b*).

The heavy fraction (HF) organic C is mineral associated fractions with a slower turnover rate and a higher degree of chemical protection (Llorente et al., 2010). More than 90% of organic C in Mollisol was stored in HF (*Fig. 5*). Although the storage of HFC increased by 5.47% after 13 years with continues straw return, it accounts for more than 70% of all increased SOC in the bulk soil. Likewise, Liu et al. (2008) found that the contribution rate of HFC to SOC improvement was 70.7% in the treatment with maize straw return in a 10-year experiment in Northeast China. This indicates that a larger proportion of the SOC storage derived from external C inputs was sequestered in HFC pool. Therefore, the response of HFC to soil management was slow, but it played a crucial role in maintaining the stability and quantity of SOC (Llorente et al., 2010).

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DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

Effect of straw return on SOC humic fractions

In order to clarify the stable characteristics of SOM, we further fractionated HF to different humic fractions (i.e. HA, FA and HM). The C storage in humic substances were characterized as HM>HA>FA (*Fig. 4d, e, f*), which is consistent with published results in Mollisol (Zhang et al., 2019a). Crop residue is one of the major sources of humic substances in farmland soil (Guimarães et al., 2013). Hence all of the three humic fractions were significantly increased by continuous straw return to soil. Humic-like substances in organic amendments have been documented to contribute to the accumulation of native soil humic substances (Brunetti et al., 2007). Crop straw contained a certain amount of humic-like substances (Adani and Ricca, 2004). During the straw decomposition process, this humic-like substance could be adsorbed by soil minerals (Simonetti et al., 2012), meanwhile, some released labile biomolecules (e.g., polysaccharides, peptides and aliphatic compounds) could be incorporated into native soil humic substances throw chemical protection. When the soil was in a net loss of SOC, its humic fractions was always in a decreasing state (Guimarães et al., 2013). Therefore, the treatment with no mineral fertilization significantly decreased the C storage in HA and FA (*Fig. 4d, e*). On the other hand, mineral fertilizers alone did not significantly affect the C storage in humic fractions (*Fig. 4d, e, f*), which implies that the humic composition of Moillsol is always in a stable state under the conventional management.

The ratio of HA/FA could provide information on the humification rate and accumulation regularity of SOC under different management (Guimarães et al., 2013). The HA/FA ratio significantly increased after 13 years of combined application of straw return and mineral fertilizers compared with initial soil, mineral fertilizers alone and no fertilizers in this study, indicating that long-term straw application was more conducive to the accumulation of HAC. This could be further proved by the highest contribution rates of HAC (47%) to the SOC improvement (*Table 3*). Previous studies conducted in Fluvisol (Shindo et al., 2006), Inceptisol (Zhang et al., 2011) and Hapludoll (Song et al., 2019) also reported that application of straw or organic manure in combination with mineral fertilizers could increase the HA/FA ratio of SOM. A plausible explanation is that FA was more soluble and reactive than HA (Dou et al., 2020). In the early stage of crop residues decomposition, the formation of FA fractions is rapid, however, the FA later would be easily transferred into more stable HA fractions under microbe's activity (Ingelmo et al., 2012; Dou et al., 2020). Thus, our result suggested that medium-term application of straw is favorable for the formation of HA fraction. The HA fraction would play a major "sink" role in the process of SOC sequestration.

Conclusions

A 13-year field experiment demonstrates that, due to the higher C input by straw, the combined application of straw return and mineral fertilizer significantly increased SOC in the top 0-20 cm layer of a Mollisol under soybean-maize cropping system. There was a positive linear relationship between SOC content and increasing years with straw return, which indicates that Mollisols do not reach saturation point in C sequestration over the experimental period. The storage of organic C in density (i.e. fLF, fLF and HF) and humic fractions (i.e. HA, FA and HM) were also generally higher after the returning of straw. Moreover, straw return clearly increased the proportion of fLFC, oLFC and HAC, but decreased the proportion of HMC, resulting in the HA/FA ratios being higher than the application of no fertilizer and mineral fertilizers. The HA fractions contributed the

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DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

highest rates to the SOC improvement after 13 years straw return. The storage of SOC and its fractions did not change under treatment with application of mineral fertilizers alone, indicated an equilibrium state of SOC. It was concluded that SOC fractions in response to straw return were different. In addition, this study was only over a medium-term period of 13 years, a long-term study is needed to verify the effects of crop straw return on SOC fractions in a soybean–maize rotation system in Northeast China.

Acknowledgements. This research was funded by the National Key Research and Development Program of China (2016YFD0200309), the National Natural Science Foundation of China (41807094, 41771327, 41807085), China Agriculture Research System (CARS-04), applied Technology Research and Development Program of Heilongjiang (GY2017ZB006).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82678284

DIFFERENT SOWING METHODS INCREASING THE YIELD AND QUALITY OF SOIL WATER CONSUMPTION OF DRYLAND WINTER WHEAT ON THE LOESS PLATEAU OF CHINA

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(Received 11th Aug 2020; accepted 28th Oct 2020)

Abstract. A field experiments was conducted at the Agricultural University, Wenxi, Shanxi China. During the winter season of 2017-2019 in which three sowing methods were applied: wide space sowing (WSS), furrow sowing (FS) and drill sowing (DS) along with four nitrogen levels (150 kg·hm^{-1}, 225 kg·hm^{-1}, 300 kg·hm^{-1} and 375 kg·hm^{-1}). The present study was designed to evaluate the effect of tillage condition on nitrogen, production and consumption, protein content/yield and grain yield of dryland wheat. The wide space sowing method has extremely significant effects on the number of ears, thousand-grain weight and yield of winter wheat and also it significantly increased nitrogen absorption efficiency by 50%, nitrogen fertilizer production efficiency by 51%, the number of ears and yield were also significantly increased by 22%, 46% and the content of protein, globulin, gliadin and gluten in grains were the highest in furrow sowing, while the yield of albumin and grain protein were the highest in wide space sowing. The wide space sowing WSS and a nitrogen level of 300 kg·hm^{-1} was beneficial to increase water consumption during the growth period, also to increase the tiller dynamics, improve the dry matter quality of the plant, and to improve nitrogen absorption and nitrogen fertilizer production efficiency by increasing grain protein.
Keywords: *WSS, biomass, leaf ratio, grain yield, grain protein*

Introduction

The dryland winter wheat (*Triticum aestivum* L.) is the most important crop of the Loess Plateau with approximately 4.3 million hectares cultivating area (Wang et al., 2010). Winter wheat in this region is usually cultivated as a single crop per year followed by more than three months of summer bare fallow. Most of the precipitation (50-60%) in the Loess Plateau falls in summer from June to September (Zhang et al., 2009). Furthermore, there is a large inter-annual variation in precipitation, such as a wet year may receive two to five times more rainfall than the dry year. Therefore, the production of winter wheat and other crops in the Loess Plateau varies greatly with the distribution pattern and rate of rainfall (He et al., 2014).

Therefore, precipitation stored in the soil during the summer fallow period after wheat harvest is utilized by the subsequent crop and crucial for the success of cropping in the Loess Plateau (Schlegel et al., 2017). Water storage in soil has been affected by the different management practices such as tillage and fertilizer application (Grigoras et al., 2012). Winter wheat yield has been increased by the application of fertilizer but it also resulted in increasing soil water depletion and formation of dry subsoil layer especially in the high land areas (Yan et al., 2015). Hence for sustainable wheat production, it is crucial to seek management practices for improving water-and N-use efficiency (Fu et al., 2014). Improving yield and quality has always been an important task for wheat cultivation. The yield and quality of wheat depends on many factors such as varieties, environment, and cultivation practices (Liang et al., 2019; Xue et al., 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

The wide space and furrow sowing methods, with wide space (22-25 cm wide base and 12 cm height) and furrow by using an all-in-one machine for ridging, fertilization and sowing, is being promoted not only for conserving precipitation and decreasing soil water evaporation, but also for avoiding contamination of soil environment with plastic (Sun et al., 2015; Li et al., 2018). It has been observed that early sowing gives high yield than late sowing due to longer growing period (Munir et al., 2002). The work of sowing method along with the improvement of soil water status and quality, seedling establishment, root growth and crop yield are also being increased mainly through improving water infiltration and retention (Yan et al., 2008). The effect of furrow treatment straw or plastic mulch on maize and wheat crop yields has been persistent (Roelcke et al., 1994).

For dryland wheat production the major task is to use such methods which could effectively enhance the soil moisture consumption. Furrow sowing method has been used for dryland wheat production and results have shown that compared with drill sowing, the furrow sowing can increase the growth of wheat at various growth stages and improve the efficiency of water uptake and increase yield. Kumar et al. (2011) and Dong et al. (2018) showed that compared with flat field sowing, the use of furrow sowing in dryland wheat could increase stem height, extend the duration of functional green leaves, increase spike quality, and increase yield by 53%. Yue et al., 2006 showed that compared with flat sowing, soil moisture is not easily lost and water retention in soil is higher under furrow sowing. With furrows, the surface temperature of 0-5 cm cultivated soil layer is increased by 1-2 °C (Yue et al., 2006). The work of sowing method along with the improvement of soil water status and quality, seedling establishment, quality and crop yield can also be increased mainly through improving water infiltration and retention (Yan et al., 2008). The tiller number and percentage of productive tillers, leaf area index, dry weight, and yield were increased by DS without decline in grain protein. Furthermore, it also enhanced the N use efficiency in wheat (Liu et al., 2018).

Fertilizers constitute an integral part for improved crop production technology. Nitrogen (N) is an essential mineral nutrient for plant growth, expands soil fertility and crop productivity (López et al., 2012; Wang et al., 2012). Proper amount of N fertilizer application is considered a key for high crop production (Liang et al., 2019). Increase in grain yield is often associated with low protein content (Triboi et al., 2002). Applying N fertilizer promotes wheat root elongation and increase soil water consumption, it also promotes wheat plant growth and then significantly improve wheat yield and water use efficiency. Grain protein content was also affected by the soil moisture content and decreased in the year with low precipitation, while increased in the year with high precipitation (Sun et al., 2014). The yield of these two crops was compared with that of conventional cultivation methods may be due to ideal coordination between soils humidity and temperature (Li et al., 2013).

On the other hand, rainfall accounts for 25-40% of the water requirement for winter wheat growth and cannot meet its full demand (Ahmadzai et al., 2017). Available water and nitrogen (N) are considered the most limiting factors in wheat production in most parts of the world, especially in arid and semi-arid regions (Gonzalez et al., 2010). Supplemental irrigation and Nitrogen fertilizer application are required to match soil water stress and stabilize yields (Tavakkoli et al., 2004). The highest Nitrogen uptake in the growth period occurred from reviving stage to anthesis stage. The proportion of Nitrogen accumulated in leaf and stem was high before the anthesis stage and the accumulated nitrogen rate in stem reached peak at the anthesis stage (Zhao et al., 2006).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Recent technological advances have focused on the simultaneous and synergistic improvement of several factors including water use, nitrogen efficiency, yield, and grain quality (Parry et al., 2011). The presence of compacted soil above and loose soil below not only prevents air leakage but also reduces water evaporation. The tridimensional sowing pattern is known (Zhao et al., 2016). Sowing methods influenced wheat yield due to changes in soil water storage and water-use efficiency on the Loess Plateau in China. However, it remained unclear how different sowing methods would influence soil bacterial diversity and abundance that contribute to the changes in soil quality and micro-environment (Mann et al., 2019).

The objective of this study was to find the best sowing method of nitrogen level to increase the yield and quality of winter wheat crop. Wide space sowing (WSS) with a nitrogen level of 300 $kg \cdot hm^{-1}$ enhances dry matter accumulation, to achieve high yield in addition, it was showed that improved, which was beneficial to the improvement of nitrogen uptake at different growing stages, ultimately improving quality, increasing yield and grain protein content.

Materials and methods

Experimental site and meteorological condition

Field experiment was conducted during the winter wheat growing season from 2017 to 2019 at the experimental station of Shanxi Agricultural University. The experimental area was geographically located in Wenxi (35°200N, 111°170E), Shanxi Province, China. It was semi-arid, hilly, dryland area where precipitation was the sole source of moisture with an average annual rainfall of 450-630 mm, 60-70% of precipitation of the year was concentrated in July-September. This region is characterized by semiarid climate which receives 491 mm of average annual precipitation, 12.9 °C annual mean temperature, and 2242 h of annual sunshine. The soil in the test site belongs to calcareous cinnamon soil according to the Chinese soil classification standard (*Table 1*). The basic nutrient properties of soil from 0-20 cm depth were determined and presented in *Table 1*, the pH of the soil was 7.93.

Table 1. *Soil nutrient properties from experimental location Wenxi*

Year	Organic matter ($g \cdot kg^{-1}$)	Alkaline N ($mg \cdot kg^{-1}$)	Available P ($mg \cdot kg^{-1}$)	Available K ($mg \cdot kg^{-1}$)
2017-2018	12.61	44.07	10.71	188.87
2018-2019	8.93	40.05	14.16	200.24

Precipitation and temperature (°C) distribution

The annual precipitation during 2015-2016 and 2016-2017 was 59.7 mm and 40.2 mm, which is less than the average long-term precipitation. In 2014-2015, most of the precipitation occurred during the fallow season with 97.2 mm higher than the long-term precipitation. In 2015-2016, total precipitation was 416.6 mm, and precipitation during growth was 198.3 mm, In 2017-2018 wintering stage precipitation was 152.40%, The precipitation during the growing seasons of winter wheat during 2016-2019 at the experimental site were 240.9 mm, 218.3 mm and 68.9 mm. Precipitation from sowing to wintering and jointing to maturity were abundant. The yearly average in the last 35 years (1981-2016) was 490.90 mm. Based on the generalized precipitation classification

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

(Ren et al., 2019), the annual precipitation pattern is divided into three types: dry year ($P \leq 25\%$), normal year ($25\% < P < 25\%$) and wet year ($P \geq 25\%$). The P was calculated as follows:

$$P = (\text{the year average precipitation} - 490.90) / 490.90$$

Temperature values (°C) in the fallow period and growth stage from 2012 to 2013. Were 24.9, and 10.4, respectively, from 2013-2014 the values were 26.7, and 10.7, respectively, from 2014-2015 the values were 24.8 and 10.5, respectively, from 2015-2016 the values were 24.7 and 10.0, respectively. The detailed temperature values (°C) are given in *Figure 1*.

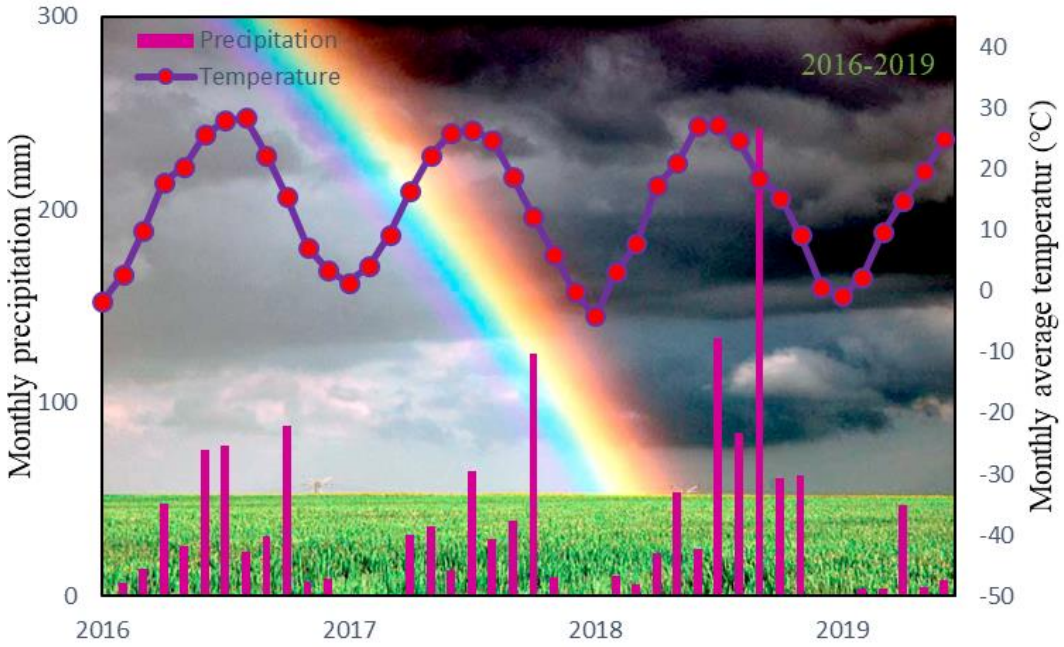

Figure 1. *Precipitation and mean temperature during the fallow period in the study year (2016-2019) in different growth stages of wheat at the experimental site in Wenxi, China. Fallow period: (22 June - 30 September), sowing to wintering (1 October - 30 November), wintering to jointing (1 December - 10 April), jointing to anthesis (11 April - 10 May), and anthesis to maturity (22 May - 10 June). (Source: Meteorological Observation of Wenxi County, Shanxi Province, China)*

Experimental design and treatments

Field management and experimental design

An experiment was started with the winter wheat season of October 2017, and ended after the winter wheat was harvested in [15]June 2019, covering 2 successive wheat crops at the same experimental plot. The seeds of winter wheat (*Triticum aestivum* L.) cultivar 'Yunhan 20410′, were obtained from Wenxi Agriculture. Wheat stubble of 20-30 cm from the previous wheat crop was left in field to reduce evaporation and to increase organic carbon content in the soil. The experiment was set as split-plot design with a single-factor completely randomized design. Experiment comprised of three different sowing methods: wide space sowing (WS), furrow sowing (FS) and drill sowing (DS).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The area of each plot was 40 m^2 (5 m × 8 m) and each treatment was repeated 3 times. The details of the machinery and sowing techniques are given in *Figures 2* and *3*. After the harvest of the former corn four nitrogen levels (150 kg·hm^{-1}, 225 kg·hm^{-1}, 300 kg·hm^{-1}, 375 kg·hm^{-1}) were applied. Before planting, 150 kg N hm^{-1} (Urea, 46%), P_2O_5 (38 kg ha^{-1}) and K_2O (75 kg ha^{-1}) were broadcasted evenly on the surface of plots. There was no top dressing during the growing seasons. The plot area was 30 m^2 (5 m × 6 m). The planting density was 315 × 10^4 plant ha^{-1} and each treatment was repeated 3 times. The straw was returned to the field after the previous stubble corn was harvested and planted on the October, 2018. Weeds and pests were well controlled by hand and no irrigation was applied across the two experimental years.

Figure 2. *(a) Furrow sowing (FS), (b) wide space sowing (WSS), (c) drill sowing (DS)*

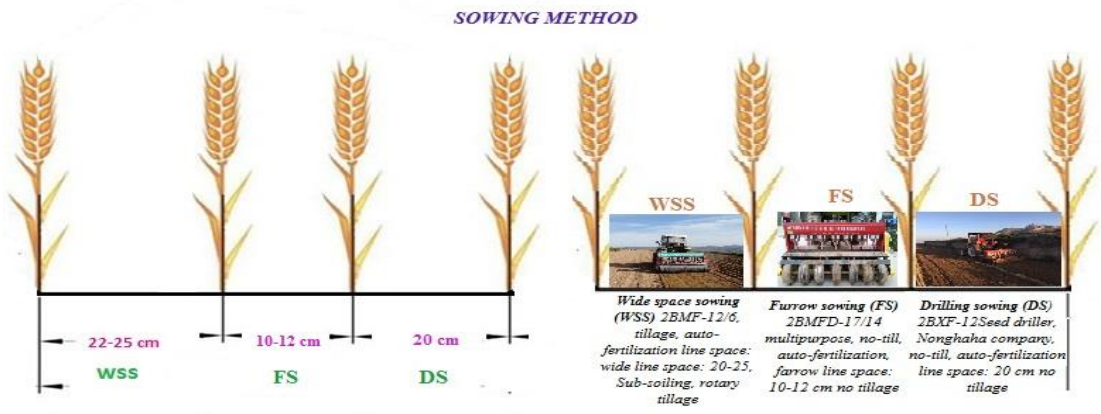

Figure 3. *Crop arrangement of wheat in configurations with different sowing methods, wide space sowing (WSS), furrow sowing (FS), drill sowing (DS)*

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

Measurements

Soil water storage

A soil sample of 0-200 cm (each 20 cm was a soil layer) was drilled at each growth stage and the soil moisture was measured after drying. Due to the flat terrain the groundwater depth below 15 m and the precipitation infiltration depth not exceeding 2 m, the surface runoff groundwater recharge and apparent depth leakage are all zero. Soil water storage were calculated by using the following formula (Liang et al., 2019):

$$\text{Soil water storage (mm)} = [(\text{wet soil weight} - \text{dry soil weight})/\text{dry soil weight} \times 100\%] \times \text{soil thickness} \times \text{soil bulk density} \quad \text{(Eq.1)}$$

Changes in soil water storage (ΔSWS) for a specific stage of wheat is calculated as the difference between the soil water storage at the beginning (SWS1) and end of the growth stage (SWS2) as follows:

$$\Delta SWS = SWS1 - SWS2 \quad \text{(Eq.2)}$$

The water consumption (CA, mm), percentage of CA to total water consumption (CP, %), and daily water consumption (CD, mm) were calculated as follows:

$$CA = P + I - \Delta SWS \quad \text{(Eq.3)}$$

$$CP = CAG / CA \quad \text{(Eq.4)}$$

$$CD = \text{the } CAG/d \quad \text{(Eq.5)}$$

where P is precipitation (mm) during this period, I is the irrigation amount, CAG refers to water consumption at a certain stage, and d is the number of days in the growing stage.

$$WUE = Y / ET \quad \text{(Eq.6)}$$

where WUE is water use efficiency (kg h^{-1} mm^{-1}); Y is the yield of wheat (kg h^{-1}).

Determination of spike number

Comparison of the spike of interest with the model spike occurs in an n-dimensional vector space, which dimensions are defined by the total spikelet number of the spike of interest. The geometrical difference in GYDAS between the two spikes is based on the scalar product of these two vectors and were calculated by using the following formula (Noor et al., 2020):

$$\cos\measuredangle\left(\vec{a}, \vec{b}\right) = \frac{\vec{a} \cdot \vec{b}}{|\vec{a}| \cdot |\vec{b}|} \quad \text{(Eq.7)}$$

Nitrogen use efficiency (NUE)

At maturity, the total N concentration in the aboveground tissue was determined by H_2SO_4-H_2O_2 digestion and analysis of the digestate by the automatic Kjeldahl

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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method (FOSS8400, Foss, Hilleroed, Switzerland). The N accumulation (NA) was calculated as the product of N concentration and aboveground biomass. The N uptake efficiency (NUE) was calculated as the ratio of N uptake by the aboveground crop at maturity to the amount of N fertilizer applied. The N fertilizer productivity (NFP) was calculated as the ratio of the grain yield to the amount of N fertilizer applied. NUE was calculated as the ratio of the grain yield to the total N uptake (Guo et al., 2014).

Determination of plant agronomic traits

Three rows of wheat plants within 0.667 m2 of a fixed point were taken at each growth stage to investigate the number of stems in the population. Plant height and leaf area of 20 plants with sowing growth and representativeness were taken at each growth stage and the plant height was measured; at the same time the length, width and number of green leaves of the second leaf were measured. Leaf area index for the determination of leaf area, the length and width of the second leaf and total number of leaves were calculated. Leaf area was measured using the following formula (Noor et al., 2020):

$$\text{Leaf area} = \text{length} \times \text{width} \times \text{number of green leaves} \times 0.85 \quad \text{(Eq.8)}$$

where 0.85 was the adjustment factor. Then leaf area index (LAI) was calculated by dividing the leaf area (cm^2) by the ground surface area.

Grain to leaf area ratio

Grain to leaf ratio was calculated according to Feng et al. (1999), using *Equations 9* and *10*:

$$\text{Grain number to leaf area ratio} = \frac{\text{total number of grain per unit area}}{\text{total leaf area on the same plot at booting stage}} \quad \text{(Eq.9)}$$

$$\text{Grain weight to leaf ratio} = \frac{\text{grain weight (mg) per unit area}}{\text{total leaf area on the same plot at booting stage}} \quad \text{(Eq.10)}$$

Total leaf area was taken from the same plot at booting stage (cm^2).

At maturity period 30 ears were burned at 105 °C for 30 min, dried at 80 °C to constant weight, crushed with a DE-100 g mini high-speed universal grinder produced (Zhejiang Hongjingtian Industry and Trade Co., Ltd) and using a continuous extraction method to determine the protein content of the grains. The MJZ-II type gluten index tester was used to determine the wet gluten content and the micro dough LAB 2800 micro flour analyzer was used to determine the flour characteristics.

Grain yield, grain protein yield

At maturity, plants were randomly sampled from three 1 m^2 areas from each plot to determine grain number $spike^{-1}$ and 1,000 grain weight. All plants from the plots were harvested on the 16th June 2018, and the 15th June 2019. Grains were air-dried whereas aboveground plant parts were oven dried until constant weight to determine the grain yield (kg ha^{-1}) and dry biomass. The harvest index (HI) was calculated dividing the grain yield by the aboveground dry biomass.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Statistical analysis

Two-way analysis of variance (ANOVA) was performed to determine the significance of sowing methods, statistical analysis was carried out using DPS 7.05 software and the significant difference among treatments were calculated by least significant difference test (LSD) at the significance level of $p \leq 0.05$. Pearsons correlation coefficients were calculated in order to determine the relationship between water consumption and yield.

Results

Production and composition yield quality formation

The effect of sowing methods on production and composition yield quality formation of winter wheat can be seen from *Figure 4*. Compared with (*DS*) the number of spikes, thousand seed weight and yield under wide space sowing, and even sowing increased from 2017 to 2019. From 2017 to 2018 the number of ears increased by 22% 16% and 17%, from 2018 to 2019 it increased by 46% 16% and 32% and the differences between the treatments were significant with WSS being the highest. The thousand grain weight increased by 3% 7% and 4%, respectively. The difference between the treatments was not significant and it increased by 2%, 5% and 0% respectively and the highest was the trench sowing followed by the *WSS*, the difference between *DS* was not significant; Compared with *DS* the number of panicles for *WSS* increased from 2017-2018 by 25% 17% and 11%, and in 2018-2019, it increased by 55% 22% and 40%, respectively and the differences between the treatments were significant.

Effects of different sowing methods on growth and development of population dynamics at each stage characteristics of winter wheat

Development population dynamics at each stage characteristics of winter wheat, the number of tillers in each growth period increased then decreased and reached the maximum at the jointing stage (*Fig. 5*). The tiller number of drill group was higher in the early and middle stage, but lower in the late stage than in other sowing methods. In different growth periods, compared with drill, the tiller number of the population under wide drill increased by 6%, 27%, 9% and 22%, respectively and was higher than in other sowing methods. The tiller number of the population under *FS* in the flowering stage and maturity stage increased by 3% and 16%, respectively compared with that under *DS*. The number of tillers in the mature stage increased by 7%. *WSS* can promote the tiller process in different growth periods, especially in the jointing period, the effect was more significant, followed by the *FS* effect, mainly increasing the number of tillers in the mature period (*Fig. 5*). That during the wintering maturity period the number of tillers in the population first increased then decreased and then decreased slowly and the number of tillers in the jointing period reached the highest value. Compared with *DS* the number of tillers in the wide wintering maturity stage increased by 34%, 11%, 25%, 40%, and 46%, respectively and the jointing maturity period was significantly higher than in other sowing methods. The tiller number of the population at the growing stage was increased by 5%, 8%, 12%, 13% and 16%, mature stages were increased by 48%, 18%, 26% and 32%. It can be seen that *WSS* can increase the number of tillers in each period.

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

Figure 4. *Effects of different sowing methods on yield and components of WSS; wide space sowing; FS: furrow sowing; DS: drilling sowing; winter wheat. Different letters indicate significant difference among treatments at the significance level of $p \leq 0.05$*

Effect of different sowing methods on plant height and leaf area index at different growth stages

Effects of different sowing methods on plant height were observed at different growth periods (*Fig. 6*). From 2017-2018 no significant differences were observed between sowing methods at the wintering and jointing stages, in wintering, jointing and booting stage, the plant height was maximum at FS flowering and maturity stage, the plant height was higher in DS than FS. Leaf area index (LAI) in each growth period increased and then decreased, reaching the maximum at booting stage. At different growth stages, the leaf area index of WS was higher than in other sowing methods, and increased as compared with DS. Sowing methods especially WS improved LAI and the effect was more significant at the later growth stage. From 2018-2019 with the progress of fertility the plant height under different sowing methods showed a trend of rising first and then gradually flattening. The plant height of each sowing stage was higher than that of other sowing methods. The plant height of WSS was higher than that of FS and DS. It can be seen that different planting methods can increase plant height and WSS has the best effect. Leaf area

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

index (LAI) first shows a trend of increase and then decrease and the difference between booting and maturity is obvious. During the booting and maturity stage the leaf area under WSS was significantly higher than in other sowing methods the leaf area was significantly higher than that of FS and DS and the leaf area under FS was significantly higher than that of DS. It can be seen that different sowing methods could increase the leaf area of winter wheat at the later growing stage and wide space sowing was better followed by even sowing.

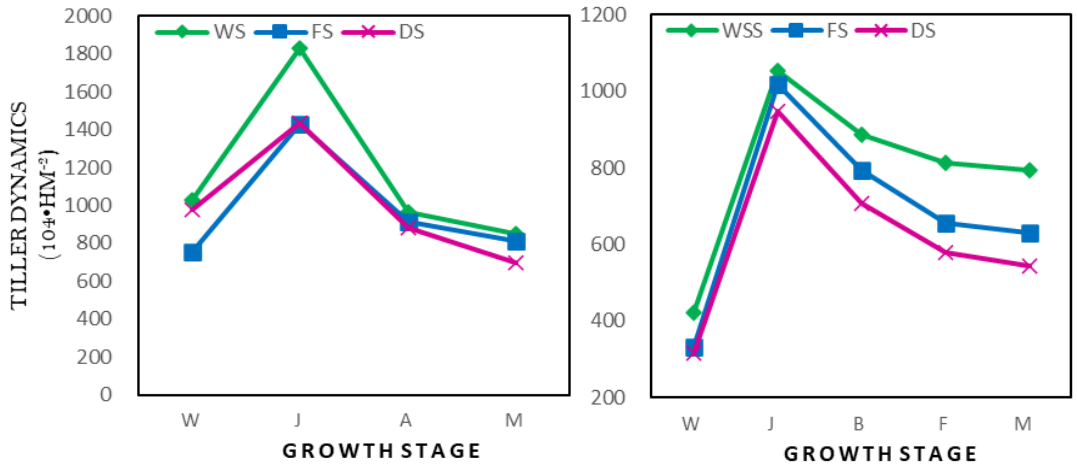

Figure 5. *Effect of sowing method on tiller dynamics at different growth stages of winter wheat. W, J, B, F, A, and M indicate wintering, jointing, bolting, Flowering, anthesis, and maturity stages W-J: Wintering stage to jointing stage, Oct 1 to Apr 10; J-B: Jointing stage to Booting, Apr 11 to May 10; B-A: Booting stage to anthesis, May 11 to May 21; A-M: Anthesis to maturity and flowering to maturity may 22 to Jun 10. Different letters indicate significant difference among treatments at the significance level of* $p \leq 0.05$

Effects on soil water consumption

The water consumption and water storage consumption ratio in the growth period under wide space sowing and drill sowing were significantly increased, while the water consumption ratio of precipitation and irrigation water consumption ratio were significantly reduced as can be seen from *Table 2*. Compared with drill sowing, the water consumption and water storage consumption ratio of furrow sowing (*FS*) and even sowing during the growth period were significantly increased, while the water consumption ratio of precipitation and irrigation water consumption ratio were significantly reduced, while water consumption of precipitation and irrigation decreased, but there was no significant difference between the two treatments. It can be seen that wide space sowing (*WSS*) and drill sowing (*DS*) can increase the water consumption and water storage consumption ratio significantly during the growth period and reduce the water consumption ratio of precipitation and irrigation water consumption ratio development of winter wheat.

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

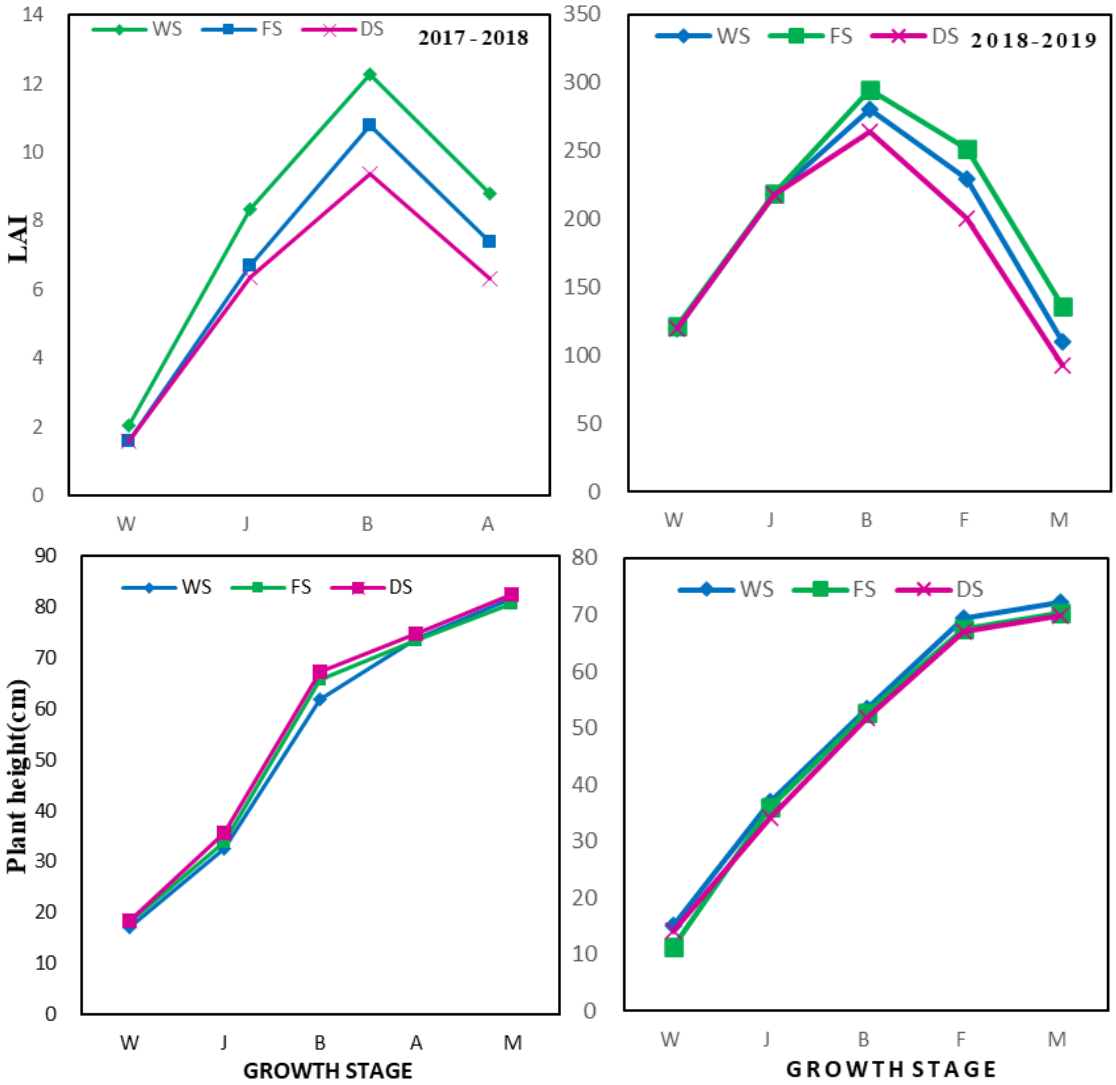

Figure 6. *Effect of sowing method on plant height and leaf area index (LAI) at different growth stages of winter wheat. W, J, B, F, A, and M indicate wintering, jointing, bolting, Flowering, anthesis, and maturity stages; WS: wide space sowing; FS: furrow sowing; DS: drilling sowing; W-J: Wintering stage to jointing stage, Oct 1 to Apr 10; J-B: Jointing stage to Booting, Apr 11 to May 10; B-A: Booting stage to anthesis, May 11 to May 21; A-M: Anthesis to maturity and flowering to maturity may 22 to Jun 10. Different letters indicate significant difference among treatments at the significance level of* $p \leq 0.05$

Effects on the proportion of soil water consumption in different growth stages

As can be seen from *Table 3,* water consumption ratio of the soil in the 0-200 cm soil layer during wintering jointing stage and flowering maturity stage was higher than that during sowing wintering stage and jointing flowering stage. Compared with drill sowing the water consumption ratio of 0-200 cm soil layer increased during the period of wintering and maturity under wide drill sowing, it was significantly higher in wintering and jointing period than in other sowing methods. During the sowing and wintering stage, the water consumption ratio of the soil in the 0-200 cm soil layer was the highest followed by *FS*, wide and drill. During the winter jointing stage, followed by *FS* the

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

wide and drill sowing was the highest and followed by *WS* flowering and maturity, furrow sowing was the highest. Wide and drill sowing can increase the proportion of water consumption in the soil layer of 0-200 cm during the wintering period and the jointing stage, which was conducive to the vegetative growth and reproductive growth of winter wheat.

Table 2. *Effects of sowing method on soil water consumption ratio of 0-200 cm depth from different water for winter wheat*

Sowing method	Soil water consumption in the growing stage (mm)	Soil water consumption ratio (%)	Precipitation consumption ratio (%)	Irrigation consumption ratio (%)
WSS	435.67 $_{a}$	36.12 $_{a}$	50.11 $_{c}$	13.77 $_{c}$
FS	410.78 $_{b}$	32.25 $_{b}$	53.15 $_{b}$	14.61 $_{b}$
DS	372.42 $_{c}$	25.27 $_{c}$	58.62 $_{a}$	16.11 $_{a}$

Table 3. *Effects of sowing method on soil water consumption ratio of 0-200 cm depth at different growing stage for winter wheat*

Sowing method	W	J	A	M
WSS	11.60 $_{d}$	40.32 $_{a}$	15.61 $_{b}$	32.47 $_{c}$
FS	18.03 $_{b}$	24.06 $_{b}$	13.01 $_{c}$	44.91 $_{a}$
DS	14.76 $_{c}$	29.75 $_{c}$	15.19 $_{b}$	40.30 $_{b}$

Sowing rates on water content in 0-200 cm soil layer at different growth stages of dryland wheat. W, J, A, and M indicate, (W), wintering, (J), jointing, (A), anthesis, and (M), maturity stages; WS: wide space sowing; FS: furrow sowing; DS: drilling sowing; W-J: WS: wide space sowing; FS: furrow sowing; DS: drilling sowing

Effects on nitrogen operation before flowering and nitrogen accumulation after flowering

Nitrogen accumulation after flowering can be seen from *Table 4*. Compared with drill sowing (*DS*) 2017-2018, the operation amount of nitrogen accumulation before flowering was significantly increased by 74%, 44% and 66%, respectively. The contribution rate of operation of nitrogen accumulation before flowering to grains was 72.58% and the operation rate of wide drill sowing was 69.03%, higher than that of groove sowing and drill sowing. Compared with drill sowing, the nitrogen accumulation after flowering was significantly increased by 76%, 52% and 39%, respectively. It can be seen that different sowing methods mainly promote the operation of nitrogen accumulation before flowering to increase the contribution rate to grains, among which *WSS* was better than other methods. In 2018-2019 compared with the drilling, drill and wide before flowering of nitrogen accumulation amount of operation and contribution rate on the grain were significantly increased, among them, the accumulation of nitrogen run before flowering amount increased by 35%, 16% and 11%, respectively. For the grain the contribution rate increased by 10%, 4% and 4% respectively and contributed to wide and drilling of grain most significantly. Compared with drill sowing, the nitrogen accumulation after flowering was reduced under the treatments of wide sowing drill sowing, furrow sowing, but there was no significant difference between the treatments of *FS* and *DS*. The contribution rate of nitrogen accumulation to kernels decreased significantly after flowering, which shows that different sowing methods mainly promote the accumulation of nitrogen in grains by

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

promoting the operation of nitrogen accumulation before flowering, especially the wide sowing.

Table 4. *Effects of different sowing method on pre-anthesis accumulated nitrogen translocation and nitrogen accumulation amount after flowering of winter wheat*

Sowing method	PANT		NAAA	
	Amount ($kg \cdot hm^{-2}$)	Contribution to N in grains (%)	Amount ($kg \cdot hm^{-2}$)	Contribution to N in grains (%)
2017-2018				
WSS	150.12 $_{a}$	81.04 $_{a}$	35.18 $_{b}$	19.00 $_{c}$
FS	128.96 $_{b}$	76.27 $_{b}$	39.69 $_{a}$	23.37 $_{b}$
DS	110.80 $_{d}$	73.52 $_{c}$	40.10 $_{a}$	26.60 $_{a}$
2018-2019				
WSS	117.16$_{a}$	69.03$_{b}$	49.10$_{a}$	28.97$_{a}$
FS	96.97$_{c}$	68.07$_{b}$	42.35$_{b}$	29.93$_{a}$
DS	67.26$_{d}$	68.97$_{b}$	27.94$_{c}$	29.03$_{a}$

PANT, Pre-anthesis accumulated nitrogen translocation amount from vegetative organs to grains after anthesis: NAAA, Nitrogen accumulation amount after anthesis

Effects on nitrogen use efficiency of winter wheat

Nitrogen use efficiency can be seen from *Table 5*. Compared with *DS* in 2017-2018 the nitrogen absorption efficiency of *WSS* and *DS* was significantly improved by 50%, 28% and 38% and the production efficiency of nitrogen fertilizer was significantly improved by 51%, 20% and 36%, respectively. The nitrogen utilization efficiency of *WSS* and *DS* was higher than that of drill, but the difference was not significant and the nitrogen utilization efficiency of *FS* and *DS* was significantly lower than that of *DS*. The yield index of nitrogen was the highest in wide and drill, but not significantly different from that in *FS* and *DS* the yield index of drill was the lowest. Different sowing methods can improve nitrogen absorption efficiency, nitrogen fertilizer production efficiency and nitrogen harvest index, among which WS is better. 2018-2019 nitrogen absorption efficiency and nitrogen production efficiency were significantly improved under WSS treatments compared with drill, among which nitrogen absorption efficiency was increased by 21%, 12% and 7%, and nitrogen production efficiency was increased by 15%, 9% and 2%, respectively. Moreover, there were significant differences among different sowing treatments. The nitrogen utilization efficiency of WSS, DS, FS and DS was significantly lower than that of drill. Different sowing methods are beneficial to the improvement of nitrogen absorption efficiency and nitrogen production efficiency, especially the wide sowing is the best.

Effects on protein and component contents in mature grains of winter wheat

Component contents in mature grains (*Table 6*) shows that compared with drilling, wide furrow sowing and information processing of grain of albumin, gliadin and glutelin, total protein content and protein yield increased significantly in 2017-2018, improved the grain albumin respectively 13%, 9%, 5%, 14%, 4%, 4% alcohol soluble protein, gluten 17%, 5%, 6% and the grain total protein were 14%, 4%, 2%, valley/alcohol ratio

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increased by 3%, 1% and 2% respectively, but no significant difference was observed between the groups. Compared with drill, the content of globulin in seeds increased under wide and drill, but decreased under furrow and drill. It can be seen that different sowing methods are beneficial to increase the content of protein and components in grains, among which, the content of protein and components in grains and the grain/alcohol ratio are the highest under wide space sowing 2018-2019 that compared with drilling, *WSS* and *FS* and information processing, the mature grain protein content and the content of albumin, gliadin glutelin and protein yield were improved, wide globulin increased under drilling and furrow sowing processing, protein content increased by 13%, 15% and 4% respectively, protein yield increased by 42%, 35% and 16% respectively and significant difference was observed between the sowing methods. The content of protein, globulin, gliadin and gluten in grains was the highest in furrow sowing, while the yield of albumin and grain protein was the highest in wide space sowing.

Table 5. *Effects of different sowing method on nitrogen use efficiency of winter wheat*

Sowing method	N uptake efficiency ($kg \cdot kg^{-1}$)	N use efficiency ($kg \cdot kg^{-1}$)	N productive efficiency ($kg \cdot kg^{-1}$)
2017-2018			
WSS	1.09 $_{a}$	40.37 $_{a}$	44.70 $_{a}$
FS	0.93 $_{c}$	36.73 $_{c}$	35.45 $_{c}$
DS	0.73 $_{d}$	40.20 $_{a}$	29.62 $_{d}$
2018-2019			
WSS	1.23 $_{a}$	40.11 $_{b}$	50.34 $_{a}$
FS	1.15 $_{b}$	40.69 $_{b}$	47.58 $_{b}$
DS	1.02 $_{d}$	43.64 $_{a}$	43.65 $_{d}$

Wide space sowing (WSS): 2BMF-12/6, tillage, auto-fertilization line space: wide line space: 20-25, sub-soiling, rotary tillage
Furrow sowing (FS): 2BMFD-17/14 multipurpose, no-till, auto-fertilization farrow line space: 10-12 cm no tillage
Drill sowing (DS): 2BXF-12Seed driller, Nonghaha company, no-till, auto-fertilization line space: 20 cm no tillage

Table 6. *Effects of different sowing method on grain protein and component contents for winter wheat*

Sowing method	Albumin (%)	Globulin (%)	Gliadin (%)	Glutenin (%)	Glu/Gli	Protein content (%)	Protein yield ($kg \cdot hm^{-2}$)
2017-2018							
WSS	2.15_{a}	1.96_{a}	4.07_{a}	4.74_{a}	1.17_{a}	14.14_{a}	1159.98_{a}
FS	2.09_{b}	1.78_{b}	3.70_{b}	4.25_{b}	1.15_{a}	12.89_{b}	831.09_{c}
DS	1.91_{d}	1.79_{b}	3.57_{c}	4.06_{c}	1.14_{a}	12.37_{c}	652.78_{d}
2018-2019							
WSS	2.27 $_{a}$	1.87 $_{ab}$	4.18 $_{a}$	4.83 $_{a}$	1.15 $_{a}$	14.18 $_{b}$	1297.97 $_{a}$
FS	2.25 $_{a}$	2.00 $_{a}$	4.25 $_{a}$	4.84 $_{a}$	1.14 $_{a}$	14.43 $_{a}$	1235.03 $_{b}$
DS	1.93 $_{a}$	1.82 $_{ab}$	3.52 $_{b}$	4.11 $_{b}$	1.17 $_{a}$	12.54 $_{d}$	917.20 $_{d}$

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

Discussion

The Loess Plateau in China covers about 0.65 million km^2 area and has a 108 million population (Wang et al., 2010). The Loess Plateau has a semiarid climate with low and variable rainfall from 300-700 mm (Li et al., 1992). Despite this productivity, precipitation can be unpredictable and insufficient for the winter wheat crop. Water is the most important limiting factor in wheat production (Zhang et al., 1999). For winter wheat, yield and biomass are dependent on water availability (Kang et al., 2002a). Irrigation is often required for augmentation of rainfall, especially during the drier winter and spring months, though this practice often exerts harmful environmental effects including drops in the water table and an associated reduction of groundwater supplies (Li et al., 2008). Some previous research also indicated that water stored at sowing time may be an important complement to the seasonal rains in dryland areas since this stored water may be more effective in promoting yield (Sun et al., 2010).

Effect of different sowing methods on soil water consumption and yield characteristics

For dryland wheat it is very important to form strong seedlings before winter and ensure safe wintering. Sowing quantity directly regulates population size, affects stability of population growth and stability of individual growth and then determines the final panicle number (Troccoli et al., 2005). Sowing methods should be combined with other cultivation measures, such as irrigation. Ridge making and furrow sowing mainly affect the surface shape and concentrate water in the trench. On the one hand, surface runoff is regulated to increase infiltration on the other hand, irrigation water can be reduced. As an effective water-saving seeding method, it has been widely used at home and abroad. Wiyo et al. (1999) showed that on the basis of conventional irrigation, the water use efficiency could be improved by reducing the irrigation amount by 30% without affecting the growth and development of wheat (Lu et al., 2003). Furrow sowing could change the flow direction and speed of surface runoff, promote water infiltration, and thus promote water absorption by roots. Many studies at home and abroad on the yield-increasing mechanism of furrow sowing show that, to a certain extent, furrow sowing eliminates many negative impact factors of traditional sowing method, improves the internal light, temperature, water and microbial environment of soil and creates suitable environmental conditions for wheat growth, thereby promoting the growth and development of wheat (Randall et al., 1990). Wheat is a crop that requires a lot of water. Water is an important factor affecting wheat production. The water saving effects of different farming and sowing methods are also different (Raun et al., 2002). Wheat yield in semiarid dryland areas is highly affected by the variation in the amount and distribution of seasonal precipitation (Wang et al., 2015). Precipitation is an important meteorological factor which affect soil water content. In the Loess Plateau and other dryland areas, the soil water content at time of sowing is important for early growth of wheat and highly dependent on the precipitation during fallow season of dryland wheat (Kang et al., 2002b; Rossato et al., 2017). The wheat yield is linearly related to the soil water content (Musick et al., 1994; Qin et al., 2013). The use of wide range precision sowing method under the condition of corn straw mulch significantly improved reduced production and composition (Liu et al., 2015). The soil water storage capacity of 0-300 cm soil layer with full membrane soil hole sowing increased during

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sowing jointing and grouting and the water consumption intensity increased during sowing jointing water use efficiency increases (Hou et al., 2017).

This study shows that compared with drill sowing (DS), wide space sowing (WSS) and furrow sowing (FS) can significantly increase water consumption during the growing period water storage and water consumption ratio significantly reduce the water consumption ratio of precipitation and irrigation water consumption. The effect is better and the percentage of soil water consumption in the 0-200 cm soil layer under wide sowing is significantly higher than in other sowing methods and the water consumption during the growth period under wide space sowing increased. The increase of 17% and 33% may be due to the uneven distribution of the roots in the upper layer of drill sowing (DS). This caused the soil moisture to be lost in the manner of soil evaporation. The water consumption of the plant increases and the ineffective water consumption decreases which is beneficial to the growth and development of wheat. The individual growth and nutrient movement of wheat during the whole growth period has a very important influence on the formation of grain yield (Chu et al., 2018). Nitrogen absorption efficiency, nitrogen fertilizer production efficiency and nitrogen harvest index significantly improved, but the mechanism that affects nitrogen operation by wide space sowing needs further study. The number of spikes, grains per spike and thousand grain weight are the three elements of yield formation. Su et al. (2007) and Dang et al. (2015) showed that compared with the traditional sowing method, the use of wide sowing increased the number of ears by 5%, the weight per thousand by 3%, and the yield increased by 12%. The number of ears and yield showed a very significant positive correlation. There was a significant positive correlation between ears number and thousand-grain weight and yield. It can be seen that WSS mainly increased yield by increasing ear number which is the same as that of Li et al. (2012). The yield is significantly positively related to the water consumption during the growth period and the nitrogen accumulation before flowering. WSS has high water consumption during the growth period and high nitrogen before bloom which promotes nutrient absorption and operation and increases yield. In addition, the broad soiled sowing population structure is reasonable, significantly increasing the number of tillers in the middle and late stages of reproductive growth, increasing ear length. The accumulation of dry matter is conducive to the increase of yield. Different sowing models have their own characteristics and only by supporting different seeding rates according to different seeding models can it be more beneficial to increase wheat yield (Sweeney et al., 2000). Studies by Wang et al. (2016) showed that in the wheat cotton intercropping mode the DS technique should be used and the seeding rate should be controlled at 225.0-262.5 $kg \cdot hm^{-2}$. It is most beneficial to improve wheat yield (Zhang et al., 2012). The precision DS method the sowing spring wheat can sow 525 $kg \cdot hm^{-2}$ and can reach a high yield of 9 499.5 $kg \cdot hm^{-2}$. Liu et al. (2012) found in the study of dry land wheat that the sowing amount in open field was 354 × 104 hm^{-2}, and 245 × 104 hm^{-2} on the film was good for improving grain yield (Feng et al., 2013), Research showed that the suitable seeding rate for wide space sowing is 105 $kg \cdot hm^{-2}$. At this time, the highest yield of wheat is 13% higher than that of conventional precision sowing and the highest yield was 8643 $kg \cdot hm^{-2}$ Cao et al. (2018). In this experiment the interaction effects of the sowing method and the sowing amount were analyzed the sowing method the sowing amount the sowing method × the sowing amount had a significant effect on the number of ears grain number, and thousand-grain weight yield of winter wheat. The suitable sowing capacity for WSS is 300 $kg \cdot hm^{-2}$, the yield at this seeding amount is

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7158 kg·hm^{-2} and the suitable seeding capacity for trenching is 300 kg·hm^{-2}. The suitable sowing capacity for DS is 225 kg·hm^{-2}, the yield at this sowing capacity is 5832 kg·hm^{-2}. It can be seen that the appropriate seeding amount should be selected according to different seeding methods. Under this experimental condition, the matching sowing method of WSS is 300 kg·hm^{-2}, which is conducive to the improvement of grain yield. It is suitable for the local sowing method and volume.

Effect of different sowing methods on quality nitrogen use efficiency formation

Agronomic practices need to ensure optimal N fertilization. These are conducted when the plant can still incorporate the N into its grain and does not limit NUE. The protein change in response to N and late-season application time of N is generally greater and more reliable under irrigation than dryland production, because the N is usually incorporated with irrigation, which increase N uptake (Jones et al., 2012). There is a coupling effect between water and nitrogen fertilizer operation in agricultural production and the reasonable combination of water and nitrogen has a significant effect of increasing yield. On the one hand, the law of soil water transport and accumulation and consumption affects the absorption and utilization of N elements by crops (Yang et al., 2008). On the other hand, proper application of N fertilizer can, to a certain extent, make up for the material loss caused by soil deficit. Jia et al. (1995) showed that compared with drill sowing wide space sowing can improve nitrogen accumulation nitrogen absorption and nitrogen utilization efficiency, the yield and nitrogen accumulation were significantly positive. There was a significant negative correlation between nitrogen accumulation and nitrogen use efficiency, a significant positive correlation was observed between nitrogen absorption efficiency and nitrogen use efficiency. This study shows that wide space sowing can significantly increase plant nitrogen accumulation at various growth stages and the contribution rate to grains is mainly increased by the amount of nitrogen before flowering, which is consistent with previous research results (Xue et al., 2017). Moderate drought is conducive to increasing the protein content of grains and is beneficial to increase the grain, alcohol ratio, while too much water will reduce the grain protein content (Xu et al., 2003; Zhou et al., 2006). But the heavy application of N fertilizer represents a significant cost and also cause serious environmental problems due to the loss of a large amount of applied N into the environment (Ma et al., 2019). Optimizing the input of N is difficult under rain-fed cropping system due to the highly variable weather and rainfall. Less N supply may limit grain yield and grain protein content and excessive application of N may increase water use in the early growing season leading to water deficit stress during flowering and grain filling, resulting in poor grain set (He et al., 2014). The relative water content of wheat in the grain filling stage was in the range of 50% - 80%. With the increase of soil moisture the protein content of grains showed a gradual decline Zhang et al., 2006 carried out potted test results. It was shown that the soil moisture content during the whole growth period was within the range of 50% to 80% of the maximum water holding capacity in the field. As the soil moisture increased the protein content of the grains gradually decreased. Under the conditions of this test, the water content in the growing period showed a significant positive correlation with the protein content of the grains. The wide space sowing had high water consumption in the growing period, high grain protein content and high grain/alcohol ratio (Zhang et al., 2014). Research showed that the use of wide stubble sowing can make wheat individuals grow robustly which can significantly increase the bulk density and

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hardness of wheat grains significantly improve water absorption wet gluten content and sedimentation value and significantly increase the maximum resistance of dough, the standard value of power and flour quality, but has no significant effect on flour protein content and dough ductility (Wang et al., 2012). Some studies have shown that the amount of nitrogen before flowering affects wheat grain protein content. It has a large regulatory effect (Desai et al., 1978) and some studies have shown that the contribution of nitrogen accumulation and operation amount before and after flowering to grain protein varies from species to species. The protein content has a regulating effect and the nitrogen accumulation after flowering mainly has a regulating effect on the grain protein content in medium protein varieties. There was a significant positive correlation between protein content in grains and nitrogen accumulation before flowering, and a significant or very significant positive correlation with grain/alcohol ratio, wet gluten content, water absorption and quality of powder maps. The grain/alcohol ratio is an important index for evaluating the quality of wheat (Zou et al., 2006). The grain alcohol ratio is related to water consumption during growth, nitrogen accumulation before flowering, wet gluten content, water absorption, dough formation time and flour. There is a significant or very significant positive correlation between the mass numbers in the prime image. It can be seen that increasing the water consumption during the growth period and the amount of nitrogen accumulation before flowering are conducive to increasing the protein content of the grain, increasing the grain/alcohol ratio, and ultimately facilitating the formation of quality. Under this test condition, the use of wide space sowing is the most beneficial to improve the grain quality indicators such as protein and component content, grain, alcohol ratio, wet gluten content, water absorption, dough formation time and stabilization time.

Effect of different sowing methods on yield and quality

Studies have shown that if the local conventional sowing amount is increased by 10% - 20%, the yield and quality can be increased by 2-15%. Gaju et al., 2011 showed that an appropriate increase in sowing amount could promote wheat root binding, increase in deep roots, enhance the absorption and transport function of roots and thus enhance the absorption and utilization capacity of nitrogen. Blankenau et al. (2001) believed that appropriate sowing amount could promote wheat nitrogen consumption (Gao et al., 2009). Constructing a reasonable population structure is that individuals can fully absorb water, light, heat and nutrient resources, promote the healthy growth of individuals, coordinate the contradictions between individuals, groups and extremely important for the coordination of wheat yield and quality (Bhatta et al., 2017); Lin et al. (1996). Too much sowing more tillers in the group leads to more water consumption in the early stages of development, drought intensification in the later stages and lower yields; too little sowing, fewer tillers in the groups, insufficient panicles and lower yields (Lei et al.,2017; Shi et al.,2017). Appropriate sowing amount can optimize yield components and increase yield. Hai et al. (2002) showed that Xiaoyan 503 was in the range of 75 kg hm^{-2} to 165 kg hm^{-2} and the yield increased first and then decreased with the increase of seeding amount and it was the highest at 105 kg hm^{-2} (Liu et al., 2017). According to them with the increased seeding volume, the leaf area index of Guomai 301 in the flowering stage gradually increased, the dry matter accumulation in the flowering stage increased first and then decreased and the number of ears and yield increased first and then decreased. Under the conditions of this experiment, the relevant analysis of factors related to yield formation shows that the water consumption during

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

the growth period, the number of ears, the 1000 grain weight and the yield have a significant or very significant correlation and the seeding rate is 300 kg·hm^{-2}. In the period the water consumption and the proportion of stored water consumption increased and the water consumption and irrigation water consumption decreased and the output gradually increased with the increase of the sowing amount. 300 kg·hm^{-2} was the highest output and the water use efficiency increased. Increasing the sowing volume within a certain range increased the nitrogen accumulation in the above ground, the nitrogen utilization efficiency decreased and the nitrogen utilization increased first and then decreased (Tao et al., 2018). A research by Shuli et al. (2012) showed that increasing planting density within a certain range increased nitrogen accumulation and absorption efficiency in the above-ground area and reduced nitrogen use efficiency. Wang et al. (2012) showed that with the increase of planting density, the nitrogen harvest index, grain increased but accumulation and nitrogen content gradually decreased, and the contribution rate of nitrogen transport from vegetative organs to the grain during the flowering period showed an upward trend. Plant nitrogen accumulation wave, nitrogen absorption and utilization rate and nitrogen fertilizer partial production efficiency showed a trend of rising first and then decreasing (Xue et al., 2017). In the range of 245~330 × 104 hm^{-2}, the grain protein content increased first and then decreased with increasing planting density. Under the conditions of this test a correlation analysis was performed and the protein content was extremely significantly positively correlated with the nitrogen accumulation and the grain/alcohol ratio before flowering. The change trend of first increase and then decrease reached the highest at 300 kg·hm^{-2}, (Wang et al., 2014). The increasing sowing amount increased the amount of nitrogen accumulation in various organs before flowering and the contribution of nitrogen accumulation in leaves glumes and cobs to flowering increased before flowering. This study showed that with the increase of seeding rate the nitrogen accumulation of plants in each growth stage showed a trend of first increase and then decline in the contribution rate of nitrogen accumulation before flowering and the amount of nitrogen accumulation before flowering showed first contributions to grains. After the increased the nitrogen absorption efficiency, nitrogen fertilizer production efficiency increased first and then decreased. It reached the highest at 300 kg·hm^{-2}. It can be seen that when the wide space sowing rate is 300 kg·hm^{-2}, it is beneficial to improve accumulation of nitrogen in each growth period, to increase the contribution rate of nitrogen operation before flowering with grain, and to improve nitrogen absorption efficiency.

Conclusion

Our study showed that the different sowing methods: wide space sowing (WSS), furrow sowing (FS) and drill sowing (DS) and four nitrogen level (150 kg·hm^{-1}, 225 kg·hm^{-1}, 300 kg·hm^{-1} and 375 kg·hm^{-1}) have different effects on the yield and quality. Wide space sowing (WSS) in Wenxi area was beneficial to the growth and development of winter wheat, improving nitrogen uptake at different growing stage, ultimately increasing yield and improving quality. Although increasing nitrogen accumulation in each growing period and improving flowering. Plant height decreased the tiller amount of winter wheat at anthesis. In summary, the wide space sowing and nitrogen level of 300 kg·hm^{-1} was beneficial to increase water consumption during the growth period, increase the tiller dynamics, promote nutrient operation, increase yield

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and grain protein content, it was beneficial to improve accumulation of nitrogen in each growth period, increase the contribution rate of nitrogen operation before flowering with grain, improve nitrogen absorption efficiency and nitrogen fertilizer production efficiency was beneficial to plant nutrient operation.

Acknowledgements. "Modern Agriculture Industry Technology System Construction" (No. CARS-3-124); The National Key Research and Development Program of China (No. 2018YFD020040105); The Sanjin Scholar Support Special Funds Projects; National Natural Science Foundation of China (No. 31771727); The "1331" Engineering Key Innovation Cultivation Team-Organic Dry Cultivation and Cultivation Physiology Innovation Team (No. SXYBKY201733).

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DOI: http://dx.doi.org/10.15666/aeer/1806_82858308

BIODIVERSITY AND BIOACTIVITY OF STROMATIC XYLARIALES COLLECTED FROM MU KO CHANG NATIONAL PARK, THAILAND

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(Received 13th Aug 2020; accepted 19th Nov 2020)

Abstract. Ko Chang Island is a tropical rain forest island of Mu Ko Chang national park, Thailand, which has a wide variety of macro-fungi. The objective of this research was to study the biodiversity of Xylariales and to investigate the bioactive activity of ethyl acetate crude extracts. The fungi were collected from the forest area of the waterfalls all over the island. The crude extracts of selected fungi were tested for antibacterial activity by agar well diffusion method and the cancer cell viability was assessed using the MTT assay. Three genera of Xylariaceae and Hypoxylaceae were found including *Xylaria*, *Daldinia* and *Hypoxylon*. The crude extracts inhibited the growth of Gram positive bacteria more than that of Gram negative bacteria and showed broad spectrum inhibition to all cancer cell lines. The crude extract of *Xylaria* sp.3 showed the highest potential of antibacterial activity and selectively inhibited gastric carcinoma (Kato III) and T cell leukemia (Jurket) with the percentage survival of 26.12 ± 0.90% and 35.37 ± 0.40% respectively. The molecular identification of *Xylaria* sp.3 was identified as *Xylaria* sp.TP5BS101. These results show that these macromycetes are an excellent repertoire of antibacterial activity and exhibit anticancer activity against Kato III and Jurket by inducing DNA fragmentation.
Keywords: *Xylariaceous fungi, diversity, antibacterial activity, anticancer activity*

Introduction

The Xylariaceae and Hypoxylaceae include some of the most diverse and interesting fungi in the Xylariales order. *Xylaria* is the largest genus in the family Xylariaceae, covering about forty genera. These are mainly wood-decaying fungi, but some species are classified as plant pathogenic fungi (Whalley, 1996; Edwards et al., 2003). This family is widely distributed in tropical and temperate climate regions, and can be found on many substrates such as dead wood, leaf litter, dung and soil (Ramesh et al., 2012; Velmurugan et al., 2013). The teleomorph stages of the Xylariaceae and Hypoxylaceae are often formed on stromata such as *Xylaria*, *Daldinia*, *Rosellinia* and *Hypoxylon* (Orachaipunlap et al., 2016). Moreover, they are also known as fungal endophytes that live inside healthy plant tissue for at least part of their life cycle without causing any disease symptoms in the host plants (Petrini and Petrini, 1985). The endophytic Xylariaceae are especially common endophytes in many tropical plants including Dipterocarpus plants, Mangrove plants, palms, ferns and orchids (Rodrigues, 1994; Fróhlicha et al., 2000; Sutjaritvorakul et al., 2011). They can produce many kinds of bioactive secondary metabolites with great potential for biotechnological applications such as antimicrobial, anticancer, antioxidant, antiviral, nematicidal and anti-inflammatory activity (Osmanova et al., 2010;

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DOI: http://dx.doi.org/10.15666/aeer/1806_83098319

Orachaipunlap et al., 2016; Helaly et al., 2018). Fungal endophytes have been discovered to be latent pathogens or decomposers, waiting for host plant weakness or death to colonize and decompose plant tissue. Therefore, the biodiversity of wood-decaying Xylariaceae should be spatially coupled with the distributions of those woody plants (Edwards et al., 2003; Thomas et al., 2016).

Ko Chang Island is a major island of Mu Ko Chang national park, Trat province, eastern Thailand (12º6'13" N & 102º21'7" E). Unlike other provinces in the East, Trat province is located in the eastern tip of the east of Thailand and has a tropical monsoon climate. Ko Chang Island is characterized by an average temperature of 27 °C. In the rainy season the maximum rainfall averages 4700 mm in May – October. This island is far from the river streams of the mainland, and the sea water around the island. In addition, Ko Chang Island has many waterfalls with a wide variety of woody plants because this island is one of only a few islands where a tropical rainforest is distributed over 70% of the inland area (Pumijumnong and Payomrat, 2013). However, there has been no report on the biodiversity of Xylariales in Ko Chang Island. This report is therefore the first report on biodiversity and their bioactivity of fungi in this family. Thus, the ultimate goal of this research was to investigate the biodiversity of saprophytic Xylariales collected from the forest area of waterfalls around Ko Chang Island and to investigate the bioactive activity of ethyl acetate crud extractsagainst both Gram negative and Gram positive human pathogenic bacteria and human cancer cell lines.

Materials and methods

Sampling site

Stromata of Xylariaceae and Hypoxylaceae were collected from the forest area of the waterfalls all over the island including Klong Plu waterfall (west coast), Klong Jao Leuam waterfall (northwest coast), Than Mayom waterfall (east coast) and Kheeri Petch waterfall (southeast coast) (*Figure 1*). This research was performed in June 2018.

Isolation and identification

Fresh stromata of Xylariaceae and Hypoxylaceae were identified and authenticated on the basis of macro- and microscopic characteristics according to Ju and Rogers (1996) and Koyani et al. (2016). The pure cultures were isolated from ascospores under light microscopy by single-spore isolation technique (Velmurugan et al., 2013) and were cultivated on potato dextrose agar (PDA) to obtain pure cultures. Molecular identification of the selected fungi was undertaken following inoculation of pure culture disks into 100 ml of potato dextrose broth (PDB) and incubated at 25°C for 7 days. The mycelial biomass was harvested and extracted by using the CTAB method (cetyltrimethyl ammonium bromide) to obtained fungal DNA following the standard protocols used in Elias et al. (2018). The polymerase chain reaction (PCR) used the primers which are ITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and PCR amplifications were performed by following the condition of Sutjaritvorakul et al. (2016). The homology studies of the fungal DNA sequences obtained were compared with the sequences of known species in the NCBI's GenBank database.

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DOI: http://dx.doi.org/10.15666/aeer/1806_83098319

Diversity analysis

The value of diversity analysis was expressed in Simpson's diversity index (D). It was determined according to the following equation (Simpson, 1949).

$$D = 1 - \left(\frac{\sum(ni - 1)}{N(N - 1)} \right) \quad \text{(Eq.1)}$$

ni = Total number of organisms of a particular species
N = Total number of organisms of all species
D = Simpson's diversity index

Fungal cultivation for biological assay

Disks of cultured fungi were cut from the edge of an actively growing colony on PDA with a flamed cork borer (6 mm) and transferred into 500 Erlenmeyer flasks containing of malt extract broth (MEB) (200 ml). All cultures were incubated for one month at room temperature (25°C) under static conditions. After the incubation period the mycelium was separated by filtration and the cultured filtrate was extracted with 300 ml of ethyl acetate. Each extracted solution was evaporated in a rotary vacuum evaporator at 40°C (BUCHI, V-800). The ethyl acetate crude extracts were dissolved in dimethylsulphoxide (DMSO), filtered through a syringe filter 0.22 µm and stored at 4°C as stock solution for bioassays (Orachaipunlap et al., 2016).

Screening for antibacterial activity

In this research, human pathogenic bacteria, including two Gram-positive bacteria, *Staphylococcus aureus* ATCC 25623, *Bacillus cereus* ATCC 6633 and two Gram-negative bacteria, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 8739 were tested for antibacterial activity by agar well diffusion method. The tested bacteria were normalized to the solution turbidity of 0.5 McFarland standard solutions and were spread on mueller-hinton agar (MHA). The wells of 4 mm diameter were punch with sterile capillary glass and 100 µl of each sample was added to the agar well (Sutjaritvorakul and Chutipaijit, 2018). The plates were incubated at 37 °C for 24 h. The magnitude of antimicrobial activity was assessed by the diameter of halo inhibition zone relative to those of the positive control (Streptomycin).

Evaluation of anticancer activity

The effect of ethyl acetate crude extract on cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide assay (MTT) against 4 human cancer cell lines including gastric carcinoma (Kato III) (ATCC no HTB-103), liver hepatoblastoma (HepG2) (ATCC no HB-8065), colorectal adenocarcinoma (SW620) (ATCC no CCL-227) and acute T cell leukemia (Jurket) (ATCC no CRL-2063). These cell lines were obtained from Department of Microbiology, Faculty of Science Chulalongkorn University, Thailand. Cell suspensions in RPMI 1640 medium were pipetted into flat-bottomed 96 well plats at density of 1×10^5 cells/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. As much as 50 µl of the ethyl acetate crude extract was added to wells and incubated under the same conditions. MTT solution was added to each well and incubated under the same conditions for 4 h. The formazan salt was

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dissolved by 150 µl of DMSO and the absorbance at 540 nm was measured using a microplate reader. The percentage of cell viability was calculated using following formula (Rezk et al., 2015).

$$Percentage\ of\ cell\ viability = \left(\frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ control\ cells} \right) \times 100 \qquad \text{(Eq.2)}$$

Investigation of DNA fragmentation

The extracts of selected fungi were tested for inducible apoptotic cell death detected by staining of DNA with Hoechst 33342 fluorescence dye. The cell lines suspension at 1×10^6 cells/ml of cancer cell were plated on the coverslip in Petri dishes and incubated at 37°C, with 5% CO_2 and at 95% relative humidity for 24 h. After that, the cancer cells were treated with 50 µl of ethyl acetate crude extract. DMSO and etoposide (10 µM) were used as negative and positive control respectively. The medium was removed and washed with phosphate buffered saline (PBS) at pH 7.4. The nucleus of cancer cells were stained with Hoechst 33258 fluorescence dye (1 µg/ml in PBS) and covered with a glass slide. Finally, cell apoptosis was observed under fluorescence microscopy (Olympus, IX71) (Pharamat et al., 2013).

Statistical analysis

The data represent mean of three replicates. Results were subjected to Completely Randomized Design (CRD) and analyaed by one-way analysis of variance (ANOVA). The mean comparisons were performed by Dancan's Multiple Range Test (DMRT) at a a significant level of $P \leq 0.05$. Statistical analyses were conducted using SPSS (SPSS for Windows version 15, SPSS Inc., Chicago, USA).

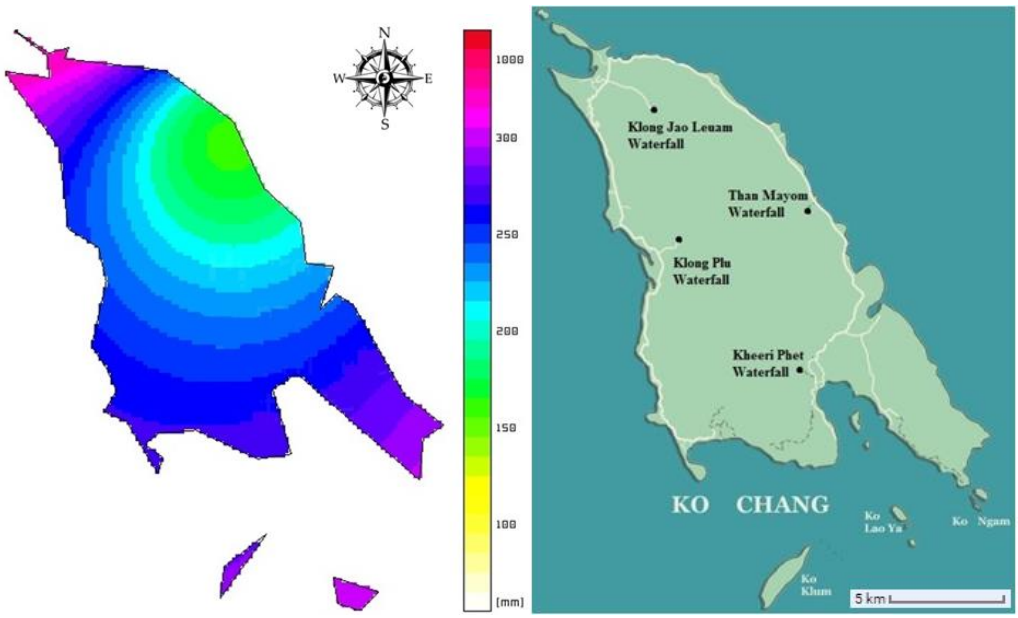

Figure 1. *The average rainfall of Ko Chang Island, Mu Ko Chang National Park, Trat Province in June 2018, Study site (●)*

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DOI: http://dx.doi.org/10.15666/aeer/1806_83098319

Results

The average rainfall of surveyed period (261.5 mm.) and study sites are shown in *Figure 1* (Data obtained from Hydro and Agro Informatics Institute, Ministry of Science and Technology, Thailand). Three genera and eleven species of Xylariaceae were found in the vicinity of four waterfalls around Ko Chang Island, Trat province (*Table 1). Figure 2* exhibits different morphotypes of xylariaceous fungi collected from Ko Chang Island. *Xylaria* sp.5 can be found at every waterfall site while *Xylaria* sp.1, *Xylaria* sp.4 and *Xylaria* sp.8 can be found only by Klong Plu waterfall. They were mostly found on dead wood, except *Xylaria* sp.3 and *Xylaria* sp.4 which were found on leaves and soil respectively. Moreover, *Daldinia concentrica* can be found near Klong Plu, Klong Jao Leuam and Than Mayom waterfalls. Two species belonging to the genus *Hypoxylon*, could not be identified at the species level by morphological characters. The diversity index of Xylariales was presented in *Table 1*. The Simpson's diversity index (D) ranges between 0 and 1 (Eq.1). With this index, 1 represents infinite diversity and 0 represents no diversity. The Klong Jao Leuam waterfall showed the highest Simpson's diversity index (0.80), while Than Mayom showed the lowest diversity index (0.55) compared with the other waterfall.

Table 1. *The Xylariales collected from Ko Chang Island, Mu Ko Chang National Park*

Fungal strain	Observed number			
	Klong Plu	**Kheeri Phet**	**Klong Jao Leuam**	**Than Mayom**
Xylaria sp.1	5	-	-	-
Xylaria sp.2	24	16	26	-
Xylaria sp.3	-	36	16	-
Xylaria sp.4	2	-	-	-
Xylaria sp.5	19	13	18	22
Xylaria sp.6	-	7	-	-
Xylaria sp.7	-	11	21	-
Xylaria sp.8	32	-	-	-
Daldinia concentrica	7	-	10	2
Hypoxylon sp.1	2	-	-	6
Hypoxylon sp.2	-	-	3	4
Simpson's diversity index	0.76	0.74	0.80	0.55

The ethyl acetate crude extracts of each fungal isolates were tested for antibacterial activity (*Table 2*). The results showed that crude extracts of all fungal isolates exhibited antibacterial activity against all tested human pathogenic bacteria. The crude extracts could inhibit the growth of Gram positive bacteria such as *S. aureus* and *B. cereus* to a greater degree than that of Gram negative bacteria such as *P. aeruginosa* and *E. coli*. *Xylaria* sp.3 could inhibit the growth of *S. aureus* and *B. cereus* with the highest inhibition zone at 20.83 ± 1.04 and 15.50 ± 0.86 mm respectively.

The anticancer activity test was performed on various cancer cell lines by using MTT method. *Table 3* shows the results of anticancer activity of ethyl acetate crude extracts against human cancer cell lines including gastric carcinoma (Kato III), liver hepatoblastoma (HepG2), colorectal adenocarcinoma (SW620) and acute T cell leukemia (Jurket). The ethyl acetate crude extracts of each fungal isolates displayed broad spectrum inhibition to all cancer cell lines. Interestingly, *Xylaria* sp.3 specifically inhibited gastric

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carcinoma (Kato III) with the percentage survival of 26.12 ± 0.90% , and it also inhibited T cell leukemia (Jurket) with the percentage survival of 35.37 ± 0.40%. In order to confirm that the cell death is induced by the ethyl acetate crude extract, apoptosis can be observed on Kato III and Jurket when treated with the crude extract of *Xylaria* sp.3. *Figure 3* showed different morphology of Kato III and Jurket cell lines treated with crude extract. The ethyl acetate crude extract of *Xylaria* sp.3 exhibits potent anticancer activity against both cancer cell lines by inducing DNA fragmentation (arrow). The results indicated that *Xylaria* sp.3 exhibited considerable biological activity. Therefore, it could produce great potential bioactive secondary metabolites. The molecular identification based on 18S rRNA, comparing results of ITS sequences indicated that *Xylaria* sp.3 had 99% homology with *Xylaria* sp.TP5BS101.

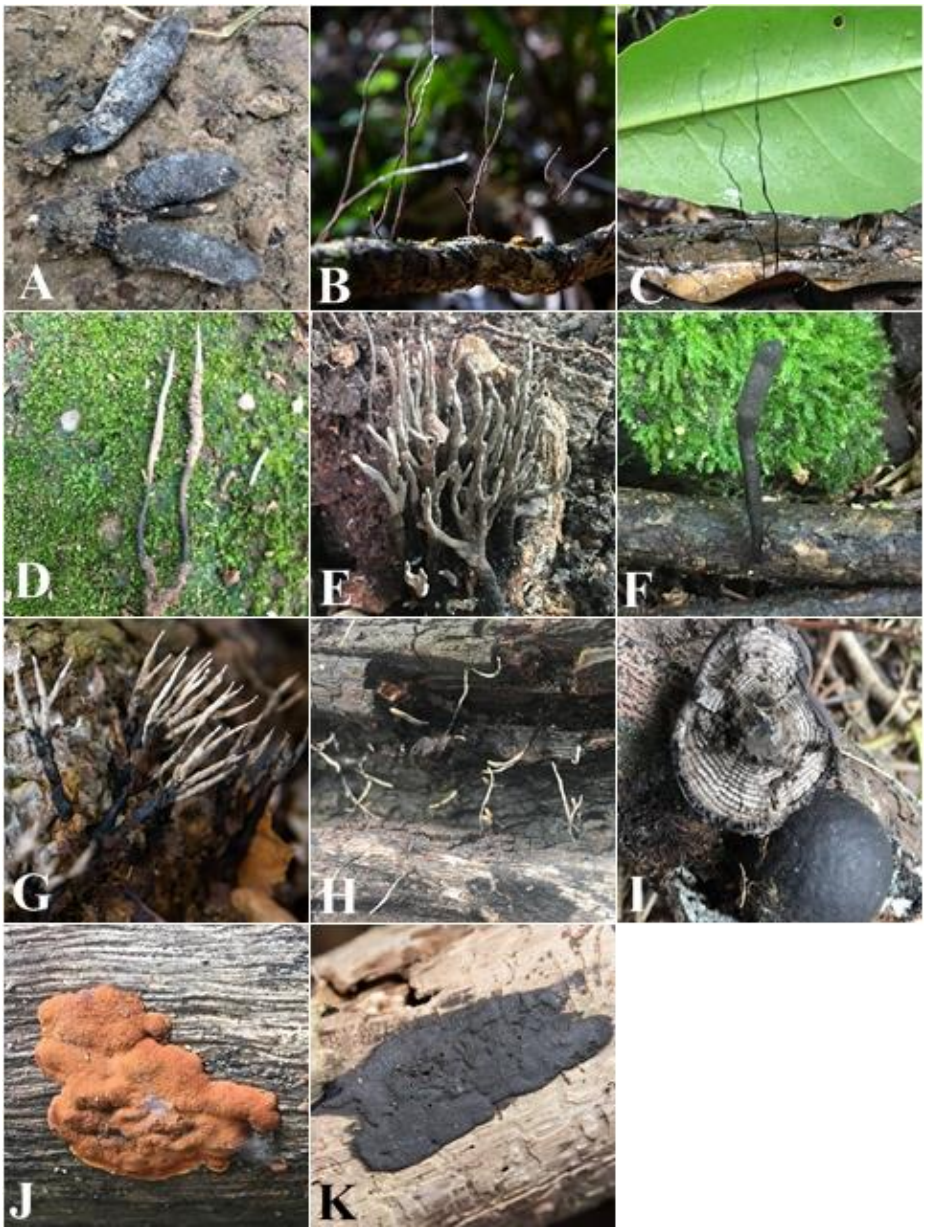

Figure 2. *Stromata of the Xylariales collected from Ko Chang Island ; Xylaria sp.1(A), Xylaria sp.2 (B), Xylaria sp.3 (C), Xylaria sp.4 (D), Xylaria sp.5 (E), Xylaria sp.6 (F), Xylaria sp.7 (G), Xylaria sp.8 (H), Daldinia concentrica (I), Hypoxylon sp.1 (J), Hypoxylon sp.2 (K)*

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DOI: http://dx.doi.org/10.15666/aeer/1806_83098319

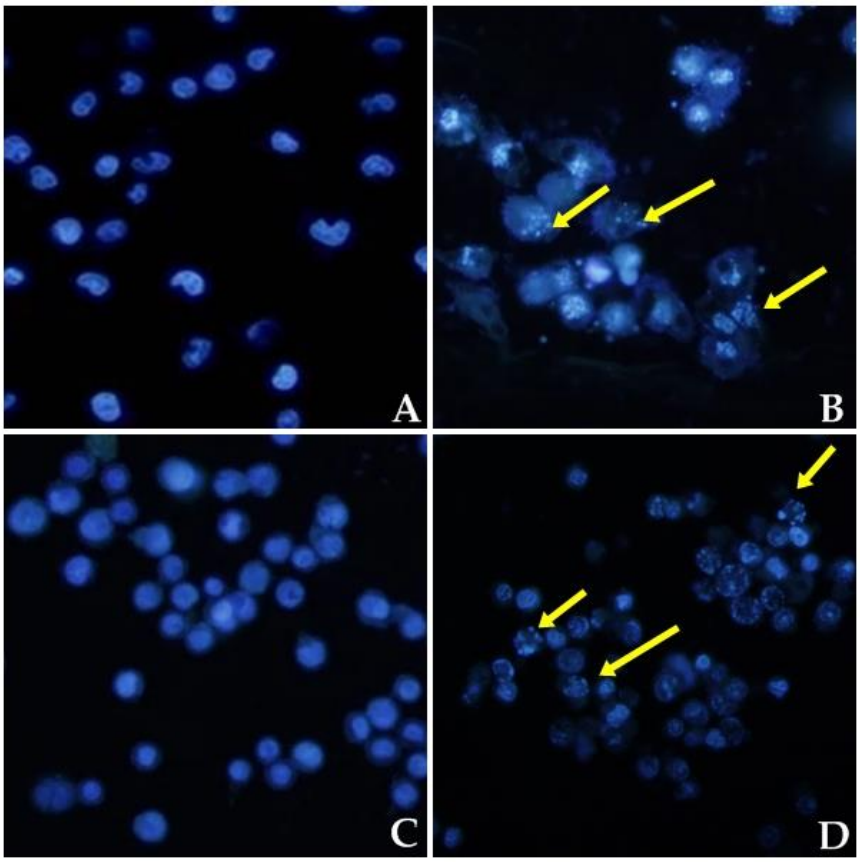

Figure 3. *Xylaria sp.3 ethyl acetate crude extracts induced apoptosis in human cancer cell lines for 24 h.; Kato III (A ,B), Jurkat (C, D). Image A and C show untreated cells. The arrows show apoptosis cells (magnification, ×400)*

Table 2. *Screening for antibacterial activity of ethyl acetate crude extracts against human pathogenic bacteria*

Fungal strain	Inhibition zone diameter (mm)			
	S. aureus	***B. cereus***	***P. aeruginosa***	***E. coli***
Xylaria sp.1	18.50 ± 1.50 [b]	12.66 ± 1.60 [c]	10.50 ± 0.86 [c]	9.16 ± 1.04 [b]
Xylaria sp.2	-	15.16 ± 0.57 [a]	8.50 ± 0.50 [e]	-
Xylaria sp.3	20.83 ± 1.04 [a]	15.50 ± 0.86 [a]	11.83 ± 0.28 [b]	10.66 ± 0.76 [a]
Xylaria sp.4	18.16 ± 3.54 [b]	14.00 ± 0.50 [b]	7.83 ± 1.04 [e]	-
Xylaria sp.5	8.50 ± 0.50 [d]	12.00 ± 0.86 [c]	-	9.33 ± 1.04 [b]
Xylaria sp.6	18.00 ± 1.32 [b]	7.50 ± 0.22 [f]	12.66 ± 0.76 [a]	9.83 ± 1.25 [ab]
Xylaria sp.7	8.83 ± 1.04 [d]	-	9.50 ± 0.50 [d]	7.90 ± 0.36 [c]
Xylaria sp.8	13.16 ± 0.76 [c]	12.66 ± 1.04 [c]	-	-
Daldinia concentrica	8.16 ± 0.28 [d]	9.66 ± 1.52 [d]	-	7.33 ± 0.76 [c]
Hypoxylon sp.1	7.33 ± 0.28 [e]	8.16 ± 1.60 [e]	7.50 ± 0.50 [e]	-
Hypoxylon sp.2	7.50 ± 0.86 [e]	12.50 ± 0.50 [c]	6.33 ± 0.28 [f]	-

Different small letters indicate significant differences, (-) no clear zone

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DOI: http://dx.doi.org/10.15666/aeer/1806_83098319

Table 3. *Anticancer activity of ethyl acetate crude extracts against human cancer cell lines*

Fungal strain	Percentage of cell viability			
	Kato III	**HepG2**	**SW620**	**Jurket**
Xylaria sp.1	36.87 ± 1.65 ^{g}	45.35 ± 1.34 ^{h}	48.85 ± 0.91 ^{e}	38.62 ± 0.38 ^{g}
Xylaria sp.2	41.03 ± 1.09 ^{f}	51.14 ± 0.67 ^{g}	43.26 ± 1.47 ^{g}	46.55 ± 1.10 ^{d}
Xylaria sp.3	26.12 ± 0.90 ^{h}	42.25 ± 0.74 ^{i}	46.24 ± 1.12 ^{f}	35.37 ± 0.40 ^{h}
Xylaria sp.4	42.66 ± 1.12 ^{f}	58.59 ± 1.10 ^{f}	56.32 ± 0.47 ^{c}	44.00 ± 1.51 ^{e}
Xylaria sp.5	52.15 ± 0.83 ^{c}	63.74 ± 0.41 ^{d}	66.23 ± 0.71 ^{a}	45.20 ± 0.72 ^{d}
Xylaria sp.6	56.67 ± 0.61 ^{b}	66.31 ± 0.18 ^{b}	58.71 ± 1.08 ^{b}	46.39 ± 1.31 ^{d}
Xylaria sp.7	48.92 ± 0.02 ^{d}	66.17 ± 1.41 ^{b}	42.55 ± 1.06 ^{h}	49.23 ± 0.79 ^{c}
Xylaria sp.8	51.36 ± 0.69 ^{c}	62.89 ± 0.88 ^{de}	47.82 ± 1.00 ^{ef}	43.45 ± 0.94 ^{e}
Daldinia concentrica	44.42 ± 1.51 ^{e}	65.23 ± 1.82 ^{c}	55.02 ± 0.93 ^{d}	62.26 ± 0.89 ^{a}
Hypoxylon sp.1	58.17 ± 1.27 ^{a}	74.18 ± 0.42 ^{a}	67.35 ± 1.29 ^{a}	54.75 ± 1.06 ^{b}
Hypoxylon sp.2	55.36 ± 2.79 ^{b}	64.90 ± 1.02 ^{cd}	43.33 ± 0.32 ^{g}	42.29 ± 1.89 ^{f}

Different small letters indicate significant differences

Discussion

Than Mayom exhibited the lowest diversity index. It could be suggested that the difference in rainfall could affect the number and diversity of Xylariaceous fungi. The average rainfall of Klong Plu, Klong Jao Leuam and Kheeri Petch waterfall during sampling period was higher than Than Mayom waterfall (*Figure 1*). Priyamvada et al. (2017) suggested that the environmental factors such as temperature, humidity and rainfall have directly effect on macro fungi diversity and the ascomycetous fungi have a tendency to appear after a rainfall. However, the appearance rate of the Ascomycota after rainfall is relatively lower when compared to the Basidiomycota.

Ethyl acetate was used for bioactive metabolites extraction because it is easy for evaporation and low toxic (Minarni et al., 2017). The ethyl acetate crude extracts inhibited the growth of Gram positive bacteria greater than that of Gram negative bacteria. These results were in concordance with the results of Canli et al. (2016) who found that the crude extracts of *Xylaria hypoxylon* has antimicrobial activity against several Gram positive and Gram negative microorganisms. *Xylaria hypoxylon* samples showed the highest inhibition zone of 16 mm against *S. aureus* ATCC 25923. Nikaido (1998) reported that Gram positive bacteria are more susceptible to a larger number of chemotherapeutic agents than Gram negative bacteria. These results may be due to the fact that the cell wall of Gram positive bacteria have a single layer, whereas Gram negative bacteria have multi-layered structure and quite complex (Yao and Moellering, 1995). The crude extract of *Xylaria* sp.3 showed the highest potential of antibacterial activity and selectively inhibited gastric carcinoma (Kato III) and T cell leukemia (Jurket). It could be suggested that *Xylaria* sp.3 crude extract might represent a promising agent for anticancer activity such as isopimarane, diterpene, glycosides, flavonoids and phenolic compounds. These bioactive compounds were reported to be high potential anticancer agents produced by Xylariaceae (Ramesh et al., 2015; Gouda et al., 2016; Abotaleb et al., 2019). Orachaipunlap et al. (2015) reported that ten selected crude extracts were examined for cytotoxicity against 5 human cancer cell lines [Human colorectal adenocarcinoma (SW620), Human acute T cell leukemia (Jurkat), breast cancer

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(BT474), Human gastric carcinoma (Kato-III), and Human liver hepatoblastoma (HepG2)] and *Xylaria* TR25 possessed the highest anticancer activity against Kato-III. Furthermore, *Xylaria curta* collected from tropical evergreen forest in India exhibited significant cytotoxic activity of 58.50% against human lung cancer cell lines (A-549) (Ramesh et al., 2015). Moreover, truncatones A and C produced by *Annulohypoxylon viridistratum* (Hypoxylaceae) exhibited high potential against human breast AC (MCF-7) (Becker et al., 2020). These results show that different cancer cell lines might exhibit different sensitivities when treated with different extracts. Furthermore, the level of inhibition has been found to be dependent on the fungal strains used and the solvent used for the extraction (Synytsya et al., 2017).

DNA fragmentation as an indication of apoptosis is a commonly used assay in drug-cell interaction studies. Cellular physiological and morphological change associated with apoptosis process steps include cell density decreasing, condensation of chromatin and fragmentation of nucleus (Elmore, 2007; Minarni et al., 2017). Bioactive compounds that inhibit the growth of cancer cells by inducing apoptosis may show a useful mechanistic approach to cancer chemotherapy, chemoprevention and preventing unfavorable side effects and resistance (Shafi et al., 2009). It could be developed for cancer treatment in the future. *Xylaria* sp.TP5BS101 has been reported by Osono et al. (2011), during studies on the decomposition of wood, petiole and leaf litter by *Xylaria* sp.TP5BS101. However, there was no report on the antibacterial and anticancer activity of this xylariaceous fungus.

Conclusion

In conclusion, eleven strains in three genera of stromatic Xylariales were isolated from the forest area of the waterfalls in Ko Chang Island. Three genera of Xylariaceae and Hypoxylaceae were found including *Xylaria*, *Daldinia* and *Hypoxylon*. They are a promising repertoire of bioactive metabolites. *Xylaria* sp.3 showed the highest potential of antibacterial activity and anticancer activity, and the crude extract exhibited specifically exhibits anticancer activity against Kato III and Jurket cancer cell lines by inducing DNA fragmentation. However, further studies are necessary to investigate the chemical structure of the ethyl acetate crude extracts.

Acknowledgements. The authors gratefully acknowledge climatic data support from the Hydro and Agro Informatics Institute, Department of Microbiology, Faculty of Science, Chulalongkorn University to provide the cancer cell lines for anticancer activity assay. This research receipted financial supported by Government Budget Grant (2560A17402004), Pathumwan Institute of Technology (001/2560) and King Mongkut's Institute of Technology Ladkrabang (Grant Number A118-0361-055 and KREF046105).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83098319

SPECIES COMPOSITION AND SEASONAL VARIATION OF PERACARIDS (CRUSTACEA: PERACARIDA) OF THE ISTANBUL STRAIT (TURKEY)

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(Received 18th Aug 2020; accepted 22nd Oct 2020)

Abstract. The Istanbul Strait connects the Black Sea with the Sea of Marmara and it has a two-layered water system. In this study, we determined the diversity of the Peracarid Crustacean fauna and the ecological characteristics of the area. The data produced in this study revealed 76 peracarid species in the Istanbul Strait, of which five species (*Animoceradocus semiserratus* (Spence Bate.1862), *Echinogammarus stocki* G. Karaman, 1973, *Leptocheirus bispinosus* Norman, 1908, *Cymodoce spinosa* (Risso, 1816), *Gnathia dentata* (G. O. Sars, 1872)) were reported for the first time in the Sea of Marmara. The physicochemical properties of the seawater as well as total organic and inorganic carbon, and mud percentages of the sediment were analyzed at the sampling stations. In these stations, no significant relationship was determined between temperature, salinity, dissolved oxygen parameters and species or individual numbers. However, a positive relationship was found between the mud percentage and species and individual numbers. In contrast, at the hard bottom stations the number of individuals was positively associated with temperature and negatively with dissolved oxygen.
Keywords: *Crustacea, species diversity, hard and soft bottom habitats, ecology, Sea of Marmara*

Introduction

The Istanbul Strait (also known as the Bosphorus) connects the Black Sea with the Sea of Marmara. This strait and the Dardanelles are located at opposite ends in the Sea of Marmara. These straits create a series of passages connecting the Aegean and Mediterranean (via the Dardanelles) to the Sea of Marmara, and ultimately the Black Sea via the Istanbul Strait (Beşiktepe et al., 1995). The Istanbul Strait, like the Sea of Marmara has a two-layered water system. The low-salinity waters of the Black Sea (17.86 PSU) are transported to the Mediterranean Sea through the straits, while the salty Mediterranean waters (37.3 PSU) move as underflow toward the Black Sea (Ünlüata et al., 1990; Orhon, 1995).

The Istanbul Strait has long coastal sides, which are both residential and social facilities are highly concentrated (Usluer and Alkan, 2016). Moreover, the Istanbul Strait is the narrowest strait used for international navigation in the world and the maritime traffic is dense (Birpınar et al., 2009). This means that pollution by sea is visible in the strait. In addition, as a large metropolis, Istanbul is a focal point of terrestrial pollution (Orhon, 1995). Therefore, monitoring of marine fauna and flora of this region is of critical importance.

The superorder Peracarida is a major component of marine benthic ecosystems and has a regionally high population density (Thiel and Hinojosa, 2009). They also dominate other groups of organisms in terms of individual number and species diversity (Guerra-García et al., 2009). Some species are highly sensitive indicators due to their predominance and sensitivity to pollutants and are frequently used as bioindicators in biological monitoring studies (Chintiroglou et al., 2004; Dauvin and Ruellet, 2007).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83218342

Therefore, the monitoring of peracarid fauna is useful for understanding the effects of ecological changes on benthic fauna (Moreira et al., 2008).

Studies involving peracarids in the Istanbul Strait begin with Sowinsky (1897). Subsequent studies were conducted by Demir (1952), Caspers (1968), Băcescu (1982), Topaloğlu and Kihara (1993), Balkıs et al. (2002), Uysal et al. (2002), Kalkan et al. (2006), Kalkan et al. (2007), Aslan-Cihangir and Panucci-Papadopolou (2011), Öktener, Trilles (2004) and Bakır et al. (2016). Balkıs et al. (2016) conducted an extensive review of studies on Malacostraca crustaceans in the Turkish Strait System in which they listed 274 species of peracarid crustacean.

This study determined peracarid species diversity in the Istanbul Strait and explored its relationship with several environmental variables. Moreover, this study will elucidate the seasonal variation of peracarid biodiversity.

Materials and methods

Sampling

Sampling was performed seasonally between 22 and 23 July 2015, 27 and 28 October 2015, 1-4 February 2016 and 1-9 May 2016 at 34 stations (*Table 1; Fig. 1*). Peracarid samples were collected from the hard bottom with a 20 × 20 cm quadrat using a spatula, and a Van Veen Grab with 0.1 m^2 sampling capacity from the soft bottom. Three replicates were collected from each station. Benthic samples were sieved with 0.5 mm mesh and stored in a 4% formaldehyde solution prepared with seawater. Hard bottom sampling was performed at 13 stations (0.5 m) near the shores of the Istanbul Strait at sites unaltered by shore filling. Appropriate stations for sampling are difficult to find due to the extensive coastal filling of the strait. Soft bottom samples were collected from 21 stations at 11 localities in the Istanbul Strait and samples were taken from two depths (18.2 m and 36.4 m). As mentioned in the introduction, there is a two-layered water system in the Sea of Marmara. The upper water layer is the Black Sea (low salinity) and the lower water layer is the Mediterranean Sea (high salinity) and this two layer don't with each other due to different densities of salinity, but form a salinity intermediate water (halocline) at 25 m depth of the Sea of Marmara (Beşiktepe et al., 1995). Depths (18.2 m and 36.4 m) were chosen to ensure sampling captured faunal differences between the Black Sea and Mediterranean waters. These two depths have different salinity and dissolved oxygen conditions.

The physicochemical parameters

Temperature, dissolved oxygen, and salinity values of the seawater were measured using a YSI brand multiparameter device. Sediment samples were collected with a plastic spoon from the upper layer of sediment at the depths specified above, placed into nylon bags and stored at -20 °C in a deep freezer. Since hard bottom samples were taken from algae and mussel rocks, mud samples were not available for total organic carbon (TOC) and total inorganic carbon (TIC) analyses. Thus, TOC, TIC and mud percentage values were obtained only for soft bottom surface sediment samples. TOC and TIC analyses were performed according to the Walkey-Blake method (Gaudette et al., 1974; Loring and Rantala, 1992). The mud percentage of sediment samples for the stations studied were determined according to the Galehouse (1971) and Mc Manus (1991) methods.

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Table 1. *Stations, coordinates, depth, and biotope properties of the sampling stations in the Istanbul Strait*

Station	Station area	Latitude	Longitude	Depth (m)	Substrate type	Sampling device
1	Garipçe Shore	41°12’799”	29°06’564”	0.5	Hard Bottom	Quadrat
2	Rumeli Kavağı Shore	41°10’670”	29°04’451”	0.5	Hard Bottom	Quadrat
3	Sarıyer Shore	41°09’792”	29°02’935”	0.5	Hard Bottom	Quadrat
4	Tarabya Shore	41°08’202”	29°03’524”	0.5	Hard Bottom	Quadrat
5	Baltalimanı Shore	41°05’820”	29°03’246”	0.5	Hard Bottom	Quadrat
6	Beşiktaş Shore	41°02’499”	29°00’623”	0.5	Hard Bottom	Quadrat
7	Anadolufeneri Shore	41°12’878”	29°09’131”	0.5	Hard Bottom	Quadrat
8	Poyraz Shore	41°12’296”	29°07’903”	0.5	Hard Bottom	Quadrat
9	Anadolukavağı Shore	41°10’3491”	29°05’308”	0.5	Hard Bottom	Quadrat
10	Paşabahçe Shore	41°07’302”	29°05’846”	0.5	Hard Bottom	Quadrat
11	Anadoluhisarı Shore	41°04’788”	29°03’899”	0.5	Hard Bottom	Quadrat
12	Kuleli Shore	41°03’613”	29°03’164”	0.5	Hard Bottom	Quadrat
13	Kuzguncuk Shore	41°02’283”	29°01’873”	0.5	Hard Bottom	Quadrat
14	Garipçe 1	41°12’828”	29°06’771”	18.2	Soft Bottom	Van Veen Grab
15	Garipçe 2	41°12’663”	29°06’907”	36.4	Soft Bottom	Van Veen Grab
16	İstinye 1	41°06’766”	29°03’619”	18.2	Soft Bottom	Van Veen Grab
17	İstinye 2	41°06’670”	29°03’772”	36.4	Soft Bottom	Van Veen Grab
18	Bebek 1	41°04’676”	29°02’941”	18.2	Soft Bottom	Van Veen Grab
19	Bebek 2	41°04’717”	29°03’056”	36.4	Soft Bottom	Van Veen Grab
20	Ortaköy 1	41°02’824”	29°01’557”	18.2	Soft Bottom	Van Veen Grab
21	Ortaköy 2	41°02’795”	29°01’609”	36.4	Soft Bottom	Van Veen Grab
22	Karaköy 1	41°01’428”	28°58’822”	18.2	Soft Bottom	Van Veen Grab
23	Karaköy 2	41°01’420”	28°58’835”	36.4	Soft Bottom	Van Veen Grab
24	Salacak	41°01’020”	29°00’202”	18.2	Soft Bottom	Van Veen Grab
25	Çengelköy 1	41°02’962”	29°03’051”	18.2	Soft Bottom	Van Veen Grab
26	Çengelköy 2	41°03’015”	29°03’027”	36.4	Soft Bottom	Van Veen Grab
27	Anadoluhisarı 1	41°04’760”	29°03’844”	18.2	Soft Bottom	Van Veen Grab
28	Anadoluhisarı 2	41°04’736”	29°03’803”	36.4	Soft Bottom	Van Veen Grab
29	Paşabahçe 1	41°07’255”	29°05’598”	18.2	Soft Bottom	Van Veen Grab
30	Paşabahçe 2	41°07’216”	29°05’322”	36.4	Soft Bottom	Van Veen Grab
31	Anadolukavağı 1	41°10’375”	29°05’273”	18.2	Soft Bottom	Van Veen Grab
32	Anadolukavağı 2	41°10’288”	29°05’222”	36.4	Soft Bottom	Van Veen Grab
33	Keçilik 1	41°11’841”	29°07’102”	18.2	Soft Bottom	Van Veen Grab
34	Keçilik 2	41°11’846”	29°07’037”	36.4	Soft Bottom	Van Veen Grab

Statistical methods

The Soyer (1970) frequency index (Fi) and the dominance index (Di) formula of Bellan-Santini (1969), respectively, were used to determine the frequency and dominance of peracarid species in the study area. The species were classified into three groups: Constant ($Fi \geq 50\%$), Common ($50\% > F_i \geq 25\%$), and Rare ($F_i < 25\%$), according to their frequency indexes. The Bray-Curtis similarity index and multi-dimensional scaling (MDS) methods were used to determine similarities between sampling stations and to resolve regional

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distribution models, respectively. R value was calculated by using ANOSIM (similarity analysis) in order to test the significant differences between the groups formed in each season for both substrates. After bulk analysis, the similarities or differences within each group and the percentage contribution of each species to the similarities and differences among the resulting groups were identified using SIMPER analysis. We used Primer 6 program for these analyses (Clarke and Warwick, 2001). Relationships with abiotic parameters were revealed using the Spearman rank correlation coefficient method with IBM Statistics Version 21 (Siegel, 1956). We calculated the Shannon-Weaver Diversity Index (*H'*) using a composite of number of species and individuals at the sampling stations (Zar, 1984).

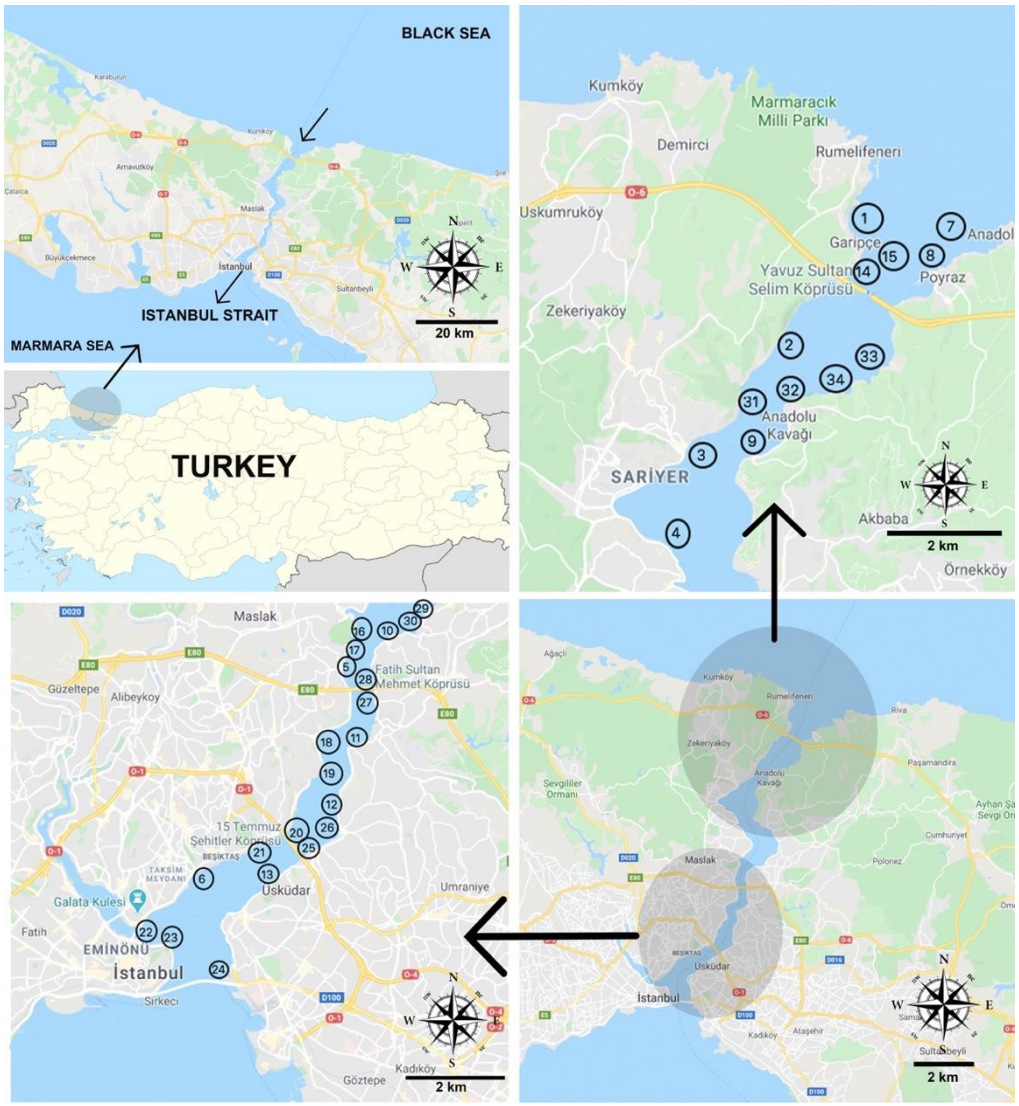

Figure 1. *Map of the sampling stations. Hard bottom station: 1. Garipçe Shore 2. Rumeli Kavağı Shore 3. Sarıyer Shore 4. Tarabya Shore 5. Baltalimanı Shore 6. Beşiktaş Shore 7. Tarabya Shore 8. Poyraz Shore 9. Anadolukavağı Shore 10. Paşabahçe Shore 11. Anadoluhisarı Shore 12. Kuleli Shore 13. Kuzguncuk Shore. Soft bottom station: 14. Garipçe 1 15. Garipçe 2 16. İstinye 1 17. İstinye 2 18. Bebek 1 19. Bebek 2 20. Ortaköy 1 21. Ortaköy 2 22. Karaköy 1 23. Karaköy 2 24. Salacak 25. Çengelköy 1 26. Çengelköy 2 27. Anadoluhisarı 1 28. Anadoluhisarı 2 29. Paşabahçe 1 30. Paşabahçe 2. 31. Anadolukavağı 1 32. Anadolukavağı 2 33. Keçilik 1 34. Keçilik 2*

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DOI: http://dx.doi.org/10.15666/aeer/1806_83218342

Results

This study identified 76 peracarid crustacean species representing five orders (*Table 2*). *Animoceradocus semiserratus* (Spence Bate, 1862), *Echinogammarus stocki* G. Karaman, 1970, *Leptocheirus bispinosus* Norman, 1908, *Cymodoce spinosa* (Risso, 1816), and *Gnathia dentata* (G. O. Sars, 1872) were new records for the Sea of Marmara. Examining the taxonomic distribution, we observed that most of the species obtained across all samples belong to the order Amphipoda (*Fig. 2*). Species belonging to the orders Amphipoda, Isopoda, and Tanaidacea were found in hard bottom samples, but not species belonging to the orders Mysidacea or Cumacea. Amphipod species are dominant on the soft bottom, but peracarid species of Isopoda, Cumacea, Tanaidacea, and Mysidacea were also found, in order of decreasing occurrence.

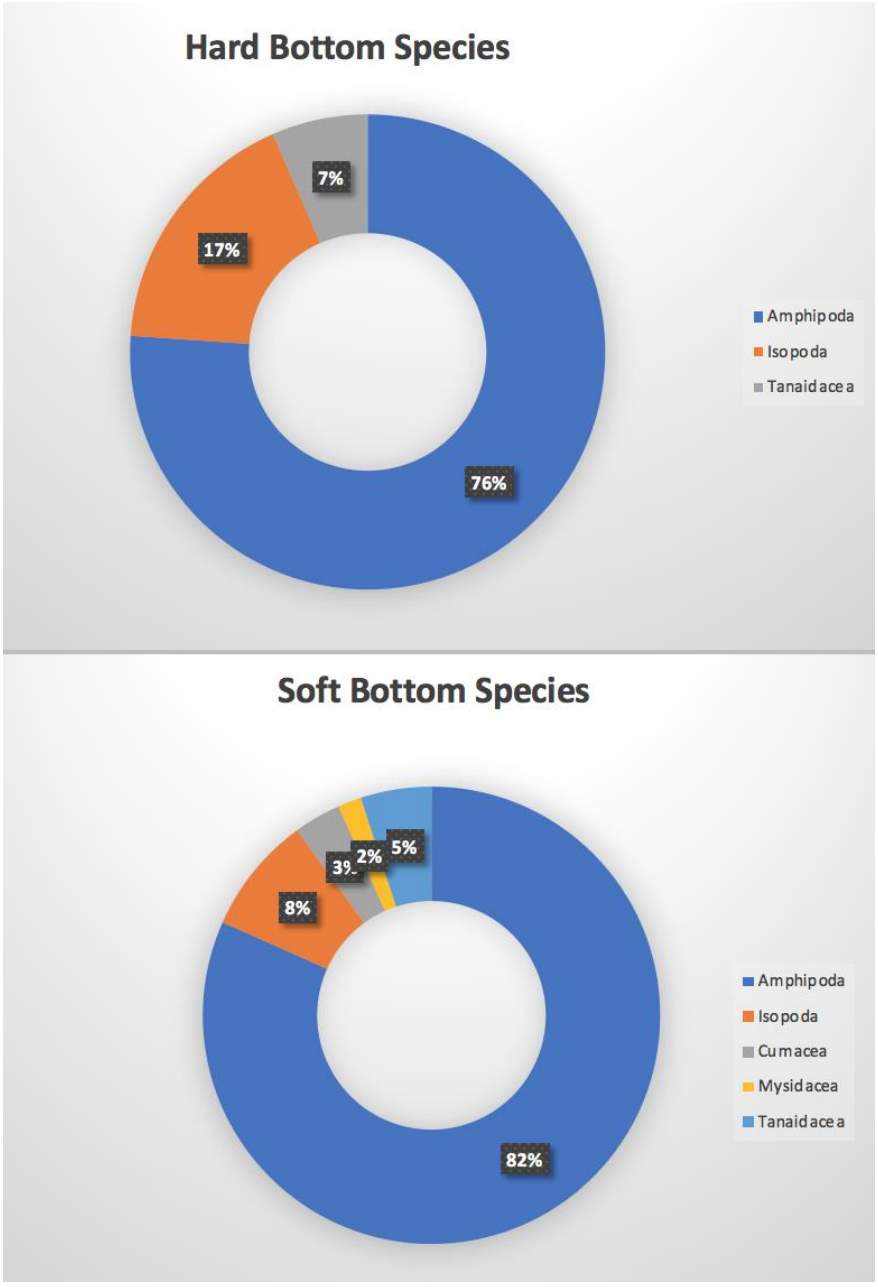

Figure 2. *The distribution of species by orders*

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Table 2. *Peracarid Crustacean species of the Istanbul Strait and average individual numbers. Habitat type: Hs: Hard substratum, Mytilus with algae Ss: Soft substratum*

SPECIES	SUMMER	AUTUMN	WINTER	SPRING
AMPHIPODA				
Ampelisca diadema (Costa. 1853)	Ss:7			Ss:3
Ampelisca multispinosa Bellan-Santini & Kaim-Malka. 1977				Ss:3
Ampelisca pseudosarsi Bellan-Santini & Kaim-Malka. 1977	Ss:3			
Amphilochus brunneus Della Valle. 1893		Ss:33	Ss:23.3	Ss: 43
Ampithoe ramondi Audouin. 1826	Hs:75039.9	Hs: 586.7	Hs:46.7 Ss:6.6	Hs:540
**Animoceradocus semiserratus* (Spence Bate.1862)	Ss:27			
Aora gracilis (Spence Bate. 1857)	Hs:6.7		Hs:6.7	
Apherusa chiereghinii Giordani- Soika. 1949				Hs:200
Apherusa mediterranea Chevreux. 1911				Hs:106.7
Apocorophium acutum (Chevreux. 1908)	Hs:3853.3 Ss:319	Hs:13.3 Ss:2094	Hs:6 Ss:263.2	Hs:286.7 Ss: 339
Apolochus picadurus (J.L. Barnard. 1962)	Ss:10			Ss:3
Caprella acanthifera Leach. 1814	Ss:67	Hs:333.3		Hs:86.7 Ss:7
Caprella danilevskii Czerniavski. 1868		Hs:6.7	Ss:13.3	
Caprella rapax Mayer. 1890		Hs:26.7		
Centraloecetes dellavallei (Stebbing. 1899)	Ss:10			
Chelura terebrans Philippi. 1839				Ss:103
Colomastix pusilla Grube. 1861	Ss:17			Ss:33
Cymadusa crassicornis (Costa. 1853)				H:13.3 Ss:87
Deflexilodes gibbosus (Chevreux. 1888)	Ss:3		Ss:6.7	
Dexamine spiniventris (Costa. 1853)	Ss:3			Hs:566.6
Dexamine spinosa (Montagu. 1813)	Hs:6.6 Ss:3	Hs:60	Ss:3.3	Hs:513.3 Ss:3
Echinogammarus foxi (Schellenberg. 1928)	Hs: 3080	Hs:120	Hs:20	
Echinogammarus olivii (H. Milne Edwards. 1830)	Hs:58513.3 Ss: 7	Hs:946.7	Hs:493.3	Hs:4366.7 Ss:6
Echinogammarus stocki G. Karaman. 1970			Hs:66.7	
Elasmopus brasiliensis (Dana. 1855)	Hs:40			
Elasmopus pocillimanus (Spence Bate. 1862)	Hs:58033.3 Ss:3			
Ericthonius brasiliensis (Dana. 1853)	Ss:13	Hs:13.3	Hs:20	Hs:40
Gammarella fucicola (Leach. 1814)	Ss:16	Ss:97	Ss:10	Ss:97
Gammarus aequicauda (Martynov. 1931)	Hs:173.3		Hs:6.7	Hs:13.3
Gammarus crinicornis Stock. 1966	Ss: 7			
Gitana sarsi Boeck. 1871				Ss:167
Protohyale (Protohyale) schmidtii (Heller, 1866)	Hs:178600 Ss:36	Hs:44206.7 Ss:6	Hs:7246.7 Ss:6.6	Hs:29260 Ss:3
Jassa marmorata Holmes. 1905	Hs:11319.8 Ss:460	Hs:7826.7 Ss:3	Hs:2853.4	Hs:8753.2 Ss:20
Jassa ocia (Spence Bate. 1862)	Hs:333.3 Ss:4363	Ss:873	Ss:113.3	Ss:70
**Leptocheirus bispinosus* Norman. 1908	Ss:123	Ss:146		
Leptocheirus pilosus Zaddach. 1844			Ss:66.7	
Maera grossimana (Montagu. 1808)			Ss:6.6	
Medicorophium rotundirostre (Stephensen. 1915)	Ss:3	Ss:3		
Megamphopus brevidactylus Myers. 1976		Ss:7		
Megamphopus cornutus Norman. 1869	Ss:7			
Melita palmata (Montagu. 1804)	Hs:579.9 Ss:206	Ss:103	Hs:13.4 Ss:753.1	Hs:280 Ss:558

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Microdeutopus algicola Della Valle. 1893		Ss:3	Ss:56.6	Hs:26.7
Microdeutopus anomalus (Rathke. 1843)	Ss:84	Ss:359	Ss:86.7	Hs:6.7 Ss:873
Microdeutopus gryllotalpa Costa. 1853	Hs:940 Ss:10	Ss:20	Ss:39.9	Hs:246.7 Ss:127
Microdeutopus obtusatus Myers. 1973			Ss:3.3	
Microdeutopus versiculatus (Spence Bate. 1857)	Ss:580	Ss:474	Ss:213.3	Hs:6.7 Ss:1823
Monocorophium acherusicum (Costa. 1853)	Ss:7	Hs:20 Ss:3	Hs:13.3 Ss:6.7	
Monocorophium insidiosum (Crawford. 1937)	Ss:1020	Ss:23	Ss:90	Hs:6.7
Nototropis massiliensis (Bellan-Santini. 1975)			Hs:6.7	Hs:6.7
Perioculodes longimanus longimanus (Spence Bate & Westwood. 1868)	Ss:17		Ss: 6.6	Ss:7
Phtisica marina Slabber. 1769	Ss:676	Hs:26.7 Ss:1587	Ss:616.6	Hs:26.7 Ss:1371
Pseudoprotella phasma (Montagu. 1804)	Ss:3			
Stenothoe bosphorana Sowinsky. 1898	Ss:7		Hs:6.7	
Stenothoe cavimana Chevreux. 1908	Hs:13.3	Hs:86.6	Ss:3.3	Hs:20 Ss:3
Stenothoe elachista Krapp-Schickel. 1975		Ss:3.3	Ss:3.3	Hs:80
Stenothoe monoculoides (Montagu. 1815)	Hs:413.4 Ss:1			Hs:2206.7
Stenothoe tergestina (Nebeski. 1881)	Hs:266.7			
ISOPODA				
**Cymodoce spinosa* (Risso. 1816)		Hs:6.7	Hs:20	
Dynamene bidentata (Adams. 1800)	Hs:673.4	Hs:926.7	Hs:20	Hs:60
Dynamene bifida Torelli. 1930			Hs: 6.7	
**Gnathia dentata* (Sars G.O. 1872)	Ss:0.7			
Gnathia maxillaris (Montagu. 1804)		Ss:187		Ss:16.7
Gnathia vorax (Lucas. 1849)	Ss:0.6			Ss:3
Idotea balthica (Pallas. 1772)	Hs:81753.3	Hs:966.8	Hs:126.7	Hs:4539.9
Idotea metallica Bosc. 1802	Hs:226.7	Hs:6.7		Hs:1166.7
Jaera (Jaera) italica Kesselyak. 1938	Hs:153.4			
Limnoria lignorum (Rathke. 1799)				Ss:207
Paragnathia formica (Hesse. 1864)	Ss:173			
Sphaeroma serratum (Fabricius. 1787)	Hs:940	Hs:546.6	Hs:26.7	Hs:133.3
Stenosoma capito (Rathke. 1837)	Hs:13.3	Hs:20		
CUMACEA				
Iphinoe trispinosa (Goodsir. 1843)		Ss:3		
Vaunthompsonia cristata Bate. 1858			Ss:3.3	
MYSIDA				
Haplostylus normani (G.O. Sars. 1877)			Ss:3.3	
TANAIDACEA				
Apseudopsis latreillii (Milne Edwards. 1828)	Ss:203.8	Ss:139 Hs: 20	Ss:953.3	Ss:67.9 Hs: 13.3
Chondrochelia savignyi (Kroyer. 1842)	Hs: 306.7	Ss:3	Ss:3.3	Hs: 6.7
Tanais dulongii (Audouin. 1826)	Hs:33119.8 Ss:3	Hs:2220	Hs: 1466.6	Hs: 2773.4

*New records for Sea of Marmara

Hard bottom

A total of 46 species were obtained from hard bottom samples. Analyzing the seasonal distribution of the species, we found that the greatest number of species are obtained in spring samples, followed by summer, autumn and final winter (*Fig. 3*). The number of

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individuals was positively associated with temperature (r: 0.384 P < 0.001). *Protohyale (Protohyale) schmidtii* (Heller, 1866) is the species recording the highest number of individuals throughout the year, with an individual count of up to 178.600 m^2 in the summer. Moreover, this species was a dominant and constant species across all seasons in the Strait, according to the dominance and frequency indices (*Fig. 4*).

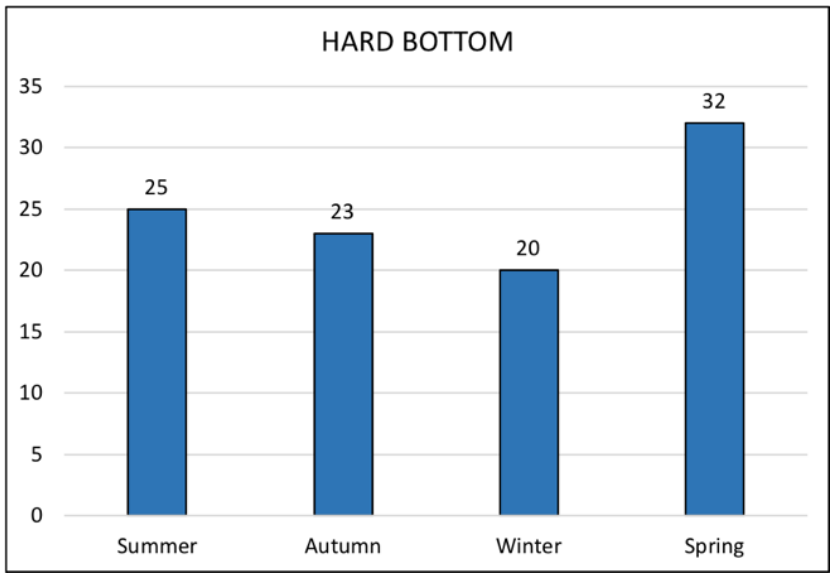

Figure 3. *Seasonal changes of species numbers on the hard bottom*

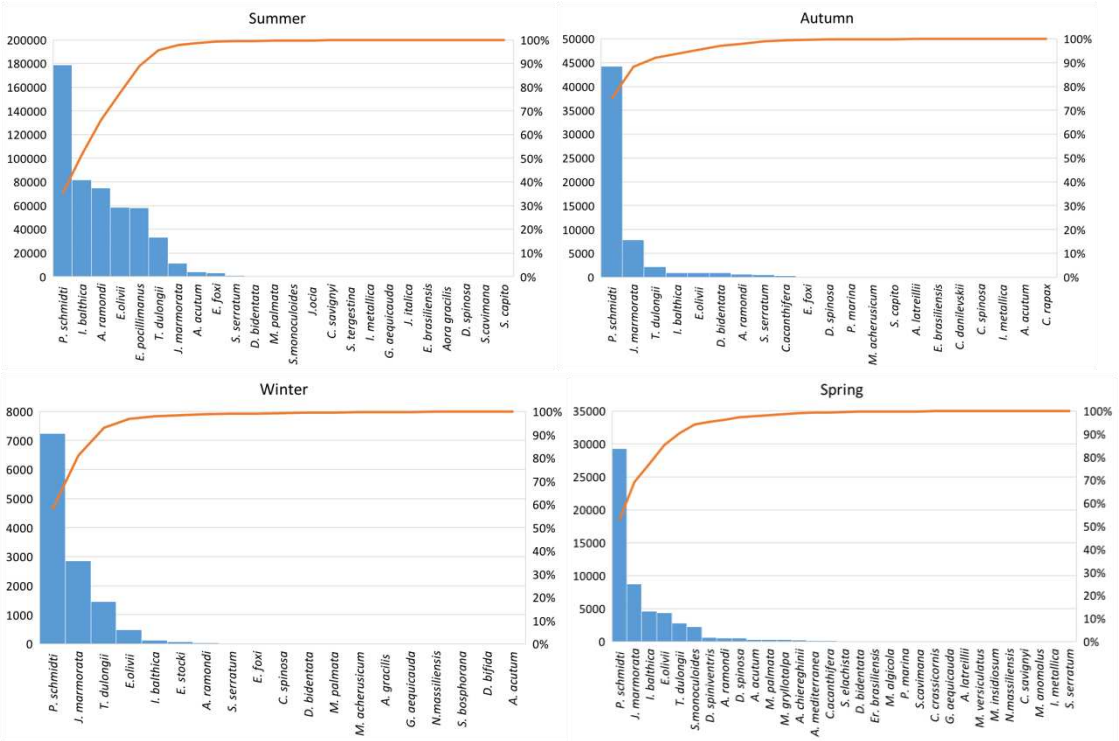

Figure 4. *Seasonal variation of the Peracarid species and individual numbers at the hard bottom stations*

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According to the Frequency and Dominancy Index values, most of 24 species are rare in the summer. Of the remaining species, 4 (*E. pocillimanus*, *M. gryllotalpa*, *D. bidentata,* and *T. dulongii*) are common and another 4 (*E. olivii*, *P. schmidtii*, *J. marmorata* and *I. balthica*) are constant. Of the 22 species obtained in autumn, 15 are rare, 3 (*A. ramondi*, *E. olivii*, *S. serratum*) are common, and 4 (*P. schmidtii, J. marmorata, I. balthica, T. dulongii*) are constant species. In winter, 19 species were obtained, of which 15 were found to be rare species, while 2, *E. olivii* and *I. balthica*, were common, and another 2, *P. schmidtii* and *T. dulongii,* were constant. In the spring, the last season of the study, 24 of 31 species were rare, 2 (*A. acutum*, *A. ramondi*) were common, and 5 (*T. dulongii*, *I. balthica*, *P. schmidtii*, *E. olivii*, *J. marmorata*) were constant species.

H' index values are determined as 0 (Autumn station 2 and winter stations 8 and 9) in stations where peracarid species were not found, while the highest *H'* value was 2.42 (Spring station 7) (*Table 3*).

Table 3. *Shannon-Weaver diversity index (H') values for hard and soft bottom stations*

HARD BOTTOM				
Station	**Summer**	**Autumn**	**Winter**	**Spring**
1	1.44	0.67	2.1	0.68
2	0.002	0	0.68	0.42
3	0.72	0.45	0.93	0.11
4	1.73	0.37	1.0	0.70
5	1.04	0.60	0.69	0.75
6	0.69	1.33	0.32	0.73
7	0.28	0.26	0.54	2.42
8	1.45	1.03	1.08	1.51
9	0.52	0.003	0	1.21
10	1.27	0.98	0	0.45
11	1.35	0.79	1.02	1.21
12	1.51	1.16	0.66	0.81
13	1.75	0.8	0.33	0.65
SOFT BOTTOM				
Station	**Summer**	**Autumn**	**Winter**	**Spring**
14	1.35	0	0	0
15	0	0	0	0.69
16	1.07	1.07	1.56	0
17	1.84	1.38	1.06	1.51
18	0	1.1	2.17	0.58
19	1.01	0	0	0.59
20	1.27	2.13	1.31	1.6
21	2.07	0	0	0
22	2.58	2.11	0	2.45
23	2.55	0	0	0
24	2.74	1.80	2.03	0.87
25	0	0.97	2.08	2.18
26	0	1.34	2.39	1.98
27	0	0	0	0.69
28	0	0	1.46	1.25
29	0	0	0	0.87
30	0.66	0	1.54	0.53
31	0	0	1.6	0.84
32	0	0	0	0
33	0	0	0	0
34	0	0	0	0

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As for similarities between stations, the station groups showing the highest similarity in summer were S4, S7, and S12, as well as S5 and S9 (*Fig. 5*). R values calculated using ANOSIM (similarity analysis) in the hard substratum and we found significant values. R values respectively for summer ($R = 0.638$, $P < 0.01$); autumn ($R = 0.797$, $P < 0.01$); winter ($R = 0.917$, $P < 0.01$); spring ($R = 0.813$, $P < 0.01$). The species *P. schmidtii*, *E. olivii* and *I. balthica* contributed greatly to the similarity of the first group, while *P. schmidtii*, *T. dulongii* and *I. balthica* contributed most to that of the of the second. Similarity percentages increased between stations in autumn, when the most similar group consisted of stations A6 and A12, the second most similar was A3, A4, and A5, and the third was A1, A8, and A9. *P. schmidtii*, *J. marmorata* and *T. dulongii* contributed most to this similarity. The highest similarity in winter was between stations W2, W4, W5, W1, and W12. The second most similar group was W3 and W7. Similar to other seasons, the resemblance was heavily influenced by *P. schmidtii*, *T. dulongii*, *J. marmorata* and *I. balthica*. The highest similarity in spring was between stations SP11, SP12, and SP13, followed by a group consisting of stations SP2, SP3, SP4, and SP5. Unlike other seasons, *H. normani*, *J. italica*, *S. tergestina* and *E. foxi* contributed most to the similarity in spring *P. schmidtii* was the species that contributed most to similarities across all seasons, except in spring at hard bottom stations.

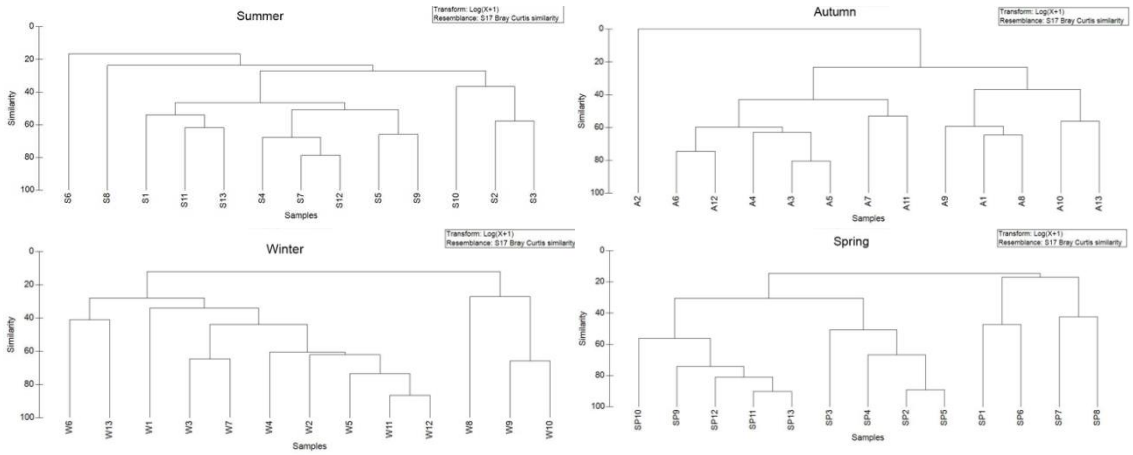

Figure 5. *The similarity of the hard bottom stations. S: Summer A: Autumn W: Winter SP: Spring*

Soft bottom

Sixty peracarid species were obtained in soft bottom sampling. Most species (38) were obtained in the summer, followed by spring (27). The number of species obtained in autumn (23) and winter (24) was nearly equal (*Fig. 6*). In analyzing frequency and dominancy index values for species in soft bottom samples, we see that the only common species is *A. acutum*, and only in spring. In all other seasons, all species were rarely obtained (*Fig. 7*).

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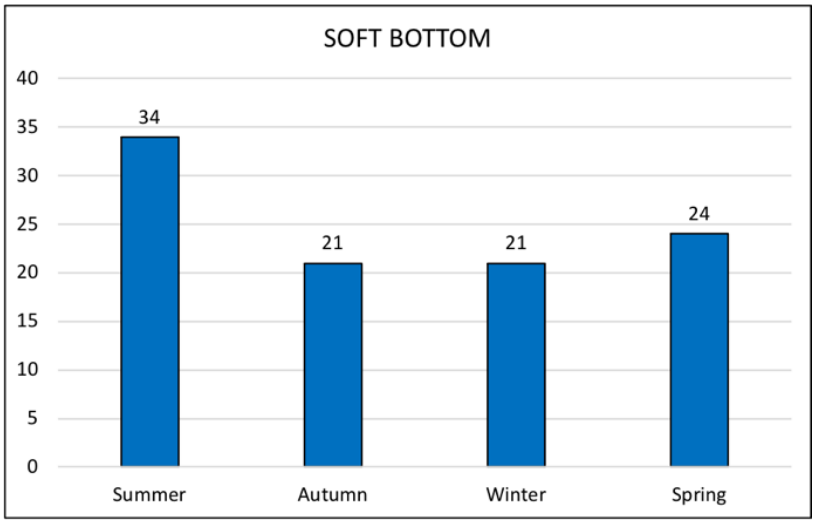

Figure 6. *Seasonal changes of species numbers on the soft bottom*

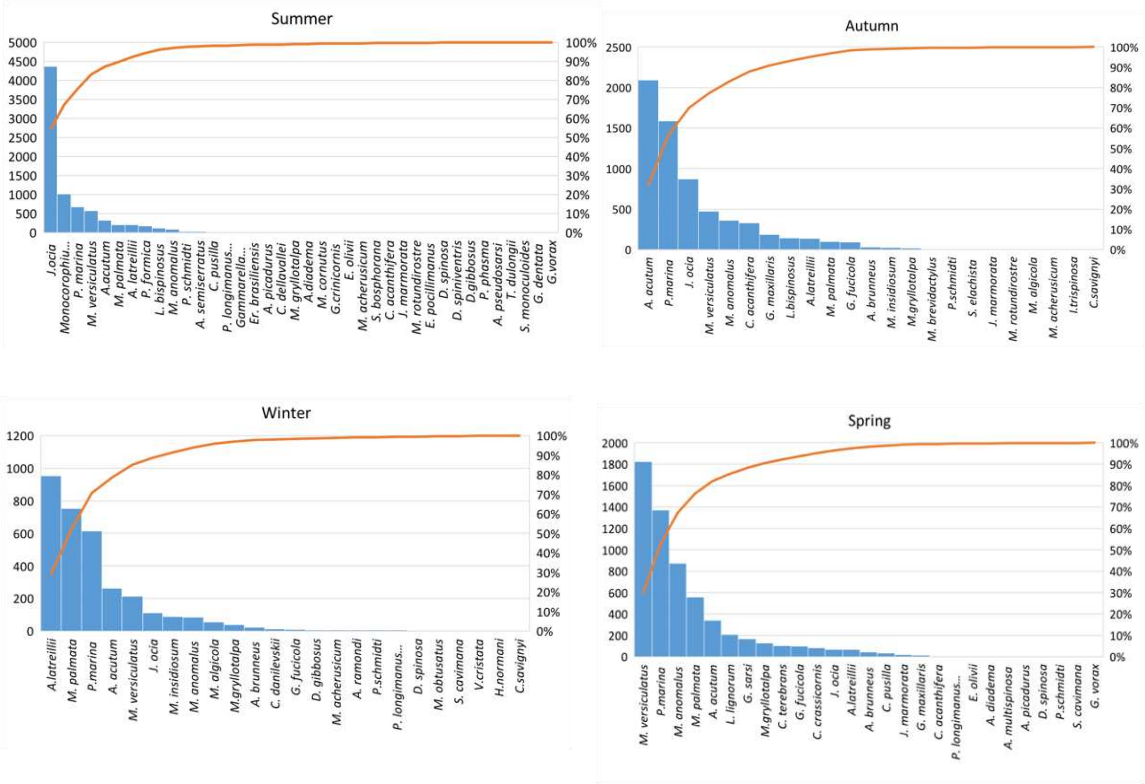

Figure 7. *Seasonal variation of the Peracarid species and individual numbers at the soft bottom stations*

H′ index values range from 0 to 2.7. Peracarids could not be obtained throughout the year at some stations (32, 33 and 34) and the *H′* value of these stations is zero. The highest *H′* values were found at 24th (Salacak) and 23rd (Karaköy) stations (*H′* = 2.7 and *H′* = 2.5) in the summer (*Table 3*).

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DOI: http://dx.doi.org/10.15666/aeer/1806_83218342

Similarity values between soft bottom stations were lower than between hard bottom stations (*Fig. 8*). For the soft bottom, there was a significant difference between the groups formed in all seasons (summer ($R = 0.791$, $P < 0.01$); autumn ($R = 0.960$, $P < 0.01$); winter ($R = 0.778$, $P < 0.01$); spring ($R = 0.958$, $P < 0.01$). The highest similarity in summer in stations with soft bottom sampling is between S14 and S16. This resemblance was caused by *P. schmidtii* and *A. acutum*. The stations A20 and A24, as well as a group of A16, A17, and A25 showed similarity in autumn. While *L. bispinosus*, *M. versiculatus* and *G. fucicola* greatly contributed to the similarity of the first group, *M. palmata* and *P. marina* contributed to the similarity of the second group. In winter, stations W16 and W17, and stations W24 and W25 were similar groups. *P. marina*, *M. gryllotalpa* and *M. palmata* contributed to the similarity of the first group and *P. marina*, *A. acutum*, *L. pilosus* and *M. algicola* contributed to the similarity of the second group. In the spring, stations SP14, SP19, 27 and stations SP24 and SP29 were similar. The species that most contributed to the first group were *A. acutum* and *P. marina*, while *M. versiculatus* and *M. palmata* contributed to the second group.

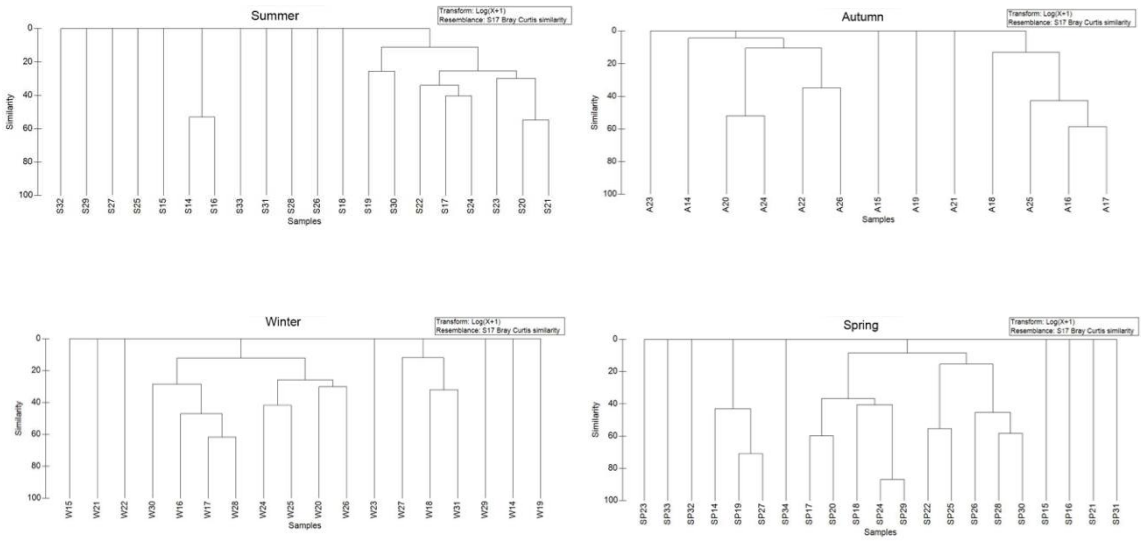

Figure 8. *The similarity of the soft bottom stations. S: Summer A: Autumn W: Winter SP: Spring*

Physicochemical parameters

At hard bottom stations, sea water temperature ranged from 7.17 °C (winter) to 24.6 °C (summer), dissolved oxygen from 7.64 (autumn) to 15.08 mg l^{-1} (winter), dissolved oxygen percentage from 89.5% (autumn) to 156.6% (summer) and salinity content ranged from 14.10 (winter) to 24.43 PSU (practical salinity unit) (winter) (*Fig. 9*).

We conducted Spearman's correlation analysis to elucidate the relationships between parameters. Accordingly, we found a negative relationship (r: - 0.702 $P < 0.001$) between temperature and dissolved oxygen. In contrast, there was a positive (r: 0.605 $P < 0.001$) relationship between species number and temperature. A negative (r: - 0.365 $P < 0.001$) relationship was observed between species number and both dissolved oxygen and number of individuals. The number of individuals was positively associated with temperature (r: 0.384 $P < 0.001$), and negatively with dissolved oxygen (r: - 0.346 $P < 0.001$).

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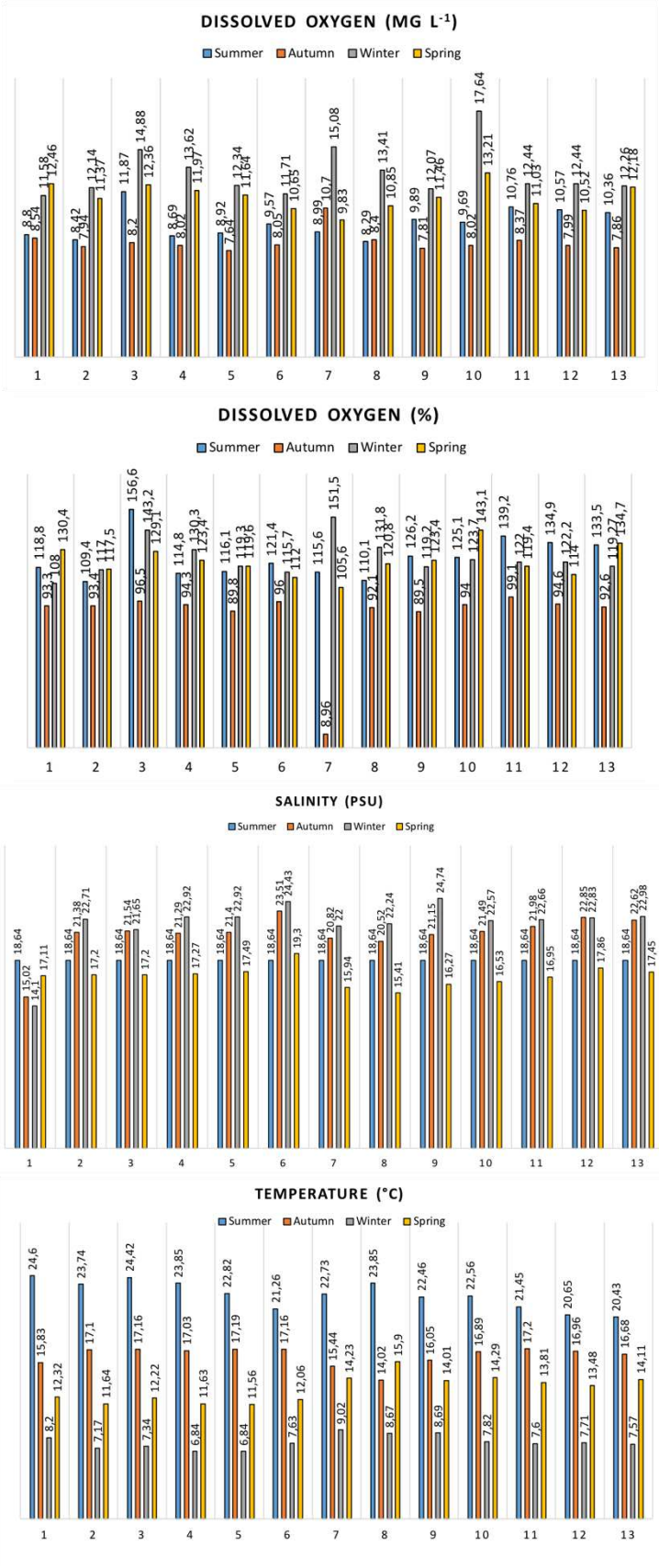

Figure 9. *Physicochemical parameters of the seawater of the hard bottom (0.5 m) stations. DO: dissolved oxygen (mg l^{-1}) DO: dissolved oxygen (%) S: salinity (PSU) T: temperature (°C)*

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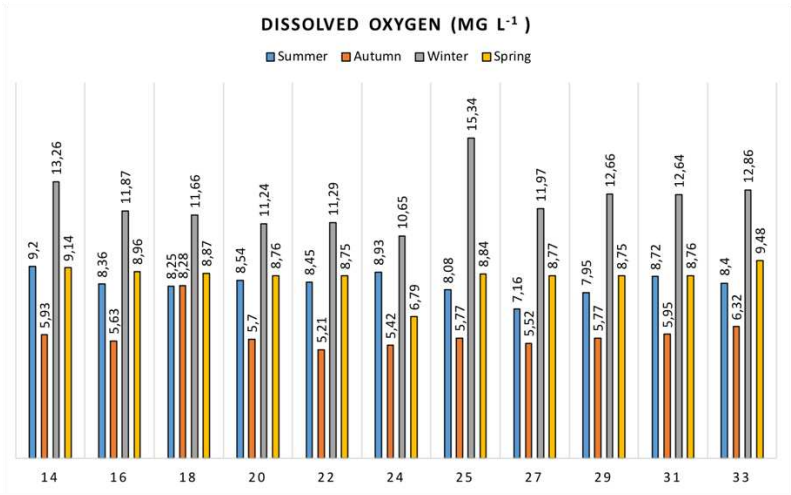
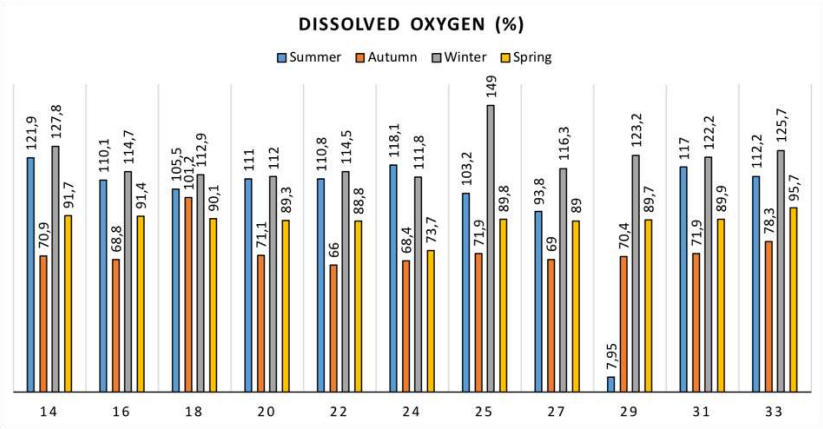
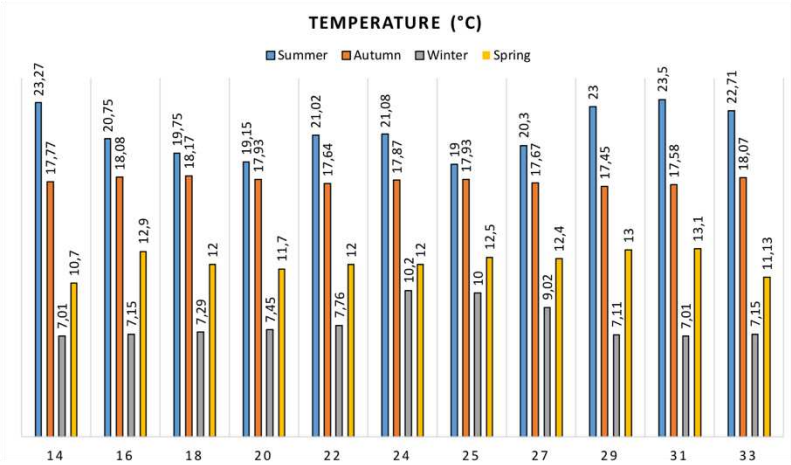
In soft bottom stations, seawater temperature values were between 6.55 °C (winter) and 23.5 °C (summer), dissolved oxygen values were between 1.84 (autumn) and 17.98 mg l^{-1} (winter), dissolved oxygen percentage values were between 7.95% (summer) and 139% (summer) and salinity values were between 16.2 (spring) and 46.44 (autumn) PSU (practical salinity unit). The lowest measured oxygen values were obtained at stations 21 (Ortaköy) and 23 (Karaköy) at 36.4 m depth for all seasons. High oxygen values were obtained at station 15 (Garipçe) (*Figs. 10* and *11*). We also found a negative relationship between dissolved oxygen values and depth as expected. (r: -0.297 $P < 0.001$). Salinity increased as expected due to the Mediterranean water below (r: 0.439 $P < 0.001$).







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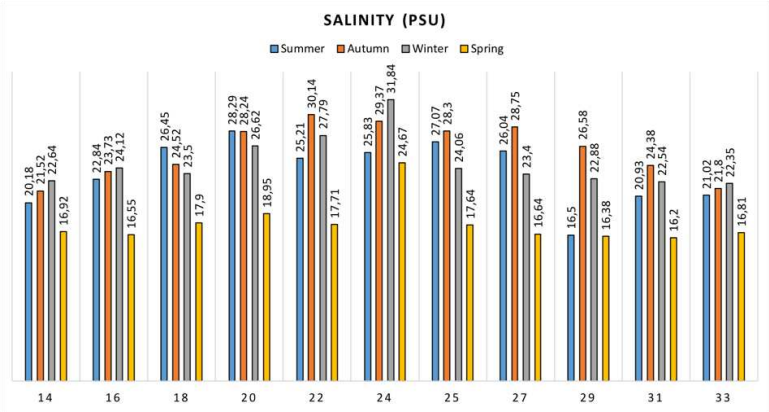

Figure 10. *Physicochemical parameters of the seawater of the soft bottom (18.2 m) stations DO: Dissolved Oxygen (mg l^{-1}) DO: Dissolved Oxygen (%). T: Temperature (°C) S: Salinity (PSU)*

The lowest Total Organic Carbon (TOC) value (0.2%) was found at stations 14 (Garipçe), 27 (Anadoluhisarı) and 31 (Anadolukavağı), while the highest values were obtained at station 23 (Karaköy) (6.4 and 5.2%). Total inorganic carbon values were between 3.09% (Çengelköy, station 25) and 56.22% (Paşabahçe, station 30) (*Table 4*). We identified statistically significant but a weak negative relationship between TOC values and DO (r: - 0.186 $P < 0.001$). A positive relationship was observed between TOC and both salinity values (r: 0.324 $P < 0.001$) and mud percentage (r: 0.360 $P < 0.001$). The relationship between TIC and mud percentage was negative (r: - 0.244 $P < 0.001$).

In this study, no statistically significant relationship was found between TOC and TIC values.

Table 4. *Physicochemical parameters determined in the soft bottom surface sediment samples of Istanbul Strait*

	TOC (%)				TIC (%)				MUD
STATION	SUMMER	AUTUMN	WINTER	SPRING	SUMMER	AUTUMN	WINTER	SPRING	PERCENTAGE (%)
14	0.36	0.62	0.84	0.21	13.90	13.90	10.81	8.3	9.50
15	2.02	0.71	0.39	2.57	32.43	7.72	30.89	32.43	16.00
16	2	1.05	1.05	0.47	6.18	4.63	20.08	21.62	24.97
17	0.82	0.75	1.51	0.23	26.25	24.5	18.53	20.08	29.03
18	0.92	0.31	0.85	0.99	21.62	17	13.90	10.81	6.47
19	0.51	0.28	0.38	0.31	9.27	4.63	15.74	16.99	5.28
20	2.68	1.11	3.24	2.5	10.81	3.09	7.72	10.81	41.94
21	2.18	4.12	1.39	2.2	9.27	10.81	7.72	10.81	20.41
22	1.63	0.58	2.5	2.7	6.18	26.25	7.72	7.1	69.9
23	2.3	2.48	6.4	5.2	6.18	6.18	7.1	7.7	72.66
24	1.05	0.56	0.65	0.83	37.07	13.90	33.98	30.89	10.86
25	2.9	1.1	2.09	1.44	3.09	24.71	15.44	25.64	17.56
26	0.9	1.1	0.63	0.9	4.63	9.27	24.71	24.69	25.99
27	0.95	0.2	0.74	0.2	3.09	4.60	4.63	3.09	1.44
28	0.62	0.84	0.61	0.2	4.63	4.63	4.63	4.63	21.26
29	0.75	0.78	0.66	0.8	13.90	12.35	10.81	13.90	25.47
30	0.8	1.78	1	1.2	12.35	44.79	15.44	56.22	13.48
31	2.04	0.51	0.2	0.69	12.35	9.27	16.98	24.69	18.23
32	0.6	0.57	0.64	0.95	7.72	8.3	4.63	6.18	84.57
33	1.23	1.02	1.11	1.55	7.72	24.71	15.44	6.18	2.52
34	3.47	3.15	2.51	0.69	6.18	18.53	38.61	24.69	16.61

TOC: total organic carbon, TIC: total inorganic carbon

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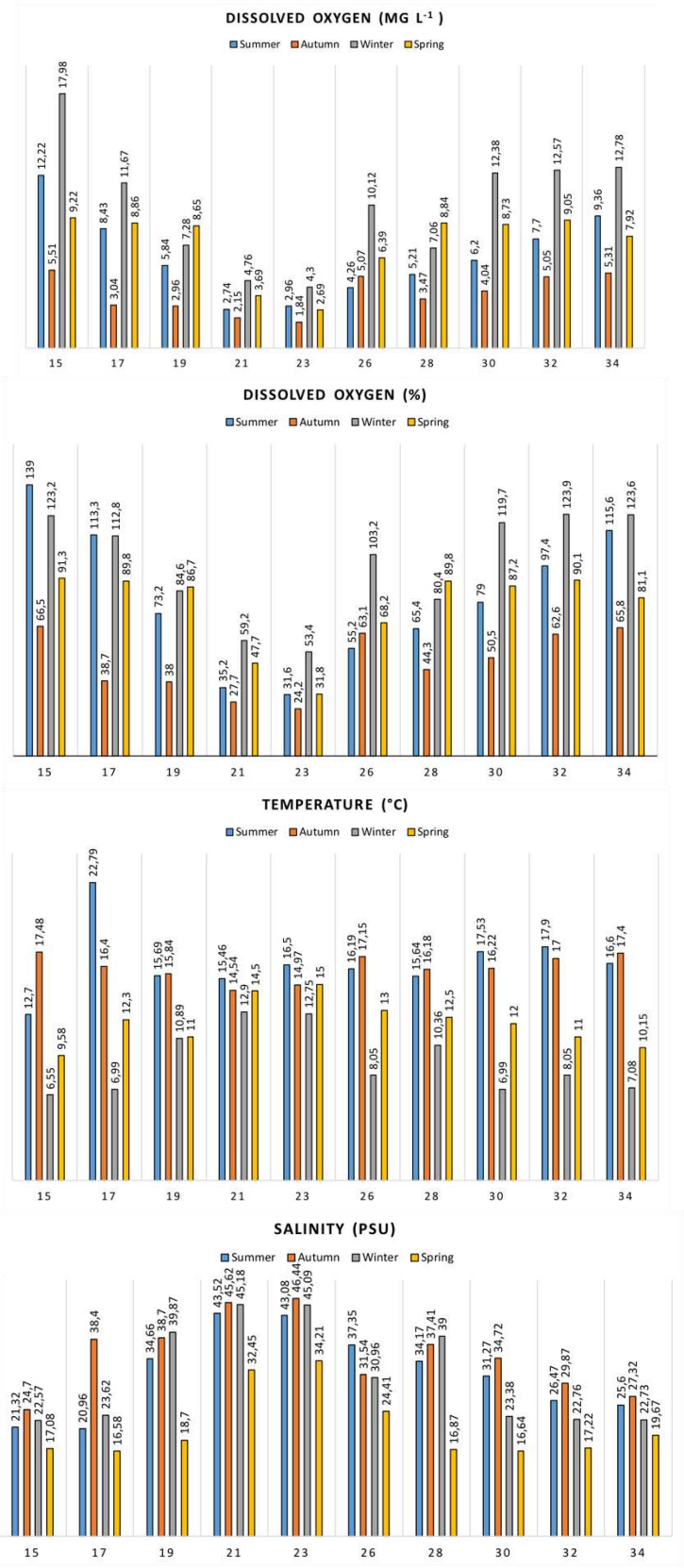

Figure 11. *Physicochemical parameters of the seawater of the soft bottom (36.4 m) stations. DO: Dissolved Oxygen (mg l^{-1}) DO: Dissolved Oxygen (%) T: Temperature (°C) S:Salinity (PSU)*

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Discussion

In this study, we found 46 species in hard bottom and 58 in soft bottom stations. Coastal filling to create artificial areas for ports, parks, and recreation areas is common on the shores of the Istanbul Strait. It has been observed that natural areas are in decline as artificial areas gradually increase in the Istanbul Strait (Onuk et al., 2017). For this reason, we experienced difficulty in finding natural stations to sample. The limited number of hard bottom stations to be found were surrounded by restaurants, residential areas, and anchored boats. Hard bottom macrobenthos is heavily influenced by habitat type and the hydrodynamic processes are particularly effective in the distribution of macrobenthic fauna, especially in the Istanbul Strait (Uysal et al., 2002). Water movements directly affect benthic organisms living in the sediment on the soft bottom, causing the transport of sediment and organic matter. In addition, hydrodynamic conditions change the distribution of the macrobenthic community in that region by changing the grain size distribution (Foulquier et al., 2020). Uysal (2002) states in his study in the Istanbul Strait that only one of the stations in the lower layer current system consists of fine sand and silt-containing mud. This station is located at the Black Sea exit of the Istanbul Strait. Researcher says that the sediments of other stations contain coarse-grained shells due to their high flow rates.

During the study, we observed that the number of species increased seasonally with temperature, and most species were collected in spring. Peracarid biodiversity depends on many factors, such as vegetation, hydrodynamics, sedimentation carbonates, habitats, and combinations of all of these features, and it has been observed in previous studies that biodiversity increases especially in spring (Wang et al., 2010). In addition to the increase in algae and mussels, which form the hard bottom communities and hosts of peracarids, this increase can also be influenced by the rising reproduction rates in the spring months when the temperature is high. According to Izquierdo and Garcia et al. (2011), peracarids reproduce year-round in the Strait of Gibraltar, but they are most abundant in spring and summer. The researchers linked this situation to seasonal changes in sea meadows, but factors such as predation and competition should also be evaluated (Izquierdo and Garcia, 2011).

The primary reason for the lack of species from the Mysid and Cumacea orders is that these peracarids prefer soft bottom habitats. However, it may also be related to the flow system and quadrat sampling on the shore (Wang et al., 2010). In this study, sampling was performed with a quadrat on hard bottom, but some researchers use a hand scoop or plankton nets to collect mysids (Porter, 2016).

The dominance of the amphipod species *P. schmidtii* in hard bottom stations is remarkable. *Ampithoe ramondi, Protohyale (Protohyale) schmidtii* and *Elasmopus brasiliensis* species were found to be dominant in a rocky community study in Gökçeada (Aslan and İşmen, 2019). Species with high frequency and dominance in this study, such as *Hyale* and *Jassa,* are tolerant of pollution (Kalkan et al., 2007). In this study, *Tanais*, which was obtained predominantly on the shores of the Istanbul Strait, was widely obtained from clean waters and harbors by various researchers. (Chintiroglou et al., 2004). In a previous study in the Istanbul Strait, *Hyale perieri*, *E. olivii*, *J. marmorata* and *T. dulongii* were dominant in discharge areas (Kalkan et al., 2007). Similarly, in this study, these species show high dominance the Istanbul Strait coast, including Beşiktaş, Sarıyer, and Tarabya, where the settlement was intense.

In this study, Cumacea, Mysid, and Tanaid species were represented by many fewer species than other orders. Cumacea and Mysid species (*I. trispinosa, V. cristata, H.*

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normani) obtained were distributed on only soft bottom stations. Only 3 tanaid obtained (A. *latreillei, C. savignyi and T. dulongi*). We obtained these tanaid species on both soft and hard bottom. However, *A. latreillei* has a high number of individuals on soft bottom and *T. dulongi* on hard bottom. Amphipods (195 species) make up the most 418 Malacostracan species in the Turkish Straits System. This is followed by decapoda (140), Isopoda (42), Cumacea (18), Mysidacea (12), Tanaidacea (7), Stomatopoda (2) and Leptostraca (1), respectively (Balkıs et al. 2016). In the studies performed in the Sea of Marmara and the Black Sea, it is seen that the Amphipods are dominant in the crustacean fauna (Sezgin et al. 2010; Bat et al., 2011; Bakır et al., 2012). This study reports the first record of the Atlanto-Mediterranean maerid amphipod *Animoceradocus semiserratus* in Sea of Marmara. This species is distributed throughout the Atlantic Ocean and Mediterranean Sea (Christodoulou et al., 2013). The existence of this species in Turkish seas was unknown until recently (Mutlu, 2020). However, considering the sampling date of the researcher, it is seen that it has been in our waters for a long time. Previous records of the species are from the Aegean and the Mediterranean Sea, and it is observed that Mediterranean origin species increase in the Sea of Marmara.

In sampling on soft bottom, stations were selected from two depths (18.2 m and 36.4 m) with a difference in salinity between them. However, according to the Spearman analysis, no significant relationship was found between depth differentiation and the number of species and individuals. While salinity is an important factor in lagoons, sediment type and amount of organic matter are more critical in the distribution of peracarids in marine ecosystems (Lourido et al., 2008; Zaabar et al., 2017). The strait's sediment has a coarse-grained structure (Uysal et al., 2002). In this study, the mud percentage values in the sampled soft bottom stations vary between 1.44% and 84.57% with an average value of 25.4%. Only a few stations had high mud percentages. A weak but positive correlation was found between the number of species and individuals and the mud percentage ($r: 0.221$ $P < 0.001$). The same relationship was also observed between the mud percentage and organic carbon. This can be explained by the fact that the amount of organic matter increases as the mud percentage increases. Fine grain sediments do not permit the entry of oxygen, increasing the absorption of organic matter. (Secrieru and Oaie, 2009). This organic material is an important parameter, especially for detritivore species, as it constitutes a food source. Two of the two stations which lack peracarids throughout the year (33 and 34) are located in Keçilik, which has vortex (Atasoy, 2008). The associated strong current is one reason why species were not collected in these stations. In the spring, high *H'* index values were obtained in the Ortaköy (Station 20), Karaköy (station 22), and Salacak (station 25) areas, which have low dissolved oxygen values and were observed to be discharge regions during sampling. This is due to the density of the species, which feed on detritus at these stations. The high organic carbon content, especially at Karaköy and Ortaköy stations supports this idea and indicates the organic contamination at these points. The strait's sediment samples show high organic carbon content values, especially in the summer. Anthropogenic organic pollution was remarkable at station 34 Keçilik (3.47%), station 20 Ortaköy (2.68%), and station 23 Karaköy (2.3%) stations, where the highest percentage of carbon was obtained. Low dissolved oxygen content in the same areas confirms this result. Again, the lowest dissolved oxygen values in seawater were measured at 36.4 m depths in Karaköy and Ortaköy in all seasons, and this is one of the results of oxygen deficiency in the bottom layers and organic pollution in the strait.

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DOI: http://dx.doi.org/10.15666/aeer/1806_83218342

Conclusion

As a result, with this study, we examined the Istanbul Strait peracarid fauna in detail in both hard and soft bottoms and reported species that were not observed in the Bosphorus and Sea of Marmara before. Thus, we ensured the update of macrozoobenthic fauna information. We have observed that most of the species we have obtained are of Atlanto-mediterranean origin, and a few species are Mediterranean endemic or cosmopolitan species. Considering that the Istanbul Strait creates a natural transition between the Mediterranean and the Black Sea, it is an expected result to obtain the species previously identified from the Mediterranean from the Istanbul Strait. We have observed that in the distribution of species in the strait, ground structure, temperature, organic carbon amount and current system are more effective than the salinity difference of Black Sea and Mediterranean waters. We think that the analysis of grain size in future studies will be useful for understanding the habitat structure and distribution. We stated that we had difficulty in finding natural coastal areas during the hard bottom sampling of the study. We think that the coastal filling creates negativity for distributing of macrozoobenthic organisms in the strait. The decrease in the amount of oxygen with depth and high carbon amount in certain areas can be considered as a warning for the pollution in the strait. However, since this study is not a pollution study, it was conducted to determine the diversity and distribution of peracarids, we recommend increasing pollution studies in the region. Moreover, this study cannot comment on the overall benthic ecological quality of the strait, since only peracarid species were examined. For this reason, the authors recommend following it up with studies on ecological quality in the future.

Acknowledgments. The author is grateful to Dr. Senem Çağlar, Ph.D., Mert Kesiktaş, Msc. Elif Yücedağ, Dr. Güley Kurt, and Ph.D., Begüm Ayfer. This study was supported by the Research Fund of Istanbul University (project number BAP 53379).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83218342

MORPHO-AGRONOMIC AND COOKING QUALITY OF COMMON BEAN (*PHASEOLUS VULGARIS* L.) GROWN UNDER DIFFERENT NITROGEN SOURCES AND NITROGEN LEVELS

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(Received 19th Jun 2020; accepted 7th Oct 2020)

Abstract. This study aimed to determine the effects of nitrogen (N) doses (0, 40, 80 and 120 kg N ha^{-1}) and two different N fertilizer sources [ammonium nitrate (AN) and calcium ammonium nitrate (CAN)] on yield, yield parameters and quality of common bean (*Phaseolus vulgaris* L.) grown under the environmental conditions of Central Anatolia, Turkey. Experimental layout was randomized blocks with four replications, and the Önceler-98 bean variety was used as the plant material. The first pod height, number of pods per plant, number of beans per pod, grain yield, 100 grain weight, protein content, wet weight, dry weight, cooking time, water absorption index and swelling capacity of grains were determined. Nitrogen fertilizer sources caused a significant difference ($P < 0.05$) in 100 grain weight and number of branches per plant. Application of CAN increased the number of branches per plant and AN fertilizer increased the 100 grain weight. The results indicated that the effect of CAN fertilizer is greater compared to AN due to the providing calcium demand of plants grown in soils with low lime content. Higher effect of CAN fertilizer on plant growth can be attributed to the slower solubility of CAN than that of the AN fertilizer. The results concluded that 40 kg N ha^{-1} may be sufficient to obtain optimum yield, yield parameters and quality of bean, and higher doses of N (>40 kg N ha^{-1}) applications are not needed.

Keywords: *bean, yield, yield components, quality, nitrogen, fertilizer source*

Introduction

A large proportion of the protein requirements of people in the world is met by plant sources (Craig, 2009). The highest amount of protein per unit area among the vegetable protein sources is obtained from legumes. Common bean among leguminous crops is rich in vitamins A, B and D and comes to the fore with protein content between 17 and 35%. (Cabrera et al., 2003). Common bean is the most commonly consumed legume and an important nutritional source in the daily diet of more than 300 million people worldwide. The cultivation of common bean is widespread in the temperate regions of the world and 94% of the production is carried mostly in developing countries of the Asian and South American continents. Application of a fertilizer within the appropriate nutrient/fertilization schedule is of great importance to obtain high yield per unit area in common bean farming.

Plant nutrients are the essential elements for plant growth and each nutrient has specific role in plant growth, thus one element cannot substitute the other. Sepetoglu (1994) reported that harvesting of 180 kg grain and 160 kg stem per hectare in bean cultivation removes 165 kg ha^{-1} nitrogen (N), 70 kg ha^{-1} phosphorus (P), 137 kg ha^{-1}

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83438354

potassium (K) and 140 kg ha^{-1} calcium (Ca) from soil. Nitrogen is one of the most important inputs in plant production. Despite the N fixing ability of legumes, N fertilizers are needed in sowing. The bean plant can meet most of the N needed by fixing the free N in atmosphere through the rhizobium bacteria, which have a symbiosis with bean plants in soil. The amount of N fixed through the symbiosis between rhizobium and edible legume varies depending on the crop type and environmental conditions and was reported ranging from 5 to 20 kg per year (Sehirali, 1988). Total amount of N, which is biologically bound on legumes in 0.3 million ha lands in Nepal, was estimated as 30 × 103 tons per year (Maskey et al., 2001), and approximately 50% of N is provided by the symbiosis between legume and rhizobium (Smil, 2002). However, nitrogen, which is needed until growing sufficient number of rhizobium bacteria in soil and establishing a symbiosis with the bean plants, should be applied to soil as the starting fertilizer. An optimum N nutrition by natural mineral N content of soils cannot be expected under insufficient N fertilization and without bacterial inoculation to the bean plants (Zahran, 1999). The N content of soils depends on many factors such as parent material of soils, soil organic matter content, microbiological condition and climate. Therefore, plant available N forms in agricultural soils are rapidly lost depending on environmental conditions.

The optimum/balanced nutrition of plants is an extremely important factor on quality as well as yield. Soil fertility management beside yield may also affect the nutritional and so cooking quality of seeds (Alamu et al., 2019) For example, the weight of chickpea grains increases by 54 to 133% after swelling in water for 18 to 24 h, and cooking and swelling for 45 to 90 min, sometimes 2 h. Calcium concentration of shells was reported effective on the cooking quality of grains. Therefore, quality of grains produced in soils with low lime content was reported poor (Sehirali, 1988). Calcium ammonium nitrate is one of the most commonly used N fertilizers in plant production in Turkey as well as in the world. However, the CAN fertilizer may have even more adverse effect locally on these values in soils with high pH value and lime content. Cultivation of the beans under such conditions and/or continuous application of CAN fertilizer, which contains Ca will negatively affect the cooking quality.

The aim of this study was to reveal the effects of two different N fertilizer sources (ammonium nitrate; AN and calcium ammonium nitrate; CAN) and increasing doses of N (0, 40, 80 and 120 kg ha^{-1}) on yield, yield parameters and cooking quality of Önceler-98 bean variety.

Material and methods

Soil and climatic characteristics of the experimental field

The field experiment was conducted under ecological conditions of Eskisehir province, Turkey in 2007. Experimental field was located in the research farm of Agricultural Faculty in Eskisehir Osmangazi University (39°48’ N; 30°31’ E, 798 m above sea level). Surface soils (0-30 cm) of the experimental field were low in organic matter content, moderate in lime content, non-saline, loamy textured and slightly alkaline (*Table 1*). The winters in the study area are usually cold and rainy and the summers are hot and dry. Long term (1928-2019) average annual total precipitation is 374.2 mm and average precipitation is 11.2 °C. The coldest month is January with an average temperature of 0 °C, while the highest average temperature (21.8 °C) was recorded in July and August (Anonymous, 2020). Total precipitation from seed sowing

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to harvest (between April and November), was 262 mm, average monthly temperature was 16 °C and relative humidity was 51.4%.

Particle size distribution of soil samples were determined according to hydrometer method (Bouyoucos, 1951) Soil pH and electrical conductivity were measured in 1:2.5 (soil:water) mixture using a pH-EC meter (Jackson, 1958), and calcium carbonate content (%) was determined using a Scheibler calcimeter (Allison and Moodie, 1965). Organic matter content (%) of soil samples were analyzed by using the modified Walkley-Black method (Nelson and Sommers, 1982). Plant available phosphorus was analyzed by the method of Olsen (1954) using Termo-aquamete UV spectrophotometer and extractable potassium was determined according to Thomas (1982) using the BWB/XP2011 model flame photometer.

Table 1. *Some physical and chemical properties of experimental field*

Soil texture	pH	Total salt (%)	Organic matter (%)	$CaCO_3$ (%)	Available (mg kg^{-1})	
					P	K
Loam	8.10	0.05	1.70	4.36	6.73	721.3

Establishment of the experiment

Local dwarf Önceler-98 bean variety (*Phaseolus vulgaris* L.) was used as a plant material of the experiment. Four different N doses (0, 40, 80 and 120 kg ha^{-1}) and two different N sources (fertilizers) [ammonium nitrate (AN) 33% N, (NH_4NO_3) and calcium ammonium nitrate (CAN) 26% N, ($5Ca(NO_3)\ 2NH_4NO_3.10H_2O$)] were applied during seed sowing. Triple superphosphate (80 kg P_2O_5 ha^{-1}) was applied during tillage before planting (Sehirali, 1988). The experimental layout was a split-plots in a randomized complete block design with three replications. The N fertilizers were placed in the main plots and N doses were in the subplots.

Seeds were manually sown on April 28, 2007 at an inter row spacing of 60 cm and an intra row spacing of 20 cm in four rows. The length of individual plots was 5 m and total area of each plot was 12 m^2. Weed control in the experiment was carried out manually using a hoe. The water requirement of plants, especially during the flowering (77 mm) and pod formation (100 mm) periods, was met by drip irrigation. The amount of irrigation water was calculated aiming to complete the water added to soil including the total rainfall in vegetation period to 439 mm which is considered the requirement of the bean plants (Silim and Saxena, 1993).

Yield and agronomic characteristics of common bean

All plants were harvested on September 2 following the ripening and drying, and the grain yield (kg ha^{-1}) was calculated after separating and weighing the grains. Above ground parts of 10 bean plants were cut in each plot to determine the effects of N doses and fertilizer sources. The first pod height (cm) per plant, number of pods per plant, number of main branches per plant and the number of beans per pod were determined in 10 bean plants. One hundred dry beans were weighed to determine the dry weight of grains.

For wet weight of bean grains, dry weights of 100 bean seeds were recorded, then dry seeds were soaked for 16 h, and extra water was poured, the seeds were dried and weighed. Water absorption capacity of beans were calculated as follows (Eq. 1):

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$$\text{Water absorption capacity (g grain}^{-1}) = (WV - DW) / 100 \quad \text{(Eq.1)}$$

In the equation: IW is the sample initial weight (g/grain); DW is the sample final dry weight.

Water absorption index was calculated as follows (Eq. 2):

$$\text{Water absorption index} = (IW)/DW) / 100) \quad \text{(Eq.2)}$$

Swelling capacity (SC) was determined as follows (Eq. 3):

$$SC = [(WV - 100) - (DV\text{-}50)] - [DV - 50/100) \times NNSG / 100\ NNSG] \quad \text{(Eq.3)}$$

In the equation: WV is the wet volume; DV is the dry volume; NNSG is the number of non-swollen grains (ml grain^{-1}).

Swelling index (%), cooking time (minutes) and protein content (using Kjeldahl Method by Bremner, 1965) using by Gerhardt Kjeldahl distillation system (Vapodest® 200 – 450) of common bean grains were also determined as quality parameters.

The treatment effects on yield, yield components and quality of common bean were assessed by analysis of variance (ANOVA) using SPSS 20.0 software. The mean values were compared by Turkey's test at 5% probability level when significant F values ($P < 0.05$) were obtained in the ANOVA test.

Results and discussion

Morphological characteristics of plants

The first pod height of bean plants, the number of pods per plant, the number of branches per plant and the number of seeds per pod were given in *Table 2*. The N resources had a significant effect ($P < 0.05$) only on the first pod height of the plants. The mean first pod height of plant was 17.47 cm in control treatment, and the highest pod height (19.11 cm) was recorded in 40 kg ha^{-1} doses of CAN fertilizer treatment. The first pod height increase in CAN (18.11 cm) application was higher compared to the AN (17.89 cm) fertilizer application. The most effective N dose for the first pod height increase in both fertilizers was obtained with 40 kg N ha^{-1}.

The first pod height, which is an important characteristic to facilitate machine harvest and reduce harvest losses (Cober et al., 2000), was increased compared to control with the N applications. Similar to the findings obtained in common bean, Oz (2008) reported the first pod height increase in soybean with the N application. The first pod heights of bean varieties were reported ranging between 13.3 and 18.1 cm (Anlarsal et al., 2000). Onder et al. (2013) reported that cultivation techniques (sowing density, fertilization, etc.) and different environmental conditions significantly affect the first pod height (Onder et al., 2013). Therefore, the first pod height should not be very low to reduce losses in the machine harvest of beans (Gunes, 2006).

The effects of N sources on the number of pods per plant was not significant while N doses and N source × N dose interaction had a significant effect ($P < 0.05$) (*Table 2*). The highest number of pods per plant was obtained in 40 kg N ha^{-1} dose (24.36 pods) and CAN fertilizer (23.32 pods) treatments. Application of CAN fertilizer at 40 kg N ha^{-1} dose had a significant effect on the number of pods per plant (25.39 pods). The highest application dose (40 kg N ha^{-1}) of the CAN fertilizer had a more pronounced effect on the

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number of pods compared to other doses, however, all doses of the AN fertilizer had an effect on increasing the number of pods per plant. Nitrogen is an important nutrient in plant nutrition that affects cell division and elongation and plays a role in pod formation (Abo-Sedera et al., 2016). Several reports published stated that the number of pods per bean plant ranges between 4 and 14 pods, and N applications has a direct impact on grain yield (Peksen, 2005; Fageria et al., 2011; Fernández-Luqueño et al., 2010).

The source of N fertilizer had a significant effect ($P < 0.05$) on the increase in number main branches per plant, and higher number of branches per plant (2.17 branches) were obtained with CAN fertilizer application. Branching is closely related to the sowing density and branching is decreased in the close inter row spacing between bean plants, while branching is increased at wide sowing distances. Branching in bean is a desired characteristic, and the increase in the number of branches increases the number of leaves per plant. The increase in plant growth increases the number of pods per plant, thus resulting in high yields (Karasu and Oz, 2010; Abo-Sedera et al., 2016). The number of branches per bean plant has been reported between 2.2 and 3.7 branch plant^{-1} (Dumlu, 2009). The increase or decrease in the number of branches per bean plant has been attributed to the differences in soil characteristics and climate of the study area, and genetic materials and fertilizer sources used in the experiment (Abo El-Yazied et al., 2012).

The N sources and doses did not have a significant effect on the number of grain per pod (*Table 2*). However, N doses increased the number of grains per pod compared to the plants in control treatment. Oz (2002) and Kacar et al. (2004) reported that the N doses increased the number of grains per pod. In contrast, Franco et al. (2008) determined that N dose did not cause a significant difference in the number of grains per pod. The number of grains per pod was reported ranging between 3.24 and 6.06 grains and this number varies depending on the characteristics of a genotype (Anlarsal et al., 2000; Peksen, 2005).

Table 2. *Effects of N sources and N doses on some of quality parameters of common bean*

	First pod height (cm)		**The number of pods per plant (pod plant^{-1})**		**The number of main branches per plant (branch plant^{-1})**		**The number of grains per pod (grain pod^{-1})**	
Rates	AN	CAN	AN	CAN	AN	CAN	AN	CAN
N0	17.47	17.83	14.47 c	23.32 ab	1.51	2.31	3.54	3.63
N40	18.19	19.11	23.31 ab	25.39 a	2.01	2.18	3.76	3.84
N80	17.74	17.71	22.15 ab	21.04 ab	1.98	2.03	3.78	3.55
N120	18.19	17.75	19.00 bc	23.52 ab	1.61	2.15	3.84	3.85
Mean	*17.89*	*18.11*	*19.74*	*23.32*	*1.78b*	*2.17a*	*3.73*	*3.72*
N0	17.65 b		18.9		1.91		3.59	
N40	18.65 a		24.36		2.11		3.81	
N80	17.73 b		21.6		2.01		3.67	
N120	17.97 ab		21.27		1.89		3.85	
F test								
Source (S)	ns		ns		*		ns	
Rate (R)	*		*		ns		ns	
S × R	ns		*		ns		ns	

*$P < 0.05$; ns: not significant

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DOI: http://dx.doi.org/10.15666/aeer/1806_83438354

The effects of N source and N doses had no significant impact on bean grain yield, while N source × N dose interaction significantly ($P < 0.01$) affected the grain yield (*Table 3*). Average grain yield varied between 1876.1 and 2089.6 kg ha^{-1} and the highest grain yield (2160.3 kg ha^{-1}) was obtained from 40 kg N ha^{-1} dose. The highest grain yield (2230.9 kg ha^{-1}), in terms of N source was recorded in 40 kg N ha^{-1} dose of CAN fertilizer application. The reports on the optimum N dose needed to obtain the highest grain yield are not consistent. Silva (2020) obtained the highest grain yield in common bean with the application of 100 kg N ha^{-1} nitrogen. In other studies, Valerio et al. (2003) stated that 80 kg ha^{-1} yields the highest grain yield, Kacar et al. (2004) stated as 90 kg ha^{-1} and Kaneko et al. (2010) stated as 180 kg ha^{-1} N. In this study, the highest number of pods per plant was determined at 40 kg ha^{-1} dose of CAN fertilizer. Similarly, others stated that the grain yield is affected by the number of pods per plant (Soratto et al., 2017; Carvalho et al., 2018; Chekanai et al., 2018).

Fertilizer sources had a significant effect on 100 hundred grain weight of bean ($P < 0.05$), while the effect of N doses on 100 grain yield was not significant. The mean 100 grain weight (42.44 g) obtained in AN application was higher than that (41.35 g) of the CAN application (*Table 3*). Some studies reported an increase also in 100 grain weight with the N applications (Oz, 2008; Nascente et al., 2017). Fageria et al. (2014) indicated that the increased doses of N increased the 100-grain weight of different bean varieties, which responded differently to the interactions of N × genotype.

Table 3. *Effects of N sources and N doses on grain yield, hundred grain weight and grain protein content of common soybean*

	Grain yield (kg ha^{-1})		**Hundred grain weight (g)**		**Grain protein content (%)**	
Rates	AN	CAN	AN	CAN	AN	CAN
N0	1876.1 cd	1857.4 d	42.25	41.62	25.27	25.69
N40	2089.6 ab	2230.9 a	42.33	41.59	24.07	26.6
N80	1995.6 bd	2017.6 bc	41.84	40.93	23.71	24.35
N120	2034.3 b	1995.1 bd	43.36	41.27	25.98	24.43
Mean	*1998.9*	*2025.2*	*42.44 a*	*41.35 b*	*24.76*	*25.27*
N0	1866.8		41.94		25.49	
N40	2160.3		41.96		25.34	
N80	2006.6		41.39		24.04	
N120	2014.7		42.32		25.22	
F test						
Source (S)	ns		*		ns	
Rate (R)	ns		ns		ns	
S × R	**		ns		ns	

*$p < 0.05$; **$p < 0.01$; ns: not significant, AN: ammonium nitrate, CAN: calcium ammonium nitrate

Quality parameters of common bean

Nitrogen sources and doses did not have a significant effect on the protein content of bean grains (*Table 3*). The CAN fertilizer caused a higher increase in protein content compared to AN fertilizer, and the highest grain protein content (26.60%) was obtained by 40 kg N ha^{-1} dose of CAN fertilizer treatment. Beans are very important nutrient

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DOI: http://dx.doi.org/10.15666/aeer/1806_83438354

sources in human nutrition in terms of protein, vitamins, minerals and fiber sources (Kutos et al., 2003). Application of organic nutrient-containing fertilizers together with mineral fertilizers reported increasing the protein content of legumes (Jagannath et al., 2002; Hegazi et al., 2011). The researchers reported that protein content varies depending on fertilization, irrigation, climate and soil characteristics (Kose et al., 2019; Gulmezoglu and Kayan, 2011). The protein content of bean grains has been reported varying between 20.11 and 28.62% (Kahraman and Onder, 2013; Chavez-Mendoza et al., 2010).

Different N sources and doses did not have a significant effect on the cooking time of bean grains ($p > 0.05$). The cooking time of bean grains ranged from 23 to 25.50 min (*Fig. 1*). The cooking time obtained in the AN fertilizer application was longer than the beans obtained in CAN fertilizer application. Longer cooking time of bean grain has been attributed to the hard shell that does not allow the grain to be soaked sufficiently or to absorb water (Saha et al., 2009). Similar to the results obtained in this study, Kigel (1999) reported that cooking the bean grains is a problem. Akdag (1996) found that the cooking time of leguminous grains are affected by the growing conditions, thickness and chemical composition of the grain shell as well as the genetic characteristics. The factors such as early harvest, cultivation in soils with high Ca and Mg concentrations and storage under improper conditions (higher than 13-14% humidity and 100 °C storage temperature levels) negatively affect the cooking quality of edible legumes. Some studies reported that the cooking time of bean grains varies between 23 and 47 min (Shimelis and Rakshit, 2005; Perina et al., 2014). Cooking of edible legume grains is defined as gelatinization of starch, as well as softening of the grain and becoming easily chewable in the mouth. The definition of cooking implies that the grain shell of a bean is affected by hot water permeability, chemical composition of the cell wall, inherited hardness of cotyledon and physical properties of the grain (Carvalho et al., 2017). In addition, factors such as grain shell composition, environmental conditions, storage conditions and chemical composition also have an effect on cooking time (Shimelis and Rakshit, 2005).

The effects of fertilizer type, N application dose and their interactions on water absorption capacity were not statistically significant. The water absorption capacity of bean grains in this study was between 0.378 and 0.430 g grain^{-1}. The highest water absorption capacity of bean grains was recorded in CAN fertilizer application and the water absorption capacity of grains decreased with the N application ratios (*Fig. 1*). The water absorption capacity of bean grains was reported ranging between 0.146 and 0.809 g grain^{-1} by Ozbekmez (2015) while between 0.168 and 0.487 g grain^{-1} by Cengiz (2007). The water absorption capacity varies depending on the composition of grains, the cell wall structure and the condition of cells in the grains. Therefore, a strong positive relationship has been reported between grain mass and water absorption capacity (Kaur and Sing, 2006).

Water absorption index ranged from 0.88-1.03%, and the highest water absorption index in the grain was obtained in CAN application. However, the effects of N sources and doses on water absorption index were not statistically significant. The water absorption index values of the bean grains obtained in this study were compatible with the findings of Cengiz (2007) (0.963-1.157%) and Ozbekmez (2015) (0.323-1.780%).

Average swelling capacity (ml grain^{-1}) varied between 0.37 and 0.49 ml grain^{-1} depending on N sources and doses. The grain swelling capacity was higher under AN application compared to that of CAN application and the swelling capacity of bean grains decreased with the N doses. The results obtained are similar to the findings of Cengiz (2007) (0.125-0.420 ml grain^{-1}) and Ozbekmez (2015) (0.104-0.574 ml grain^{-1}).

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DOI: http://dx.doi.org/10.15666/aeer/1806_83438354

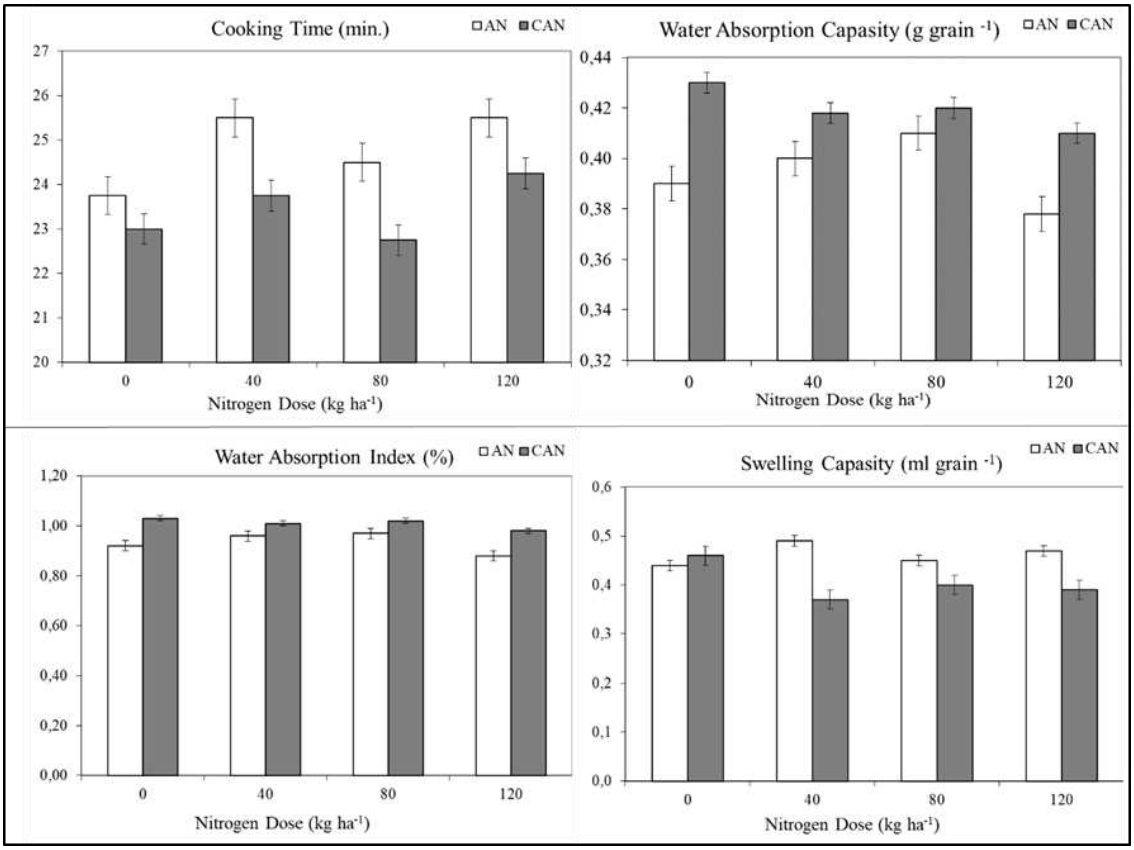

Figure 1. *Effects of N doses on cooking time, water absorption capacity, water absorption index and swelling capacity of bean grains*

Conclusions

The results revealed that application of CAN fertilizers in alkaline soils during bean production does not have a negative impact on the bean growth by the increasing soil pH, and on the bean grain quality due to the addition of Ca. The application of AN fertilizer had a significant effect ($P < 0.05$) only on 100-grain weight, while CAN fertilizer caused a statistically significant ($P < 0.05$) increase in the number of pods per plant, which is the most important grain yield parameter of the beans. In addition, although not statistically significant, the effects of CAN fertilizer on all yield components were more positive than that of the AN fertilizer. This positive effect may be related to the rapid dissolution of the AN relative to the CAN fertilizer and subsequent N losses in AN applied fields. The results obtained in this study also indicated that 40 kg N ha^{-1} application dose may be sufficient to obtain desired yield components and quality of the beans. Therefore, higher N doses than the plant requirements can cause environmental pollution as well as economic losses. The results indicated that application of 40 kg N ha^{-1} CAN fertilizer at the beginning until the bean plant can establish a symbiotic life with the natural rhizobium of the soil does not cause a decrease in grain yield. Application of CAN fertilizer even had a more positive effect than AN, however CAN fertilizer could have a slightly negative effect on cooking time of bean grains. This study should be repeated using different bean varieties, locations, N sources and in varied climatic conditions.

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DOI: http://dx.doi.org/10.15666/aeer/1806_83438354

DETERMINATION OF SHORT-TERM EFFECTS OF WILD FIRE ON SOIL PROPERTIES AND NITROGEN MINERALIZATION IN TURKISH PINE (*Pinus brutia* Ten.) IN TURKEY (THE CASE OF SARIÇIÇEK SUB-DISTRICT DIRECTORATE)

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(Received 24th Aug 2020; accepted 30th Nov 2020)

Abstract. Forest fires are one of the factors that play an important role in both global warming and nutrient accumulation in soils. The level of these effects varies according to the severity and intensity of the fire. This study was conducted to determine the one-year effects of fire on soil properties and nitrogen mineralization in Turkish Pine stands exposed to low-intensity surface fire that naturally occurred at the Sarıçiçek region the Vezirköprü district of Samsun province in 2014, in Turkey. To this end, six sampling areas were selected from both burned and unburned (control) areas in the sections. Soil samples were taken from a depth of 0-5 cm and 5-10 cm. Nitrogen mineralization was determined by the land incubation in 3 periods (April-July-October, 2014) on-site holding method. Among soil properties, texture, pH, organic matter, total nitrogen, bulk density and carbon (C) / nitrogen (N) ratio analyses were assessed. As a conclusion, it was observed that significant differences occurred in soil properties and nitrogen mineralization temporarily. Average nitrogen mineralization at a depth of 0-10 cm over the one- year period was found to be 23.56 kg /ha in the burned areas and 25.2 kg/ha in the control areas. As a result of the study, it was concluded that the fire was more effective, especially at a depth of 0-5 cm in regards to changing the soil properties. Nitrogen mineralization at a depth of 0-5 cm was greater in the burned areas compared to controls. It was determined that especially low-intensity fires were not effective toward the lower depth levels.
Keywords: *global warming, nutrient accumulation, low-intensity fire, nitrogen mineralization, surface fire*

Introduction

Although forest fires have been regarded as natural disasters for many years, they have been considered to be a part of an ecological system in recent years. Along with the understanding of the dynamics of the ecosystem, natural resource managers can preserve the natural structure of the ecosystem and also ensure its transformation into different structures and compositions by using controlled and purposeful burning applications, which are practical, economical and natural methods, as a management tool (Franklin, 1993; McKenney et al., 1994; Gauthear et al., 1996; James et al., 2018; Quigley et al., 2020).

Indeed, it has been determined in many studies that fires are not just natural events that cause damage. If there were no fires in nature, all forest areas would become monocultures, all kinds of diseases and insect damages would increase and spread due to the excess accumulation of living and dead vegetation, and there would be the excessive accumulation of flammable materials and infertility. Because of all these beneficial aspects of fires, the fire has now become one of the key elements in the management of renewable natural resources (Wright and Bailey, 1982; Alcañiz et al., 2016, 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

Forest fires are an important factor in natural forests and bushes, and they are also useful in the management of afforestation in prescribed burning (Kaye et al., 1999; Johnson et al., 2008; Boerner et al., 2009; Fonseca et al., 2017; Fuentes et al., 2018). A 5.1×10^8 ha forest area is burned mostly due to human activities every year in the world in the global process (Goldammer, 1993; Caldararo, 2002). The direct and indirect effects of forest fires on soil properties in the forest ecosystems have been extensively investigated (Fernandez et al., 1997; Mabuhay et al., 2003; Boerner et al., 2009). Some of these studies explain the effects of fires on soil organic carbon and nitrogen, which determine the soil quality.

Microbial biomass plays an essential role in nitrogen mineralization and carbon sequestration (Hernandes et al., 1997; Jensen et al., 2001; Ilstedt et al., 2003). Nevertheless, these studies do not fully indicate whether the effects of fire on microbial biomass are negative, positive or neutral (Choromanska and DeLuca, 2001; Mabuhay et al., 2003; Liu et al., 2007; Rutigliano et al., 2007; Rodriguez et al., 2009). Most of the studies indicate that there is a strong linear relationship between organic matter and soil respiration and nitrogen mineralization. In other words, the organic matter that is changeable in the soil is used in soil respiration and nitrogen mineralization (Wang et al., 2003; Haynes, 2005; Laik et al., 2009). Fire effectively changes soil respiration and nitrogen mineralization in the long or short term. It also has effects on the physical, chemical, and biological properties of the soil, such as soil moisture, nutrient availability, and microbial activities (DeLuca and Zouhar, 2000; Le Duc and Rothstein, 2007; Hamman et al., 2008). Hamman et al. (2008) indicated that prescribed burning decreased soil moisture in coniferous species, increased soil pH and significantly changed the nitrogen mineralization.

Some studies have revealed that forest fires are effective in soil respiration and nitrogen mineralization (Fernandez et al., 1997; Choromanska and DeLuca, 2001; Guerrero et al., 2005; Boerner et al., 2006). However, others state that their effects are inconsistent. Namely, while DeLuca and Zouhar (2000) stated that potential mineral nitrogen increased immediately after the fire, Weston and Attiwill (1990) determined that it decreased.

Wang et al. (2012) indicated that organic matter decreased by 20.3% and nitrogen increased by 13.1% after the fire compared to control areas. However, they reported that the fire had no significant effect on soil organic matter and total nitrogen. They stated that the highest effect of the fire occurred within the first three months after the fire. In the study, it was also revealed that the forest type and natural fires in natural zones affected total nitrogen and total organic carbon. Accordingly, while organic matter and nitrogen decreased by 25.3% and 22.7% in coniferous species, they increased by 28.0% and 28.7% in hardwood species. While fire decreased soil respiration by around 13.5 %, it decreased nitrogen mineralization by approximately 21.8%. The effect of fire on nitrogen mineralization changed as the depth of soil changed, and there was a decrease by 23.8% at a depth of 0-5 cm.

The objectives of this study were to determine the N mineralization potential, soil respiration and some soil properties in Turkish Pine (*Pinus brutia Ten.*) stands exposed to natural fire in Sarıçiçek region in Vezirköprü district of Samsun province under field conditions, in Turkey.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

Material and Method

Site description

Study area is located Vezirköprü, Samsun in the northern Turkey (41º 14′ 21′′ N - 34º 54′ 13′′ E). This study was conducted in average 60 years old, with 30% canopy cover Turkish pine (*Pinus brutia* Ten.) stands exposed to natural fire in the section numbered 28 within the boundaries of Sarıçiçek Forest Sub-District Directorate within Amasya Regional Forest Directorate, Vezirköprü Forest Management Directorate in Turkish Pine stands exposed to natural fire and the control areas next to them (*Figure 1*). The fire damaged approximately 1 hectare of the forest area. Average slope of the study area was 20-30% and its aspect was southwest.

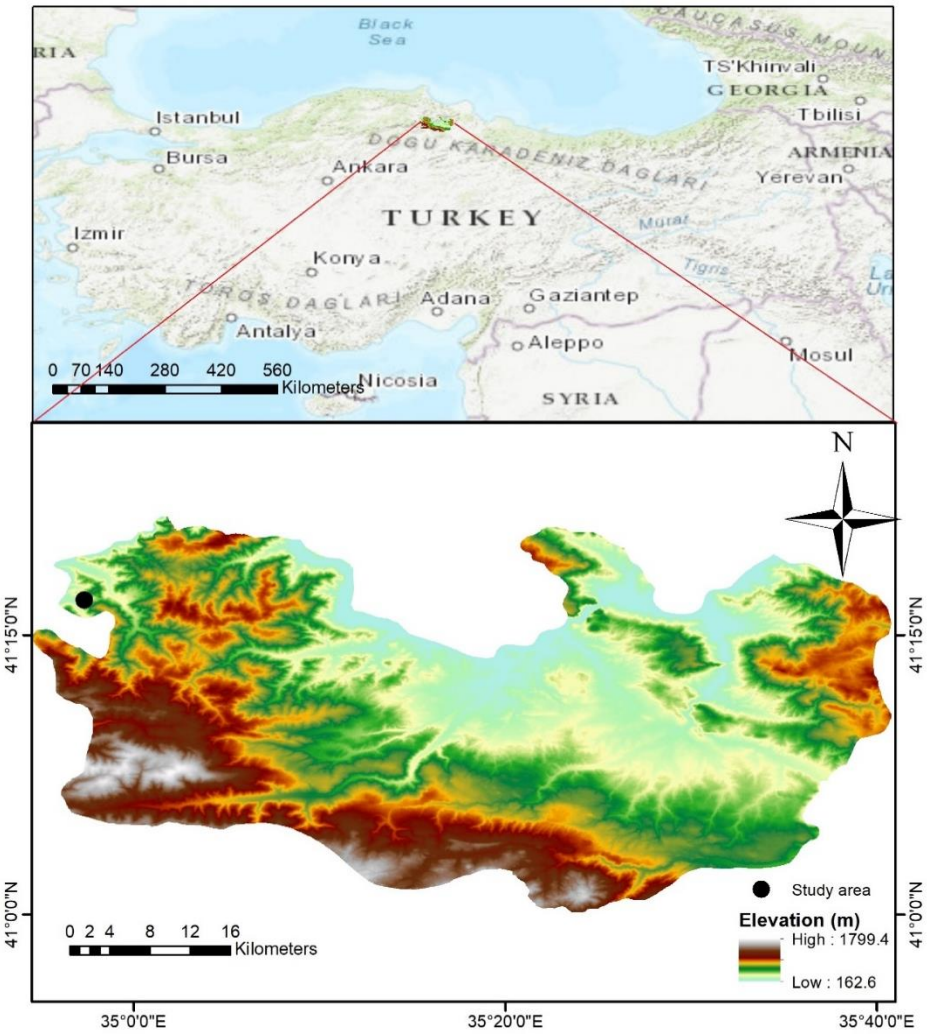

Figure 1. *Location (a) (green color) and Digital Elevation Model (ARC-GIS 9.2) (b) of the study area*

At the meteorological station where the research area is located, the highest average temperature occurs in July and August by 21.2 ºC, the lowest average temperature occurs in January by 0.9 ºC, the annual average temperature is 11.5 ºC, the lowest average humidity occurs in July by 60.3%, the highest average wind speed occurs in July by

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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2.2 m/sec, the lowest average precipitation occurs in August by 14 mm, the highest average precipitation occurs in May by 55.1 mm, and the annual precipitation is 415.6 mm.

With regard to geological structure, the Central Black Sea region consists of volcanic rocks such as basalt, andesite and granite. The soil type is generally sandy, clay soil (Anonymous, 2009).

The research area is located in the Euro-Siberian (Euxine) flora area, which is one of Turkey's three major flora regions (Anşin, 1983).

It was determined that the research area was composed of coniferous species (Scotch pine, Corsican pine, and Pinus brutia) and hardwood species (beech and hornbeam). While there are pure beech stands in the area, it was determined that they were also mixed with Scotch pine tree species.

Determination experimental plots method

The experimental plots were determined by carrying out a preliminary study on Turkish pine areas exposed to natural fire in February 2014 one month after the fire. A total of 12 experimental plots, including six plots from the burned area and six plots from the control areas, were determined (*Figure 2*). As the size of the experimental plots, 15 m * 20 m = 300 m^2 areas were chosen.

A B

Figure 2. *Views of burned area (A) and unburned area (B)*

Taking soil samples

Twenty-four soil samples (6 burned * 2 depths= 12, 6 controls * 2 depths=12, Total 24) were taken from the fire and control areas in every period. Soil sampling was performed in every three months (April 2014, July 2014, and October 2014). Analyses were conducted on a total of 72 soil samples. These soil samples were placed in double plastic bags, labeled and brought to the laboratory environment.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Net mineralization test

For nitrogen mineralization, the samples were taken from a depth of 0-5 cm and 5-10 cm from each sampling areas. The wet weights were measured by a digital weight meter. The samples were passed through 4 mm standard steel sieves, and some of them were placed in polyethylene bags, labeled, incubated under field conditions and then buried in the soil for net mineralization measurement. Some of the sieved soil was also labeled and brought to the laboratory to determine the actual mineral nitrogen. Mineralization measurements were conducted in three periods (April 2014 - July 2014, July 2014 - October 2014, and October 2014 - April 2015). Nitrogen mineralization was measured in a total of 432 soil samples, including mineralization and actual (initial) mineralization in field incubation.

Laboratory methods

The samples taken from the study area to the laboratory environment were dried until they were air-dried. The roots and stones of each sample were removed, labeled and placed in plastic bags. The dried soil samples were crushed in a mortar and sieved using a 2 mm standard steel sieve. The sieved samples were labeled and prepared for analysis. The texture analysis of the soils was performed based on the Bouyoucos hydrometer method (Gülcur, 1974). The pH of soil samples was determined by the glass electrode method using an Inolab pH level I pH meter (Gülcur, 1974). The organic matter in the soil was determined by the modified Walkley - Black wet decomposition method (Gülcur, 1974; Kacar, 2009). The Kjeldahl wet digestion method was used for the determination of total nitrogen (Steubing, 1965; Ozturk et al., 1997). The carbon/nitrogen ratio (C/N) is the ratio of organic carbon and organic nitrogen measured in percent to each other. The samples taken from the field with a soil bulk density cylinder were dried at 105 ºC, and the moisture in the soil was removed. After weighing the soil in the bulk, the bulk density was calculated by dividing it by cylinder volume (Gülcur, 1974).

Determination of mineral nitrogen

In the samples taken from the beginning of April 2014, actual mineral nitrogen and net mineral nitrogen yields were calculated. Mineral nitrogen was identified in a total of 432 soils, 144 soils in each period, at both depth levels. The micro-distillation method was used for the determination of mineral nitrogen in the soil (Bremner and Keeney, 1965; Gerlach, 1973; Güleryüz, 1992). The determination of mineral nitrogen consists of two stages. In the first stage, the amount of ammonium (NH_4^+-N) in the soil is found, and the amount of nitrate (NO_3^--N) is determined in the second stage (Öztürk et al., 1997). The net amount of ammonium was calculated by the difference between the ammonium value measured in the samples taken at the end of the incubation period and the ammonium value measured in the samples taken at the beginning of the incubation. This calculation was made for three periods. The annual net NH_4 yield was found by summing the ammonium yields obtained over three periods (*Eq.1*).

$$Net\ NH_4 = End\ of\ incubation\ NH_4 - Initial\ NH_4 \quad \text{(Eq.1)}$$

The net amount of nitrate was calculated by the difference between the nitrate value measured in the samples taken at the end of the incubation period and the nitrate value measured in the samples taken at the beginning of the incubation. This calculation was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

made for three periods. The annual net NO_3 yield was found by summing the nitrate yields obtained over three periods (*Eq.2*).

$$Net\ NO_3 = End\ of\ incubation\ NO_3 - Initial\ NO_3 \quad \text{(Eq.2)}$$

Net mineral nitrogen yield was calculated by adding the sum of the net ammonium yield and net nitrate yield.

The statistical analysis was performed on the data obtained by using the SPSS 16.0 and Windows statistical software. The analysis of variance was done to determine whether there was a temporal difference in the fire and control areas, and Tukey's test was carried out to determine where the differences were. The difference between the fire and control areas in terms of soil properties was determined by conducting the independent t-test. As a consequence of the tests, letters (such as A, B, AB, a, b) were provided as indicators of significance levels ($P<0.05$) and difference in the tables.

Results and Discussion

Results

The data obtained by some physical and chemical analyses on the soils are presented in *Tables 1 and 2*.

Table 1. *Temporal change data of some soil properties of the soils at a depth of 0-5 cm*

Soil Property	Sample Area	April 2014	July 2014	October 2014	Significance level
Sand	Control	73.70Aa	72.93Aa	77.39Aa	0.207
	Fire	71.02Aa	71.47Aa	68.68Ab	0.185
	Significance level	0.300	0.472	**0.001**	
Clay	Control	11.27Aa	6.89Ba	6.19Ba	**0.000**
	Fire	12.74Aa	8.97Aa	7.49Aa	0.131
	Significance level	0.586	0.182	0.241	
Silt	Control	15.03Aa	20.18Aa	16.42Aa	0.092
	Fire	16.24Aa	19.56ABa	23.82Bb	**0.037**
	Significance level	0.702	0.786	0.003	
pH	Control	6.76Aa	7.14Aa	7.17Aa	0.170
	Fire	7.03Aa	7.19Aa	7.46Aa	0.061
	Significance level	0.156	0.806	0.239	
Organic Matter	Control	7.91Aa	8.01Aa	9.27Aa	0.336
	Fire	9.34Aa	8.48Aa	8.33Aa	0.263
	Significance level	0.143	0.595	0.229	
Total Nitrogen	Control	0.26Aa	0.26Aa	0.30Aa	0.297
	Fire	0.25Aa	0.24Aa	0.23Ab	0.601
	Significance level	0.744	0.444	**0.008**	
C/N ratio	Control	18.0Aa	18.2Aa	18.3Aa	0.880
	Fire	22.0Ab	20.9Ab	21.0Ab	0.222
	Significance level	**0.000**	**0.007**	**0.001**	
Bulk Density	Control	1.09Aa	0.99Aa	0.96Aa	0.126
	Fire	1.06Aa	0.92Aa	0.92Aa	0.149
	Significance level	0.511	0.136	0.680	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

Table 2. *Temporal change data of some soil properties of the soils at a depth of 5-10 cm*

Soil Property	Sample Area	April 2014	July 2014	October 2014	Significance level
Sand	Control	64.5Aa	67.3ABa	73.8Ba	**0.023**
	Fire	65.5Aa	68.9Aa	66.4Ab	0.594
	Significance level	0.773	0.621	**0.029**	
Clay	Control	18.5Aa	13.5ABa	7.8Ba	**0.001**
	Fire	14.9Aa	10.2Aa	9.5Aa	0.053
	Significance level	0.288	0.125	0.088	
Silt	Control	17.04Aa	19.23Aa	18.44Aa	0.623
	Fire	19.56Aa	20.88Aa	24.12Aa	0.254
	Significance level	0.150	0.588	0.062	
pH	Control	6.43Aa	7.20Aa	7.30Aa	0.060
	Fire	6.57Aa	6.90Aa	7.48Ba	**0.000**
	Significance level	0.726	0.091	0.507	
Organic Matter	Control	4.31Aa	5.73ABa	7.24Ba	**0.048**
	Fire	6.08ABb	4.86Aa	6.97Ba	**0.026**
	Significance level	**0.030**	0.411	0.787	
Total Nitrogen	Control	0.14Aa	0.16Aa	0.19Aa	0.134
	Fire	0.16ABa	0.14Aa	0.18Ba	**0.019**
	Significance level	0.122	0.448	0.549	
C/N ratio	Control	18.1Aa	21.2Aa	21.6Aa	0.042
	Fire	21.6Ab	20.6Aa	22.1Aa	0.306
	Significance level	0.013	0.605	0.689	
Bulk Density	Control	1.12Aa	1.21Aa	1.15Aa	0.640
	Fire	1.10Aa	1.15Aa	1.14Aa	0.703
	Significance level	0.522	0.346	0.964	

According to these data, at a depth of 0-5 cm, while soil properties such as sand, nitrogen content and bulk density generally decreased with the fire, properties such as clay, silt, pH, organic matter and C/N ratio increased. Along the passage of time over the fire, sand, clay, organic matter, nitrogen, and bulk density decreased. This decrease or increase was not found to be statistically significant, except for the silt in the fire area. The difference in soil properties values between the burned area and unburned area was not generally statistically significant. The increase or decrease in soil properties were at very low levels.

At a depth of 5-10 cm, while soil properties such as sand, silt, pH, and C/N ratio generally increased with the fire, properties such as clay, organic matter, nitrogen, and bulk density tended to decrease. The difference in soil properties values between the burned area and unburned area at a depth of 5-10 cm was not statistically significant. The increase or decrease in soil properties was at very low levels. The temporal change in soils in the areas exposed to fire was found to be statistically significant in pH, organic matter, and nitrogen values.

Net nitrogen mineralization values are presented in *Table 3*. At a depth of 0-5 cm, while the amount of ammonium was lower in the fire areas than control areas, the amount of nitrate was higher. In the mineralization measurements performed in the burned areas,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

while it was revealed that ammonium tended to decrease first and then to increase and finally to decrease compared to the control areas, there was an increase, and then an increase and finally a decrease in nitrate values. The total mineralization value at a depth of 0-5 cm was found to be low in the fire area only in the October 2014-April 2015 mineralization period. The total annual mineralization value was identified to be slightly higher in the fire area. The temporal change in the fire areas was determined to be significant in ammonium and total nitrogen mineralization ($P<0.05$). The difference in all mineral nitrogen values between the burned area and unburned area was found to be statistically insignificant ($p>0.05$).

Table 3. *Mean net nitrogen mineralization values at a depth of 0-5 cm and 5-10 cm*

	Sample Area	Depth	Measurement Period			Total Annual	Significance level
			April-July 2014	July-October 2014	October 2014- April 2015		
NH_4 (kg/ha)	Fire	0-5 cm	1.58Aa	1.59Aa	2.57Ba	5.74a	**0.008**
	Control		1.64Aa	1.53Aa	2.7Ba	5.87a	**0.010**
	Significance level		0.863	0.865	0.720	0.786	
NO_3 (kg/ha)	Fire	0-5 cm	2.14Aa	1.37Aa	2.43Aa	5.94a	0.208
	Control		1.44Aa	1.27Aa	2.57Ba	5.28a	**0.012**
	Significance level		0.113	0.715	0.849	0.584	
NH_4+NO_3 (kg/ha)	Fire	0-5 cm	3.72ABa	2.96Aa	5.00Ba	11.68a	**0.032**
	Control		3.08Aa	2.80Aa	5.27Ba	11.15a	**0.001**
	Significance level		0.306	0.779	0.727	0.498	
NH_4 (kg/ha)	Fire	5-10 cm	1.50Aa	1.49Aa	3.2Ba	6.19a	**0.000**
	Control		1.45Aa	1.66Aa	3.16Ba	6.27a	**0.000**
	Significance level		0.915	0.448	0.893	0.578	
NO_3 (kg/ha)	Fire	5-10 cm	1.52Aa	1.44Aa	2.79Aa	5.75a	0.058
	Control		2.33Aa	1.61Aa	3.84Ba	7.78a	**0.000**
	Significance level		0.176	0.523	0.113	0.096	
NH_4+NO_3 (kg/ha)	Fire	5-10 cm	3.02Aa	2.93Aa	5.99Ba	11.94a	**0.003**
	Control		3.78Aa	3.27Aa	7.00Ba	14.05a	**0.000**
	Significance level		0.389	0.430	0.201	0.128	

At a depth of 5-10 cm, ammonium mineralization was revealed to be low in the fire areas, as at a depth of 0-5 cm. When the temporal change was analyzed, it was determined that the amount of ammonium decreased in the first measurement period, decreased in the second measurement period, and slightly increased in the third measurement period with the fire compared to control areas. Concerning this change in nitrate values, there was a decrease in all three measurement periods. Total mineral nitrogen values were

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

determined to be higher in the control area compared to the fire area. The temporal change in the fire areas was found to be statistically significant in ammonium and nitrate values, as at a depth of 0-5 cm ($P<0.05$). The difference between all mineral nitrogen data in the burned and unburned areas was found to be insignificant in all periods ($P>0.05$).

The values of nitrogen mineralization per hectare in the burned and unburned areas are presented in *Table 4*. According to these values, the ratio of nitrate to ammonium was found to be higher than 1 in the fire area at a depth of 0-5 cm. However, it was determined to be low in the control area. At a depth of 5-10 cm, it was greater than 1 in the control area and less than 1 in the fire area. At a depth of 0-10 cm, these ratios were calculated to be 1.08 and 0.98 in the unburned area and burned area, respectively. Nitrification values also revealed similar results. At a depth of 0-10 cm, while annual ammonium mineralization values were 11.93 kg/ha and 12.14 kg/ha in the burned area and unburned area, respectively, nitrate mineralization values were 11.69 kg/ha and 13.06 kg/ha, respectively. Finally, the total mineralization values were 23.56 kg/ha and 25.20 kg/ha, respectively. Based on these results, it was observed that mineralization values decreased with the fire.

Table 4. *Net mineralization and nitrification values and ratios in soils*

Depth (cm)	Parameter	Control	Fire	Parameter	Control	Fire
0-5	NH_4 (kg/ha)	5.87	5.74	NH_4 (mg/kg)	12.04	12.62
0-5	NO_3 (kg/ha)	5.28	5.94	NO_3 (mg/kg)	11.10	12.79
0-5	NH_4+NO_3 (kg/ha)	11.15	11.68	NH_4+NO_3 (mg/kg)	23.14	25.41
0-5	NO_3/NH_4	0.90	1.03	NO_3/NH_4	0.92	1.01
0-5	NO_3/NH4+ NO_3*100 (%)	47	51	NO_3/NH4+ NO_3*100 (%)	48	50
5-10	NH_4 (kg/ha)	6.27	6.19	NH_4 (mg/kg)	11.43	11.42
5-10	NO_3 (kg/ha)	7.78	5.75	NO_3 (mg/kg)	14.13	10.69
5-10	NH_4+NO_3 (kg/ha)	14.05	11.94	NH_4+NO_3 (mg/kg)	25.56	22.11
5-10	NO_3/NH_4	1.24	0.93	NO_3/NH_4	1.24	0.94
5-10	NO_3/NH4+ NO_3*100 (%)	55	48	NO_3/NH4+ NO_3*100 (%)	55	48
0-10	NH_4 (kg/ha)	12.14	11.93	NH_4 (mg/kg)	23.47	24.07
0-10	NO_3 (kg/ha)	13.06	11.69	NO_3 (mg/kg)	25.23	23.48
0-10	NH_4+NO_3 (kg/ha)	25.20	23.56	NH_4+NO_3 (mg/kg)	48.7	47.55
0-10	NO_3/NH_4	1.08	0.98	NO_3/NH_4	1.07	0.98
0-10	NO_3/NH4+ NO_3*100 (%)	52	50	NO_3/NH4+ NO_3*100 (%)	52	49

As a nitrogen mineralization values were calculated in mg/kg, while the total annual values were 24.07 mg/kg and 23.47 mg/kg in the burned area and unburned area, respectively, in ammonium at a depth of 0-10 cm, they were 23.48 and 25.23, respectively, in nitrate and 47.55 and 48.70 mg/kg, respectively, in total mineralization values. In the evaluation of mineralization values in mg/kg, it was observed that ammonium mineralization increased, and nitrate and total mineralization decreased with the fire at a depth of 0-10 cm. However, it was revealed that all three mineralization components increased with the fire at a depth of 0-5 cm.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

Discussion

There have been some increases and decreases in the sand, clay and silt values examined in the texture measurements performed in the soil after the fire. However, this alteration was not statistically significant. In many studies, it was concluded that there might be a difference in texture values with the breakdown of soil particles under high temperatures (Iglesias et al., 1997; De Bano et al., 1998, Hubbert et al., 2006; Chief et al., 2012). On the other hand, some researchers stated that there was no significant change in the texture structure of the fire in low-intensity fires (Granged et al., 2012; Scharenbroch et al., 2012). In the presented study, the low fire intensity confirms the findings obtained with a literature study.

Soil pH values were analyzed; they were revealed to be higher in the topsoil in the fire area compared to the control area. In many studies, it was indicated that the soil pH value increased after the fire, especially in the topsoil (Certini, 2005; Küçük, 2006; Ekinci, 2006; Scharenbroch et al., 2012; Berber et al., 2015; Muqaddas et al., 2015; Kong et al., 2019). In this study, the effect of fire on pH was not found to be statistically significant, which can be attributed to the very low intensity of the fire. Some researchers stated that the effect of fire on pH was statistically insignificant, as in the presented study (Switzer et al., 2012; Meira-Castro et al., 2014; Alcañiz et al., 2016; Valkó et al., 2016; Lucas-Borja et al., 2020)

Soil organic matter values were examined; it was observed that the effect of the fire was insignificant. The reason for it may be the low severity of the fire because changes in soil properties in low-intensity fires were less. However, in many studies, it is indicated that the fire has a significant effect on the soil organic matter (Johnson and Curtis, 2001; Choromanska and de Luca, 2001; Six et al., 2002; Muqaddas et al., 2015; Alcañiz et al., 2016). On the other hand, in some studies, researchers stated that the effect of forest fire on soil organic matter change was insignificant (Gundale et al., 2005; Lavoie et al., 2010; Valkó et al., 2016). While the soil organic matter values in the fire area were higher in the first six months after the fire compared to the control area, they were found to be lower at later times. When the overall averages were evaluated, the amount of organic matter in the fire area was found to be slightly higher compared to the control area.

The total nitrogen values in the fire area were determined to be close to each other in the first six months after the fire. Then, there was a serious decrease in the fire area. Some researchers stated that, the fire decreased nitrogen in the soil (Dzwonko et al., 2015; Muqaddas et al., 2015; Francos et al., 2019). Based on the statistical evaluation, the effect of the fire on total nitrogen was revealed to be insignificant. However, in many studies, it was concluded that the fire played an increasing role in total nitrogen in the soil (Knoep and Swank, 1993; Johnson and Curtis, 2001; Knoep et al., 2004; Scharenbroch et al., 2012; Alcañiz et al., 2016).

İt was observed that the fire generally played an increasing role in the carbon/nitrogen ratio. The effect of the fire on nitrogen and carbon-nitrogen ratio was also found to be statistically significant. In some studies, it was reported that the C/N value increased after the fire (Gundale et al., 2005; Scharenbroch et al., 2012), which is considered to be due to the fact that the increase in organic carbon in the soil after the fire led to an increase in the C/N ratio. Likewise, in the correlation analysis, the fact that we found a negative correlation between the C/N and total nitrogen supported this statement.

The bulk density values at both depths were revealed to be lower in the fire area in comparison with the control area. However, this low value was not statistically significant. It could be said that the low bulk density in the fire area was caused by higher

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DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

organic matter and ash content in the fire areas. High organic matter decreases bulk density. The effect of time on bulk density was also determined to be insignificant both in the fire area and in the control area. In some studies, researchers stated that bulk density decreased after the fire (Brye, 2006; Chief et al., 2012; Mastrolonardo et al., 2015).

Ammonium mineralization showed lower values in the fire area at a depth of 0-5 cm after the fire compared to the unburned areas, it exhibited higher values at a depth of 5-10 cm. At a depth of 0-10 cm, this value was also found to be higher in the control areas. However, it was revealed that this alteration was not statistically insignificant. Since the measurements were made on a hectare basis, changes, especially in bulk density and soil moisture content, affected these values because this change was found to be more different in the mg/kg measurement units of ammonium mineralization. Especially at a depth of 0-5 cm, ammonium mineralization values were determined to be higher in the burned areas compared to the unburned areas. The fact that the fire in the study area was in the form of a low-intensity surface fire also appeared to play a determining role in this effect. The effect of the fire occurred more significantly at a depth of 0-5 cm. The effect of time on mineralization was found to be significant. Changes in soil temperature, soil moisture, soil pH and soil organic matter, and therefore, changes in the C/N ratio over time can be shown as the factors causing it because these variables change the amount of ammonium in the soil. The periodic change of organic matter in the fire area influences mineral ammonium. The removal of litter on the soil surface in the fire areas could affect the net mineral ammonium values in seasonal changes in soil moisture. In general, the net ammonium yield and nitrate yield are high in moist soils and dry soils, respectively (Anggria et al., 2012).

Mineral nitrate yields were identified to be higher in the fire areas at a depth of 0-5 cm and in the control areas at a depth of 5-10 cm. These measurements were calculated on a kg/ha basis. However, this difference was found to be statistically insignificant. The results obtained when the measurements were evaluated on an mg/kg basis did not change the outcome of these changes a lot. Nitrate mineralization on an mg/kg basis occurred in the fire area at a depth of 0-5 cm and in the control area at a depth of 5-10 cm. Therefore, the degree of influence of the fire was also limited at a depth of 0-5 cm. In particular, nitrate mineralization is associated with pH values. Nitrification events in the soil increase as pH increases. It is indicated by some researchers that the nitrate content increases as pH increases (Robinson, 1963; Black, 1968; Sahrawat, 1982; Lopez and Martin, 1995; Paul and Clark, 1996; Neary et al., 1999; Anggria et al., 2012). In their study, Mikita-Barbato et al. (2015) found that the amount of nitrate in the fire areas was higher compared to the control areas. Fernandez-Fernandez et al. (2015) revealed that the mineral nitrate values in the fire area on a kg basis were lower compared to the control area. As a reason for it, they indicated the high intensity of the fire. Low fire intensity in our areas may not affect microorganism activities too much. The fact that organic matter is easily decomposed by microorganisms and reaches the appropriate pH environment enables us to find the nitrate yield in the fire areas in this study higher compared to the control areas. There was also a linear relationship between the amount of organic matter in the soil and nitrogen content mineral nitrate yield. High organic matter plays an increasing role in nitrate mineralization. It is considered that the fact that the organic matter in the fire area was found to be high also led to an increase in the amount of mineral nitrate.

The effect of time on total net mineralization data was determined to be significant both in the fire area and in the control area. As a reason for it, it is considered that

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1806_83558371

properties such as changes in temperature and precipitation conditions within the periods and changes in microorganism activities and physiological activities of plants explained this difference. It is also considered that different properties such as organic matter, pH nitrogen, and C/N between periods led to this difference. In many studies, it was determined that organic matter, total nitrogen, and C/N value were associated with the total net mineralization values. In various studies, it was also concluded that nitrogen mineralization increased after the fire (Kovacic et al., 1986; Kaye and Hart, 1998; Knoepp and Swank, 1993; Hamman et al., 2008). The researchers attributed this increase to the combustion products, evaporation of organic nitrogen from the soil surface, microorganism activities in the soil, and changes in pH, soil temperature, and moisture in the soil (Blair, 1997; Knoepp et al., 2004).

Conclusions

As a result of this study, the results of one-year period on the soil properties and biological activities of the fire in the region where red pine grows under extreme growing conditions were evaluated. Since the fire severity is low and it is out of the fire season at the time of the fire, the impact of the fire on the soil properties did not emerge at statistically significant levels. In order to reveal the effect of fire on nitrogen mineralization, especially in red pine ecosystems, it can be investigated by experiment fires with different fire intensities and its effects on soil properties. Again, regional differences can be revealed by planning trial fires in the Mediterranean Region and Black Sea Region in the same period. Again, according to the results of this study, considering that the fire increases the soil pH, organic matter and mineralization amounts, it can facilitate rejuvenation activities by making it easier for the seed to reach the soil by going to cover fire studies, especially during abundant seed years.

Prescribed fire practices ensure that nutrients kept in the litter are transferred to the soil, especially in areas with low litter decomposition. In this way, the seed is provided to reach the soil, and germination and sapling development are positively affected in rejuvenation studies. Practitioners can easily achieve this goal by applying low density cover fire applications in such areas. By performing these applications at regular intervals, an increase in the accumulation of substances, which will trigger the fire, can be prevented. In this way, the possibility of a fire is reduced during periods of intense fire risk.

Acknowledgements. This study has been prepared using the data in Unal Kahveci's master thesis. We would like to thank all the staff of Vezirköprü Forest Management Directorate for their support in the realization of this study.

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