ESTIMATION OF THE LOWER AND UPPER QUANTILES OF GUMBEL DISTRIBUTION: AN APPLICATION TO WIND SPEED DATA

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Abstract. In this paper, we consider different estimators of the quantiles of two-parameter Gumbel distribution. We use methodologies known as maximum likelihood, modified maximum likelihood and probability weighted moment to obtain the estimators of the quantiles. We compare the performances of the estimators with respect to bias and mean square error criteria via Monte Carlo simulation study. Their robustness properties are also examined in the presence of data anomalies. In the real data analysis part of the study, the seasonal maximum daily wind speed data from Sinop station (Turkey) in 2015 is considered. It is modelled by using two-parameter Gumbel distribution and analysed to compare the performances of the methodology presented in the study. All in all, the results of simulations and the real data application show that the maximum likelihood and modified maximum likelihood estimators, which have similar performance, provide better performance than the probability weighted moment estimator does in both obtaining estimates of the quantiles of Gumbel distribution and modelling of the data for almost all cases.
Keywords: *Gumbel distribution, quantile, modelling extreme events, efficiency, robustness*

Introduction

Estimation of the quantiles of any distribution is very important in real life problems. As examples due to Modarres et al. (2002) "*Estimates of the upper quantiles of the distribution of a risk factor or an exposure index are commonly used to assess the risk to human health as a result of exposure to chemicals and microbes in the environment, or to determine if concentration levels of contaminants exceed specified limits*" and Goel et al. (2004) "*Extreme wind quantiles are needed to calculate design values of wind load effect on structures*". Therefore, in literature, various different distributions have been considered by many authors in the context of extreme value analysis, for example Gumbel distribution, Wakeby distribution, Generalized Pareto distribution, Generalized Extreme-Value distribution, Log-normal, Log-logistic and Log-double exponential distributions and Frechet distribution (Landwehr and Matalas, 1979a; Landwehr and Matalas, 1979b; Hosking and Wallis, 1987; Martins and Stedinger, 2000; Modarres et al., 2002; Koutsoyiannis, 2004).

The Gumbel known as the Extreme Value type I distribution, first proposed by Gumbel (1941), is one of the most widely probabilistic models used in modelling the extreme events in many research studies, for example, total snowfall, maximum snow, air pollution and maximum daily flood discharges (Simiu et al., 2001; Koutsoyiannis, 2004; Graybeal and Leathers, 2006; Ercelebi and Toros, 2009; Aydin and Senoglu, 2015). On the other hand, in the literature, although the most widely used statistical distribution for modelling the wind speed data is Weibull, it may not provide better fitting for all wind regimes. For this reason, different distributions are used for modelling the wind speed data (Brano et al., 2011; Kantar and Usta, 2015; Alavi et al., 2016; Jung et al., 2017). For example, Gumbel distribution has also been used to both

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estimate extreme wind speed required for the determination of the wind turbine class in the wind power industry and evaluate the wind energy potential required designing a wind turbine (Hong et al., 2013; Kang et al., 2015). Additionally, Lee et al. (2012) reported that the Gumbel distribution is more reliable than the Weibull distribution in modelling the extreme wind speeds. Martin et al. (2014) showed that the Gumbel distribution estimates wind speed more accurately than the Weibull distribution does.

Aim of this paper is to obtain the estimators of the lower and the upper quantiles of the Gumbel distribution. The estimators of the quantiles are obtained by using the well-known and widely used maximum likelihood (*ML*) methodology. The likelihood equations, however, do not have explicit solutions. Therefore, we use two different approaches to solve them. The first approach is iterative and other one is non-iterative which is called as modified maximum likelihood (*MML*). We also use, the probability weighted moment (*PWM*), which is very popular methodology in hydrology and climatology. The reason of using *PWM* is its conceptual simplicity, implementation and good performance. Furthermore, wind speed data obtained from the Turkish State Meteorological Service is modelled by Gumbel distribution and analysed to show the performance of the considered estimation methods.

Materials and methods

The seasonal wind speed data

In this study, the seasonal wind speed data recorded at the heights of 10 m in maximum daily basis in 2015 in Sinop station (Turkey) is analysed. Geographical coordinates for this station are given as

Station	Region in Turkey	Latitude (N)	Longitude (E)	Altitude (m)
Sinop	North	42°01'44"	35°09'19"	32

In *Table 1*, descriptive statistics which are mean, minimum (*Min*), maximum (*Max*), median, standard deviation (*SD*) and range for seasonal maximum daily wind speed data (m/s) are given.

***Table 1**. Summary of the descriptive statistics for the seasonal maximum daily wind speed data*

Season	Mean	*Min*	*Max*	Median	*SD*	Range	*n*
Winter	10.0189	3.2000	24.6000	9.8000	4.2694	21.4000	90
Spring	9.5087	4.6000	18.3000	9.2000	2.9934	13.7000	92
Summer	7.8228	4.4000	17.3000	7.6000	2.0648	12.9000	92
Autumn	8.9560	4.5000	19.5000	8.3000	2.9676	15.0000	91

According to the results given in *Table 1*, range (which is defined as the difference between the highest and the lowest value) is the largest in winter (December-January), and is the smallest in summer (June-August) as expected. Similar comments can also be done for *SD* which is another measure of variability.

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Gumbel distribution

Probability density function (*pdf*) $f(x)$ and the cumulative density function (*cdf*) $F(x)$ of the Gumbel distribution are given by *Equation 1*

$$f(x) = \frac{1}{\delta} e^{-\left((x-\theta)/\delta + e^{-(x-\theta)/\delta}\right)}, \ x \epsilon \mathbb{R} \tag{Eq. 1}$$

and *Equation 2*

$$F(x) = e^{-e^{-(x-\theta)/\delta}}, \tag{Eq. 2}$$

respectively. Here, $\theta \in \mathbb{R}$ is location parameter and $\delta > 0$ is scale parameter. The location parameter θ is also the mode of the distribution. Inverse of the *cdf* in *Equation 2,* i.e. $x(F)$, is obtained as follows (*Eq. 3*)

$$x(F) = \theta - \delta \ln(-\ln F). \tag{Eq. 3}$$

The moment generating function of Gumbel distribution is given by *Equation 4:*

$$M(t) = e^{\theta t} \Gamma(1 - \delta t), t < 1/\delta. \tag{Eq. 4}$$

Mean ($E(X)$), variance ($Var(X)$), skewness ($\sqrt{\beta_1}$) and kurtosis (β_2) values of Gumbel distribution are given as follows:

$E(X)$	$Var(X)$	$\sqrt{\beta_1}$	β_2
$\theta + \delta\gamma$	$\pi^2/6\,\delta^2$	1.14	5.4

where γ is Euler's constant defined by *Equation 5:*

$$\gamma = -\int_0^\infty \ln x \, e^{-x} dx. \tag{Eq. 5}$$

Gumbel distribution is related to the Weibull distribution. In particular, if *Y* has a Weibull distribution with shape parameter ϕ and scale parameter λ, then (*Eq. 6*)

$$X = -log(Y) \tag{Eq. 6}$$

has a Gumbel distribution with the location parameter $\theta = -log(\lambda)$ and the scale parameter $\delta = 1/\phi$.

The graphs of the *pdf* of the Gumbel distribution for some selected values of the location parameter θ and the scale parameter δ are given in *Fig. 1*. It is clear from *Fig. 1* that Gumbel distribution is unimodal and skewed to the right.

Estimation of quantiles

Let X_q be *q*-th quantile of the Gumbel random variable X. It is defined as (*Eq. 7*)

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$$X_q = \theta - \delta \ln(-\ln q), 0 < q < 1, \quad \text{(Eq. 7)}$$

see *Equation 3*. Estimator of the quantile X_q, i.e. $\hat{X}_q$, is obtained by substituting the estimators of the parameters θ and δ in *Equation 7*.

In the following subsections, we briefly describe the estimation techniques mentioned before for estimating the quantiles of the Gumbel distribution.

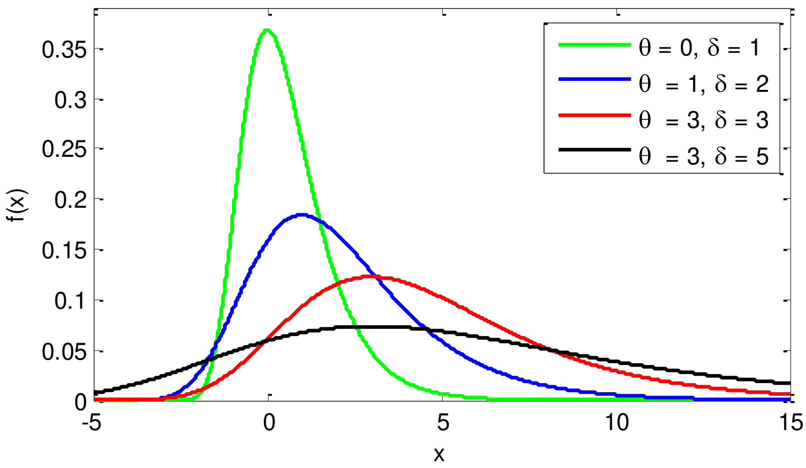

***Figure 1**. Plots of the Gumbel distribution for some selected and values*

The method of maximum likelihood

The *ML* estimators $\hat{\theta}$ and $\hat{\delta}$ of the parameters θ and δ are the solutions of the following likelihood equations (*Eqs. 8 and 9*)

$$\frac{\partial \ln L(\theta,\delta)}{\partial \theta} = \sum_{i=1}^{n} \frac{1}{\delta} - \frac{1}{\delta} \sum_{i=1}^{n} g(z_i) = 0 \quad \text{(Eq. 8)}$$

$$\frac{\partial \ln L(\theta,\delta)}{\partial \delta} = -\frac{n}{\delta} + \frac{1}{\delta} \sum_{i=1}^{n} z_i - \frac{1}{\delta} \sum_{i=1}^{n} z_i g(z_i) = 0 \quad \text{(Eq. 9)}$$

where $g(z_i) = e^{-z_i}$ and $z_i = (x_i - \theta)/\delta$. It is obvious that explicit solutions of the likelihood equations cannot be obtained because of the nonlinear term $g(.)$. Therefore, we can use two different approaches to solve the likelihood equations. One is iterative and the other one is non-iterative given in the next subsection.

The method of modified maximum likelihood

The *MML* estimators of parameters θ and δ are obtained by linearizing the non-linear term $g(z_i)$ in the likelihood equations in (*Eq. 8*) and (*Eq. 9*). We linearize the likelihood equations by using the first two terms of Taylor series expansion around the expected values of the standardized order statistics, i.e. $t_{(i)} = E\left(z_{(i)}\right)$ and $z_{(i)} = \left(x_{(i)} - \theta\right)/\delta$, (Tiku, 1967; Tiku, 1968). Solutions of these modified likelihood equations are the following *MML* estimators (*Eq. 10*):

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$$\hat{\theta}_{MML} = K + L\hat{\delta}_{MML} \text{ and } \hat{\delta}_{MML} = \frac{-B+\sqrt{B^2-4nC}}{2\sqrt{n(n-1)}}, \quad \text{(Eq. 10)}$$

where $K = \frac{1}{m}\sum_{i=1}^{n}\beta_i x_{(i)}$, $L = \frac{\Delta}{m}$, $\Delta = \sum_{i=1}^{n}\Delta_i$, $\Delta_i = (\alpha_i - 1)$, $m = \sum_{i=1}^{n}\beta_i$, $\mathrm{B} = \sum_{i=1}^{n}\Delta_i\left(x_{(i)} - \hat{\theta}_{MML}\right)$, $C = \sum_{i=1}^{n}\beta_i\left(x_{(i)} - \hat{\theta}_{MML}\right)^2$, $\alpha_i = e^{-t_{(i)}} + t_{(i)}e^{-t_{(i)}}$, $\beta_i = -e^{-t_{(i)}}$ and $t_{(i)} = -\ln\left(-\ln\left(\frac{i}{n+1}\right)\right)$, $i = 1,2,\cdots,n$.

The *MML* estimators are asymptotically equivalent to the *ML* estimators. Therefore, they are asymptotically fully efficient under the regularity conditions. They have high efficiencies even for small sample sizes. They are also robust to plausible deviations from the assumed distribution and also to the presence of the outliers in the data set (Tiku and Suresh, 1992; Vaughan and Tiku, 2000).

The method of probability weighted moment

The *PWM* estimators of θ and δ are obtained as (*Eq. 11*)

$$\hat{\theta}_{PWM} = \widehat{M}_{(0)} - \gamma\hat{\delta}_{PWM} \text{ and } \hat{\delta}_{PWM} = \frac{\widehat{M}_{(0)} - 2\widehat{M}_{(1)}}{\ln 2}, \quad \text{(Eq. 11)}$$

respectively (Greenwood et al., 1979; Landwehr et al., 1979a). Here, γ is Euler's constant and $\widehat{M}_{(k)}$ is an unbiased estimate of $M_{(k)}$ (*Eq. 12*):

$$\widehat{M}_{(k)} = \frac{1}{n}\sum_{i=1}^{n} x_{(i)}\frac{(n-i)!(n-1-k)!}{(n-1)!(n-i-k)!} \quad \text{(Eq. 12)}$$

where $x_{(i)}$ are *i*-th ordered observations and $M_{(k)} = M_{1,0,k}$ is calculated from the following *PWM*s equality for $i,j,k \in \mathbb{R}$ (*Eq. 13*):

$$M_{i,j,k} = E\left(X^i\left(F(X)\right)^j\left(1-F(X)\right)^k\right) = \int_0^1\left(x(F)\right)^i F^j(1-F)^k dF. \quad \text{(Eq. 13)}$$

Here, $F(X)$ is the *cdf* of the random variable X and $x(F)$ is the corresponding inverse distribution function.

Simulation study

To compare the performances of *ML*, *MML* and *PWM* estimators of the *q*-th quantile of the Gumbel distribution X_q, an extensive Monte Carlo simulation study is designed and conducted with respect to their biases and mean squared error (*MSE*) for different sample sizes and quantile values. Bias and *MSE* for $\hat{X}_q$ are calculated as (*Eqs. 14* and *15*):

$$Bias(\hat{X}_q) = 1/n\sum_{i=1}^{n}\left(X_q - \hat{X}_{q_i}\right) \quad \text{(Eq. 14)}$$

$$MSE(\hat{X}_q) = 1/n\sum_{i=1}^{n}\left(X_q - \hat{X}_{q_i}\right)^2 \quad \text{(Eq. 15)}$$

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respectively. Here, n is the number of replication and $\hat{X}_{q_i}$ is the estimate of X_q in i-th replication. We also calculate the relative efficiencies (*RE*) of the *ML* estimator with respect to the *MM*L and *PWM* estimators of X_q, i.e. (*Eq. 16*),

$$RE = \left(MSE(\hat{X}_q)/MSE(\hat{X}_{q,ML})\right) \times 100. \tag{Eq. 16}$$

We consider the sample sizes, $n = 5$, 10, 50, 100 and 1000 and quantile values, $q = 0.01$, 0.05, 0.10, 0.90, 0.95 and 0.99. Bias and *MSE* values of the estimators are computed based on $[\![100{,}000/n]\!]$ replications. Here, $[\![.]\!]$ indicates the greatest integer value. Without loss of generality, it is assumed that the location parameter $\theta = 0$ and the scale parameter $\delta = 1$.

Here, the quantile estimates $\hat{X}_q$ are computed by substituting the estimates of the parameters θ and δ in *Equation 7*, i.e. (*Eq. 17*),

$$\hat{X}_q = \hat{\theta} - \hat{\delta}\ln(-\ln q),\ 0 < q < 1. \tag{Eq. 17}$$

Robustness properties of the estimators

To compare the robustness properties of estimators mentioned above, the efficiencies of the *ML*, *MML* and *PWM* estimators of the quantiles of the Gumbel distribution are examined via Monte-Carlo simulation study when there exist data anomalies, such as misspecification of the model and presence of the outliers in the data set. For this purpose, Gumbel with location parameter $\theta = 0$ and scale parameter $\delta = 1$, i.e., $G(\theta = 0, \delta = 1)$ is assumed as true model, and consider the following alternative models:

(i) *Model I*: Misspecified model: $G(\theta = 0, \delta = 2)$,
(ii) *Model II*: Misspecified model: $G(\theta = 1, \delta = 1)$,
(iii) *Model III*: Contamination model: $0.90G(\theta = 0, \delta = 1) + 0.10U(-3,3)$,
(iv) *Model IV*: Mixture model: $0.90G(\theta = 0, \delta = 1) + 0.10G(\theta = 0, \delta = 2)$,
(v) *Model V*: Dixon's outlier model:
$$(n-r)G(\theta = 0, \delta = 1) + rG(\theta = 0, \delta = 2),\ r = [\![0.1n + 0.5]\!].$$

Model evaluation

The suitability of estimates of Gumbel distribution in fitting the wind speed data can be evaluated by numerical methods. For this purpose, the root mean square error (*RMSE*) and coefficient of determination (R^2) are used and they are calculated by using the following formulas (*Eqs. 18* and *19*)

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(\hat{F}(X_{(i)}) - u_i\right)^2} \tag{Eq. 18}$$

$$R^2 = 1 - \frac{\sum_{i=1}^{n}\left(\hat{F}(X_{(i)}) - u_i\right)^2}{\sum_{i=1}^{n}\left(\hat{F}(X_{(i)}) - \bar{\hat{F}}(X_{(i)})\right)^2}, \tag{Eq. 19}$$

respectively (Nash, 1970; Barrett, 1974; Jöreskog and Sörbom, 1981; Willmott, 1982). Here, $\hat{F}(X_{(i)})$ is the estimated value of the *cdf* for the i-th order statistics. u_i is the

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expected value of $\hat{F}(X_{(i)})$ and is equivalent to $i/(n+1)$. $\bar{\hat{F}}$ is the mean of the estimated *cdf*s $\hat{F}$, i.e., $\bar{\hat{F}} = \frac{1}{n}\sum_{i=1}^{n}\hat{F}$. It should be noted that lower value of $RMSE$ and the higher the R^2 indicate better fitting to the data.

Results

Simulation results

To compare the performances of the methods presented in the previous section, results of some simulation studies are presented in *Table 2*. All the computations were performed by using MATLAB R2010a. It should be noted that *Table 2* gives the bias and *MSE* values of $\hat{X}_q$ for both the lower (i.e., $X_{0.01}$, $X_{0.05}$ and $X_{0.10}$) and the upper (i.e., $X_{0.90}$, $X_{0.95}$ and $X_{0.95}$) tail quantiles. It is observed that the *PWM* estimator of X_q shows better performance than the other estimators do with respect to bias criterion for all values of *q* even for small sample sizes (Landwehr et al., 1979a). As the sample size *n* increases, all the estimators show more or less the same performance.

The *ML* estimator outperforms the other estimators almost in all cases in terms of the *MSE* criterion. It should be noted that both *MSE* and *Bias* decrease while the sample size *n* increases which signifies that all of these estimators are consistent. Especially for $n > 5$, *MSE* values of *ML* and *MML* estimators are quite close to one another as expected. Also, the *MSE*s of lower tail quantiles are smaller than *MSE*s of upper tail quantiles since the Gumbel distribution is skewed to the right, see *Table 2*.

Table 2*. Simulated Bias, MSE and RE values of* $\hat{X}_q$

		$q = 0.01, X_q = -1.53$			$q = 0.05, X_q = -1.10$			$q = 0.10, X_q = -0.83$		
n	**Method**	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***
5	*ML*	0.3274	0.4978	100.0	0.2595	0.3614	100.0	0.2106	0.2922	100.0
	MML	0.2339	0.6746	135.5	0.2259	0.4022	111.2	0.2094	0.3224	110.3
	PWM	0.0068	0.5969	119.9	0.0077	0.4025	111.3	-0.0008	0.3138	107.3
10	*ML*	0.1528	0.2093	100.0	0.1210	0.1526	100.0	0.0931	0.1277	100.0
	MML	0.1301	0.2077	99.2	0.1212	0.1535	100.5	0.1077	0.1303	102.0
	PWM	-0.0012	0.2591	123.7	-0.0046	0.1794	117.5	-0.0097	0.1432	112.1
50	*ML*	0.0452	0.0356	100.0	0.0265	0.0281	100.0	0.0190	0.0215	100.0
	MML	0.0468	0.0358	100.5	0.0315	0.0284	101.0	0.0261	0.0219	101.8
	PWM	0.0146	0.0452	126.9	0.0039	0.0342	121.7	-0.0019	0.0255	118.6
100	*ML*	0.0187	0.0186	100.0	0.0175	0.0130	100.0	0.0080	0.0108	100.0
	MML	0.0212	0.0187	100.5	0.0206	0.0131	100.7	0.0120	0.0109	100.9
	PWM	0.0001	0.0233	125.2	0.0075	0.0154	118.4	-0.0023	0.0128	118.5
1000	*ML*	0.0020	0.0018	100.0	0.0021	0.0012	100.0	-0.0059	0.0010	100.0
	MML	0.0026	0.0018	100.0	0.0026	0.0012	100.0	-0.0053	0.0010	100.0
	PWM	-0.0003	0.0022	122.2	0.0022	0.0015	125.0	-0.0084	0.0014	140.0

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***Table 2**. (Continued)*

		$q = 0.90, X_q = 2.25$			$q = 0.95, X_q = 2.97$			$q = 0.99, X_q = 4.60$		
n	**Method**	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***
5	*ML*	-0.2794	1.0948	100.0	-0.3892	1.6518	100.0	0.6387	3.4784	100.0
	MML	0.1212	1.7614	160.8	0.1196	3.0598	185.2	-0.0938	7.9007	227.1
	PWM	-0.0037	1.3347	121.9	-0.0059	2.0758	125.6	-0.0119	4.5372	130.4
10	*ML*	-0.1378	0.5360	100.0	-0.1894	0.8217	100.0	0.3291	1.6893	100.0
	MML	0.0441	0.5872	109.5	0.0328	0.8950	108.9	0.0183	1.7976	106.4
	PWM	-0.0054	0.6220	116.0	0.0029	0.9923	120.7	0.0031	2.0887	123.6
50	*ML*	-0.0235	0.1033	100.0	-0.0363	0.1693	100.0	0.0617	0.3339	100.0
	MML	0.0068	0.1053	101.9	-0.0001	0.1714	101.2	0.0127	0.3383	101.3
	PWM	0.0021	0.1240	120.0	0.0019	0.2013	118.9	0.0062	0.4175	125.0
100	*ML*	-0.0202	0.0511	100.0	-0.0241	0.0796	100.0	0.0269	0.1589	100.0
	MML	-0.0066	0.0518	101.3	-0.0080	0.0804	101.0	0.0046	0.1616	101.6
	PWM	-0.0058	0.0577	112.9	-0.0015	0.0945	118.7	-0.0013	0.1944	122.3
1000	*ML*	0.0070	0.0052	100.0	-0.0040	0.0087	100.0	-0.0202	0.0160	100.0
	MML	0.0071	0.0053	101.9	-0.0020	0.0087	100.0	-0.0233	0.0160	100.0
	PWM	0.0111	0.0061	117.3	-0.0033	0.0103	118.3	-0.0157	0.0194	120.7

Robustness results

To assess the robustness properties of the methods mentioned earlier, results of some simulation studies are given in *Table 3*. It should be noted that different values of n are used in the simulation study, however, here the results are just reproduced for $n = 50$ as an illustration.

For $q \leq 0.10$, the *MML* estimator is the best among the others for models I, IV and V, the *PWM* estimator is more efficient than the others for models II-III. For $q > 0.10$, the *ML* outperforms the other methods for almost all alternative models (except for models II and III) with respect to the *MSE* criterion. The *PWM* estimator is the best for model III and the *MML* estimator performs better than the other estimators do for model I. However, all the estimators have substantial bias for all the alternative models.

Model evaluation results

In this study, to illustrate the practical use of the considered estimation methods in the previous section, we use the seasonal maximum daily wind speed modelled by the Gumbel distribution. Before analysing the data set, we evaluated the suitability of Gumbel distribution to fit the wind speed data by using Q–Q plots (which is the graphical technique) and Kolmogorov–Smirnov (*KS*) test, see *Table 4*.

Table 4 shows that computed values of the *KS* test given by the *ML*, *MML* and the *PWM* of Gumbel distribution are less than the theoretical values (which are $KS_{0.05,90} = 0.1434$, $KS_{0.05,91} = 0.1426$ and $KS_{0.05,92} = 0.1418$). Therefore, the results of the *KS* test and Q–Q plots are showed that the Gumbel distribution provides a plausible model for the data, see *Fig. 2*.

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***Table 3**. Simulated Bias, MSE and RE values of $\ddot{X}_q$ for the alternative models when $n = 50$*

	$q = 0.01, X_q = -1.53$			$q = 0.05, X_q = -1.10$			$q = 0.10, X_q = -0.83$		
	Model I								
Method	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***
ML	1.4590	2.2803	100.0	1.0569	1.2143	100.0	0.7775	0.6903	100.0
MML	0.7675	0.7154	31.3	0.4928	0.3289	27.0	0.2963	0.1676	24.2
PWM	1.5202	2.5148	110.2	1.0999	1.3342	109.8	0.8177	0.7714	111.7
	Model II								
ML	-1.0305	1.0985	100.0	-1.0263	1.0793	100.0	-1.0204	1.0639	100.0
MML	-1.8477	3.4365	312.8	-1.8036	3.2730	303.2	-1.7724	3.1635	297.3
PWM	-0.9986	1.0451	95.1	-1.0010	1.0349	95.8	-0.9987	1.0242	96.2
	Model III								
ML	0.3116	0.1984	100.0	0.2517	0.1341	100.0	0.2129	0.0977	100.0
MML	0.2881	0.1781	89.7	0.2263	0.1170	87.2	0.1863	0.0830	84.9
PWM	0.1800	0.0966	48.6	0.1518	0.0729	54.3	0.1387	0.0585	59.8
	Model IV								
ML	0.1840	0.1194	100.0	0.1328	0.0775	100.0	0.1134	0.0592	100.0
MML	0.1669	0.1013	84.8	0.1142	0.0644	83.1	0.0941	0.0495	83.5
PWM	0.1823	0.1022	85.5	0.1319	0.0666	86.0	0.1146	0.0506	85.5
	Model V								
ML	0.1885	0.1181	100.0	0.1365	0.0753	100.0	0.1045	0.0565	100.0
MML	0.1699	0.0982	83.1	0.1176	0.0626	83.1	0.0845	0.0464	82.1
PWM	0.1880	0.0989	83.7	0.1358	0.0636	84.4	0.1075	0.0473	83.7
	$q = 0.90, X_q = 2.25$			$q = 0.95, X_q = 2.97$			$q = 0.99, X_q = 4.60$		
	Model I								
Method	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***	***Bias***	***MSE***	***RE***
ML	-2.2027	5.3105	100.0	-2.8807	8.9102	100.0	-4.4939	21.4414	100.0
MML	-1.7585	3.5304	66.4	-2.2435	5.6380	63.2	-3.3523	12.4176	57.9
PWM	-2.2650	5.6816	106.9	-2.9417	9.4012	105.5	-4.6243	22.9100	106.8
	Model II								
ML	-0.9698	1.0465	100.0	-0.9664	1.0909	100.0	-0.9671	1.2431	100.0
MML	-1.4403	2.2183	211.9	-1.3730	2.0825	190.8	-1.2232	1.8460	148.4
PWM	-0.9947	1.1133	106.3	-1.0040	1.1985	109.8	-1.0202	1.4323	115.2
	Model III								
ML	-0.2171	0.1857	100.0	-0.3361	0.3271	100.0	-0.5201	0.7733	100.0
MML	-0.2573	0.2131	114.7	-0.3806	0.3708	113.3	-0.5715	0.8483	109.6
PWM	-0.0351	0.1184	63.7	-0.0889	0.1945	59.4	-0.1578	0.4155	53.7
	Model IV								
ML	-0.2665	0.2374	100.0	-0.3518	0.3866	100.0	-0.5252	0.8493	100.0
MML	-0.2956	0.2541	107.0	-0.3837	0.4081	105.5	-0.5628	0.8738	102.8
PWM	-0.2577	0.2584	108.8	-0.3432	0.4109	106.3	-0.5089	0.8909	104.9
	Model V								
ML	-0.2543	0.2169	100.0	-0.3516	0.3819	100.0	-0.5543	0.8337	100.0
MML	-0.2840	0.2338	107.7	-0.3815	0.4003	104.8	-0.5900	0.8575	102.8
PWM	-0.2503	0.2384	109.8	-0.3373	0.4097	107.2	-0.5256	0.8639	103.6

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***Table 4**. Computed values of KS test using the ML, MML and the PWM of Gumbel distribution for each season*

	Winter		**Spring**		**Summer**		**Autumn**	
Method	***KS***	***p*-value**	***KS***	***p*-value**	***KS***	***p*-value**	***KS***	***p*-value**
ML	0.0632	0.6089	0.0474	0.6147	0.0569	0.6112	0.0682	0.6071
MML	0.0643	0.6085	0.0465	0.6150	0.0595	0.6102	0.0699	0.6064
PWM	0.0575	0.6110	0.0509	0.6134	0.0487	0.6142	0.0618	0.6094

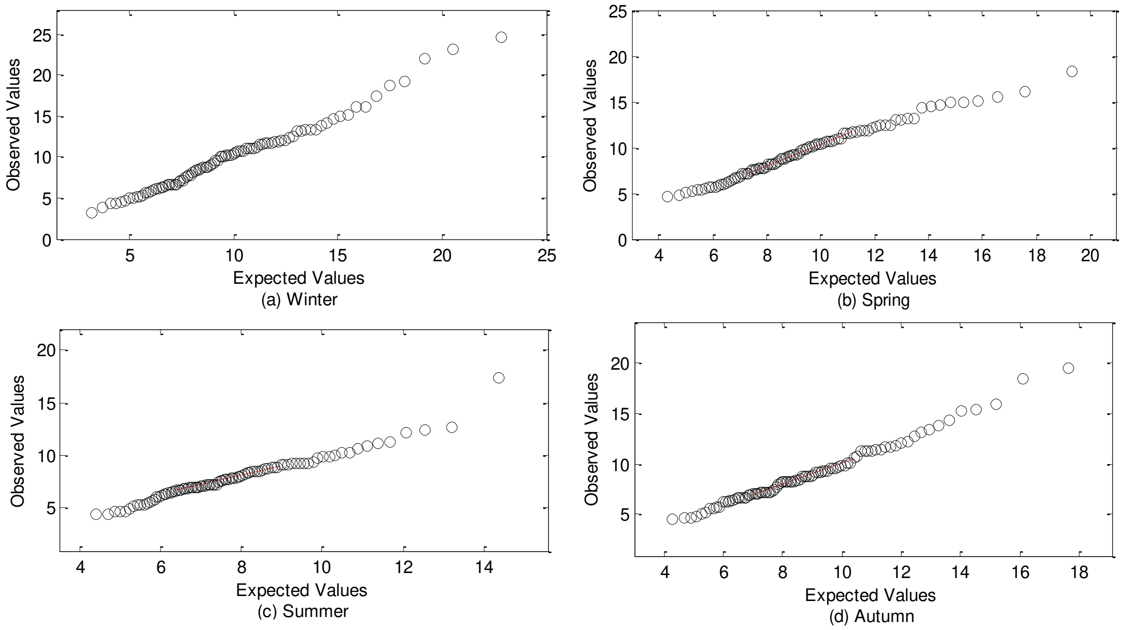

***Figure 2**. Q–Q plots of the seasonal maximum daily wind speed data for Gumbel distribution*

Then, it is purposed to determine a distribution providing better fit to wind speed data among Gumbel distribution based on the *MML*, *ML* or *PVM*. For this aim, the *ML*, *MML* and *PWM* estimates of the parameters and also R^2 and *RMSE* values of Gumbel distribution based on the estimators are calculated for each season. *Table 5* shows that the Gumbel distribution based on *MML* estimates provides the best fit to the spring and the summer, Gumbel distribution based on *PWM* estimates gives a better fit than the others for winter, Gumbel distribution based on *ML* estimates fit best for autumn, since the *RMSE* and R^2 values corresponding to these estimates are the lowest and the highest respectively, among the others.

Furthermore, in order to identify the distribution providing better fit to wind speed data by visual, histograms and fitted Gumbel probability plots for seasonal maximum daily wind speeds are used and results of analyses are presented in *Fig. 3*. It shows that the Gumbel distribution based on both *ML* and *MML* estimates also provides a better fit to the seasonal maximum daily wind speed data (except for winter) since curves of Gumbel probability plots of *ML* and *MML* estimates are almost superimposed. It should be noted that the results in *Table 4* are also consistent with graphs of the frequency histograms and fitted Gumbel probability plots based on the estimates in *Fig. 3*.

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***Table 5**. Estimates of the parameters and computed values of R^2 and RMSE corresponding the ML, MML and the PWM of Gumbel distribution for each season*

	Winter				**Spring**			
Method	$\hat{\theta}$	$\hat{\delta}$	***RMSE***	R^2	$\hat{\theta}$	$\hat{\delta}$	***RMSE***	R^2
ML	8.1074	3.2645	0.0262	0.9921	8.0938	2.4860	0.0273	0.9916
MML	8.1271	3.2842	0.0257	0.9924	8.1119	2.4981	0.0263	0.9923
PWM	8.0860	3.3487	0.0243	0.9930	8.0914	2.4554	0.0292	0.9905
	Summer				**Autumn**			
Method	$\hat{\theta}$	$\hat{\delta}$	***RMSE***	R^2	$\hat{\theta}$	$\hat{\delta}$	***RMSE***	R^2
ML	6.8945	1.6556	0.0269	0.9907	7.6448	2.2138	0.0222	0.9942
MML	6.9100	1.6601	0.0260	0.9914	7.6580	2.2257	0.0228	0.9939
PWM	6.9020	1.5954	0.0267	0.9912	7.6231	2.3094	0.0222	0.9939

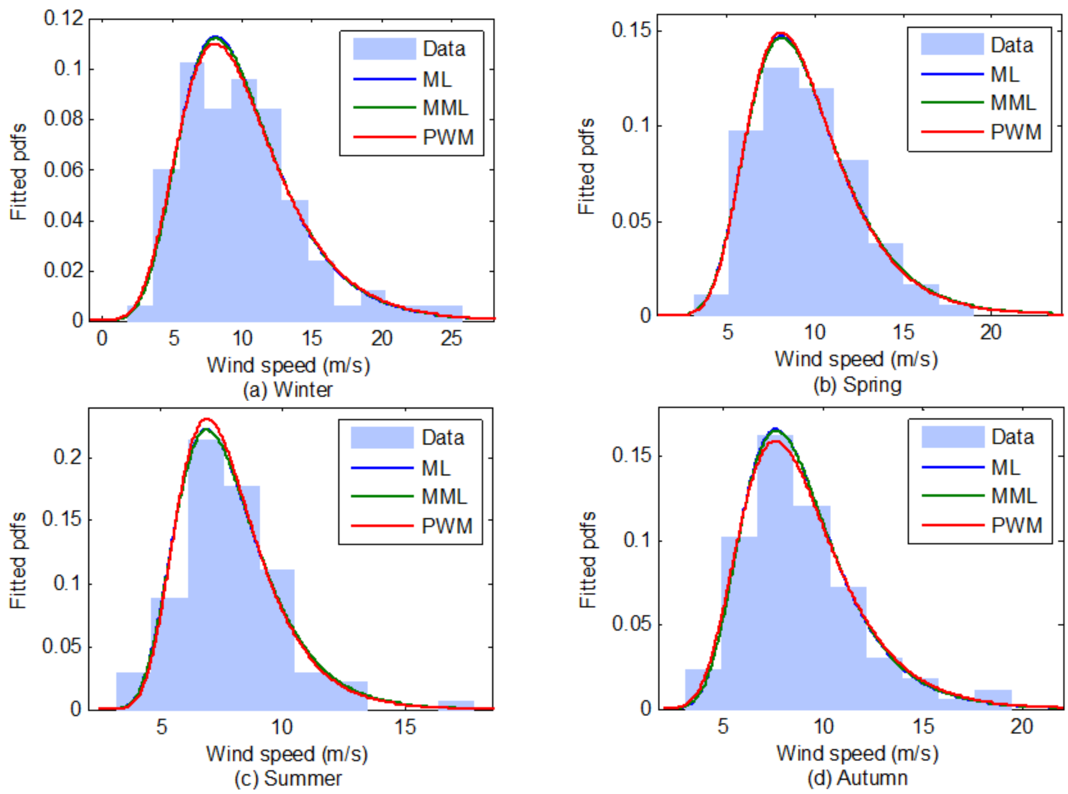

***Figure 3**. Histograms and fitted Gumbel probability plots based on ML, MML and PWM estimates superimposed for seasonal maximum daily wind speeds*

Results of quantiles estimates for wind speed data

In this part, performances of the estimators of quantiles are examined by using the considered methodologies for wind speed data recorded in Sinop. For this purpose, estimates of quantiles and their bootstrap standard deviations (*BSD*) are calculated for the values of q (i.e., 0.01, 0.05, 0.10, 0.90, 0.95 and 0.99) for each season, see *Table 6*.

According to the results presented in *Table 6*, in terms of the *BSD*, the *ML* estimate of X_q is the best with respect to *BSD* for summer (all values of q) and winter (values of q, i.e., $q \geq 0.90$) seasons. The *MML* estimate of X_q outperforms for autumn (all values

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of q), winter (values of q, i.e., $q \leq 0.10$) and spring (values of q, i.e., $q \leq 0.10$). The *PWM* estimate of X_q has the best performance for spring (values of q, i.e., $q \geq 0.90$). Additionally, its *BSD* values of *MML* are quite close to *BSD* values of *ML* because of the asymptotic equivalence of the *ML* and the *MML* estimators (Bhattacharyya, 1985; Vaughan and Tiku, 2000; Senoglu and Tiku, 2002). This result is consistent with the simulation results presented in *Table 2*.

Table 6*. Estimates of X_q and their BSD values for summer maximum daily wind speed data for all seasons*

	Winter						**Spring**					
	$q = 0.01$		$q = 0.05$		$q = 0.10$		$q = 0.01$		$q = 0.05$		$q = 0.10$	
Method	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***
ML	3.1562	0.3934	4.5790	0.3419	5.4186	0.3246	4.3358	0.2865	5.3875	0.2636	6.0368	0.2429
MML	3.1677	0.3834	4.5959	0.3351	5.4392	0.3208	4.3427	0.2755	5.4007	0.2547	6.0536	0.2370
PWM	3.0339	0.5303	4.4979	0.4381	5.3535	0.3973	4.3979	0.3640	5.4324	0.3274	6.0747	0.2905
	$q = 0.90$		$q = 0.95$		$q = 0.99$		$q = 0.90$		$q = 0.95$		$q = 0.99$	
Method	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***
ML	15.4433	0.8058	17.7021	0.9960	23.0077	1.3086	13.6726	0.5419	15.4400	0.6309	19.4613	0.9043
MML	15.5050	0.8149	17.7738	1.0049	23.1056	1.3192	13.7380	0.5638	15.5142	0.6521	19.5591	0.9375
PWM	15.5897	0.8757	17.8900	1.1216	23.2691	1.5150	13.5754	0.4964	15.3165	0.5962	19.2744	0.8461
	Summer						**Autumn**					
	$q = 0.01$		$q = 0.05$		$q = 0.10$		$q = 0.01$		$q = 0.05$		$q = 0.10$	
Method	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***
ML	4.3919	0.2280	5.0981	0.2064	5.5290	0.1866	4.2899	0.2823	5.2380	0.2382	5.8290	0.2249
MML	4.3855	0.2303	5.1023	0.2080	5.5392	0.1887	4.3066	0.2796	5.2551	0.2366	5.8458	0.2237
PWM	4.5056	0.2830	5.1776	0.2425	5.5934	0.2134	4.1419	0.3472	5.1282	0.2819	5.7450	0.2546
	$q = 0.90$		$q = 0.95$		$q = 0.99$		$q = 0.90$		$q = 0.95$		$q = 0.99$	
Method	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***	$\ddot{X}_q$	***BSD***
ML	10.5956	0.3675	11.7843	0.4359	14.4837	0.6554	12.5904	0.5502	14.2155	0.6879	17.7625	0.9858
MML	10.6788	0.3708	11.8871	0.4380	14.6203	0.6586	12.6113	0.5473	14.2369	0.6842	17.7867	0.9784
PWM	10.4600	0.4253	11.5965	0.5245	14.2128	0.8190	12.7651	0.6023	14.4534	0.7716	18.1356	1.1553

Discussion and conclusions

In this paper, we investigate the performances of different methods for estimating the several specified quantiles of the Gumbel distribution. Robustness of the estimators is also investigated. Their performances are compared via Monte Carlo simulation study with respect to the bias and *MSE* criteria.

Simulation results show that the *PWM* method outperforms the other methods even for small sample sizes with respect to the bias criterion. In terms of the *MSE*, the *ML* method has the best performance for all sample sizes and all values of q. The *MSE* values of the *MML* and *ML* estimates, however, are very close especially for $n > 5$.

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In the presence of outliers, the *ML* estimator is found to be robust to the data anomalies (except for models I and III) as expected. Also, all the estimators have substantial bias in almost all cases.

In application, seasonal maximum daily wind speed data taken from Sinop station in Turkey is modelled by using Gumbel distribution based on the *ML*, *MML* and *PWM* estimates. The results of the analyses demonstrate that the fitted densities corresponding to the *ML* and *MML* estimates provide better fit than the fitted densities corresponding to the *PWM* estimate for almost all seasons (except for winter season), see *Table 5* and *Fig. 3*. Also note that *ML* and *MML* estimators provide the best performance based on *BSD* for almost all seasons except for several *q* values of spring as shown in the *Table 6*.

On the other hand, extreme value data generally demonstrate excess kurtosis and/or heavy right tails (Pinheiro and Ferrari, 2016). Gumbel distribution is non-heavy-tailed and characterized by constant skewness and kurtosis, although it is commonly used in modelling environmental data. In this study, it provides quite well modelling in the seasonal maximum daily wind speed data according to the results of *KS* tests, *Q-Q* plots and the histograms and fitted densities superimposed. Additionally, the result of analyses of the real data shows that the *ML* and *MML* estimators provided better results than *PWM* estimator does in both modelling Gumbel distribution to the wind speed data and estimating the lower and upper quantiles of Gumbel distribution for many cases. The *MML* estimators are also numerically very close to the *ML* estimates since they are asymptotically equivalent (Tiku and Akkaya, 2004).

In conclusion, Gumbel distributions based on the *ML* and *MML* estimates can be proposed as an alternative distribution to Gumbel distribution based on the *PWM* estimate because of their superiority on modelling the peak of the wind speed distribution. Moreover, the *ML* and *MML* estimation methods can be recommended to be used in estimating the quantiles of Gumbel distribution for the data due to advantage of having the small *BSD* values.

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MORPHO-PHYSIOLOGICAL RESPONSE OF STEVIA (*STEVIA REBAUDIANA* BERTONI) TO SALINITY UNDER HYDROPONIC CULTURE CONDITION (A CASE STUDY IN IRAN)

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Abstract. Stevia (*Stevia rebaudiana* Bertoni) is a relatively unknown crop in Iran and many countries of world, with great potential as a natural sweeteners source. Stevia has a high content of sweeteners, which are up to 150 times sweeter than sugar, but virtually with no calories. An experiment was carried out to investigate the effect of salinity stress on root characteristics and physiological traits of stevia using six different concentrations of NaCl (0, 30, 60, 90, 120, and 150 mM) in Hoagland solution with four replications in open shading structures at Medicinal Plant Research Center, Shahed University of Tehran, Iran. The results showed that root characteristics (such as root fresh weight (RFW), root dry weight (RDW), root volume (RV), root length (RL), root area (RA), root diameter, root mass density (RMD), and dry root mass density (DRMD)) reduced with the intensification of NaCl. The effect of NaCl was significant on the protein content, activity of catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), and DPPH. The increasing of NaCl caused significantly enhancement of protein content, CAT activity, and total antioxidant activity. The POD activity showed a significant decrease by the increasing of sodium chloride rate. The PPO and POD activities by the increasing sodium chloride rate showed a significant decrease. Steviol glycosides (SVglys) compositions: stevioside (Stev), rebaudioside A (Reb A), rebaudioside B (Reb B), rebaudioside C (Reb C) and dulcoside A (Dulc A) and SVglys content showed changes under the influence of salinity. In 30 mM NaCl treatment was obtained the highest value of total SVglys yield and SVglys content. The findings from this study lead to the conclusion that, salinity stress caused reduces root characteristics and changes in physiological traits (protein content, activity of CAT, POD, PPO and total antioxidant activity DPPH). On the other hand, at the lowest salinity level (30 mM), the highest amount SVglys was obtained. It seems that the high level of SVglys at lower salinity levels is one of the reasons for salinity tolerance in Stevia, which requires further investigation.
Keywords: *enzyme activity, Hoagland, NaCl, peroxidase, root, steviol glycosides*

Introduction

The herb of stevia (*Stevia rebaudiana* Bertoni) also known as a honey leaf is a perennial plant and from the Asteraceae (Compositae) family native to Brazil and Paraguay (often referred to as the herb of Paraguay) (Karimi et al., 2015). The plant has a sweet taste due to the presence of diterpene SV glys. Stev and Reb-A are dominant glycoside compositions of the plant which makes that plant to be even 300 times sweeter than sucrose (Hajihashemi and Ehsanpour, 2014). This plant is of worldwide importance and high demand in many countries (such as Japan, Korea, China and South America) which are high potency natural sweeteners and low-calorie (Barbet-Massin et al., 2016).

The plant can, apparently, be successfully grown under different conditions regarding climate and soils (Hajar et al., 2014). The plant is adapted to poor soils, with low nutrient requirements. However, stevia shows some variability in what concerns the sensitivity or tolerance to salinity stress (Reis et al., 2015). There is often a tendency for

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a relation between growth and yield of crops, and salinity that is well established in the scientific literature: usually, the higher salinity levels the less growth and yield of the crop (Ityel et al., 2012; Jamil et al., 2012; Reis et al., 2015).

Salinity is one of the most hazardous and limiting environmental factors of crop product and plant growth particularly in arid and semi-arid regions (Gharsallah et al., 2016). FAO (2015) reported that 800 million ha of land and 32 million ha of agricultural land are estimated salt affected. The soil salinity of decreased water uptake by the root plants and causes osmotic stress, ion toxicity and mineral deficiencies (Munns and Tester, 2008).

Responses of plants to salinity stress (including physiological and morphological modifications) depending on the stage of growth and the genotype (Montana et al., 2014). Different types of salts exist in agricultural lands (such as Na_2SO_4, Na_2CO_3, KCl, $CaSO_4$, $MgSO_4$, $MgCl_2$ and etc) each of which can lead to the salinity stress (Bazrafshan and Ehsanzadeh, 2016). However, in salinity stress are dominant mostly Na^+ and Cl^- concentrations (Montana et al., 2014), thus NaCl is commonly the most widespread and disadvantageous salt in agricultural lands of Iran and other countries (Azizpour et al., 2010).

Salinity stress has been reported (Wu et al., 2010) to cause reactive oxygen species (ROS) formation and accumulation in the plant. Oxidative stress defenses happen through an enzymatic antioxidant mechanism including CAT, SOD, POD and non-enzymatic antioxidants as phenolics, flavonoids (Gharsallah et al., 2016).

Plant roots are the first organ to experience the effects of salt stress and essential to plant maintenance, as well as, have an important function in determining the yield of crops (Ober and Sharp, 2007). The widespread root system is positively correlated with salinity tolerance. Variations in the number of roots, root length, and its growth rate have been observed in different varieties and plants that these traits may create differences in plant characteristics such as tolerance to drought and salinity stresses (De-Oliveira and Varshney, 2011). The root of the plant's longer and more lateral roots than fewer plants that this attribute is more tolerant to salinity stress. An important factor in the tolerance to salinity how to develop their root system (Singh et al., 2000). Increased root surface is important since caused increased levels of absorption and increasing the efficiency of water and nutrients. Therefore, longer roots and more root surface can be provided possibility salinity tolerance (Aghaei et al., 2009).

The cultivation of stevia multipurpose plant caused increasing its production for medicinal and other purposes. There is much research on potential uses, micropropagation, and cell culture, secondary metabolite production of this plant. However, there are only a few reports available on biochemical modifications and without reported on root characteristics of stevia in NaCl salinity condition (Gupta et al., 2016). On the other hand, root characteristics and biochemical traits modifications are important for understanding salinity tolerance mechanism, but strategies differ among plant species (Liu et al., 2016). Therefore, there is a need to research on modifications of root parameters and biochemical traits of the plant. So, in the present research was planned with objectives the assess of six different concentrations of NaCl (0, 30, 60, 90, 120, and 150 mM) on root characteristics and physiological traits of stevia.

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Material and methods

Plant material and growth conditions

The present study was carried out based on a completely randomized design (CRD) with four replications in open shading structures at Medicinal Plant Research Center, Shahed University of Tehran, Iran. The seedling of stevia (*Stevia rebaudiana* Bertoni) propagation was carried out by tissue culture. The uniform two seedlings were transplanted into the pot (30 × 30 cm) containing a mixture of pumice and perlite (50:50 ratio). Two weeks after establishment of seedlings were subjected to salinity stress with different NaCl (Sodium chloride; Merck, Darmstadt, Germany) concentration (0, 30, 60, 90, 120, and 150 mM). Salinity treatment was applied according to the treatments 15 days after planting and continued until the end of the experiment. The pots were watered one time a day by mineral nutrients with 500 mL per pot of modified Hoagland solution (*Table 1*). The EC and pH of drainage water from pots were checked every week, and an additional 500 mL of distilled water was applied to minimize pH and EC changes in the root zone (Rahimi and Biglarifard, 2011). The whole plants were harvested at 62 days after transplanting and leaves and stems were separated and placed in the freezer (-30 °C) used for further assays.

Table 1. *Nutritional composition of used in the preparation of Hoagland solution*

Macronutrients	Chemical components	Stock solution	ml of stock solution/1 ml
KNO_3	2M KNO_3	202 g/L	2.5
H_2PO_4	1M KH_2PO_4 (pH to 6.0)	136 g/L	0.5
$Ca(NO_3)_2$	1M $Ca(NO_3)_2$•$4H_2O$	236 g/0.5L	2.5
NH_4NO_3	1M NH_4NO_3	80 g/L	1
Micronutrients			
Iron	Iron (Sprint 138 iron chelate)	15 g/L	1.5
$MgSO_4$	2M $MgSO_4$•$7H_2O$	493 g/L	1
H_3BO_3	H_3BO_3	2.86 g/L	1
$MnCl_2$	$MnCl_2$•$4H_2O$	1.81 g/L	1
$ZnSO_4$	$ZnSO_4$•$7H_2O$	0.22 g/L	1
$CuSO_4$	$CuSO_4$•$5H_2O$	0.051 g/L	1
Na_2MoO_4	Na_2MoO_4•$2H_2O$	0.12 g/L	1

Root parameters calculation

Underground organ, total roots were washed thoroughly and minimized damages with running water. Root fresh weight was measured immediately with 0.001g accuracy. RV was obtained by subtraction of root volume after inserting it in the certain volume of water to first RV with 0.1 mm accuracy. RA was evaluated by Atkinson method (Hajabbasi, 2001). Root diameter and volume was obtained by *Table 2* relations and other traits such as root fresh weight to soil volume (RMD), root dry weight to soil volume (DRMD), root dry weight to root volume, and root length to root fresh weight (RF) was calculated (Ganjali et al., 2003).

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__Table 2.__ Method of calculation parameters associated with root

Parameters	Formula	Reference
Root length (RL)	RL = (roots weight) × 0.890	Ganjali et al., 2003
Root area (RA)	$RA = 2(\text{Root volume} \times \pi \times RL)^{0.5}$	Hajabbasi, 2001
Root diameter (RD)	$RD = (4 \times \text{Root fresh weight}/(RL \times \pi))^{0.5}$	Akhavan et al., 2012
Root Surface Area Density RSD)	RSD = (RL × RD × π)	Akhavan et al., 2012

π = constant number 3.14

Protein and antioxidant enzymes assay

Samples were frozen in liquid nitrogen and stored at -30 °C. One g of frozen leaf was homogenized in a mortar with 5 mL of 50 mM potassium phosphate buffer (pH = 7.5) containing 1 mM ethylenediaminete-traacetic acid (EDTA), 1 mM dithiotreitol and 2% polyvinyl pyrrolidon (PVP). The homogenate was centrifuged at 15,000 g for 25 min and the supernatant was used for protein and antioxidant enzyme assay.

Protein content

For determining the amount (concentration) of protein, spectrophotometer with Bradford method (1976) was used. The basis of this method is binding the Coomassie Brilliant blue G-250 to protein in an acidic environment and determine maximum absorption from 595 to 465 nm. The absorbance at 595 nm had a direct comparison to protein concentration.

Catalase assay

The CAT activity assay was performed using Chance and Maehly (1995) method. Three mL reaction mixture containing 2.5 mL 0.05 mM sodium phosphate buffer (pH = 7), 30 µg protein solution was added to quettes and at the time of measurement, 30µL H_2O_2 (30%) was added to reaction mixture and the absorbance at 240 nm, at 60 s, and at 25 °C was recorded.

Peroxidase assay

POD activity was assayed adopting the method of Polle et al. (1994). According to this method, POD activity was determined at 436 nm by its ability to convert guaiacol to tetraguaiacol (€ = 26.6 $mM^{-1}cm^{-1}$). The reaction mixture contained 100 mM potassium phosphate buffer (pH = 7.0), 20.1 mM guaiacol, 10 mM H_2O_2 and enzyme extract. The increase in absorbance was recorded by the addition of H_2O_2 at 436 nm for 3 min.

Polyphenol oxidase assay

The polyphenol oxidase (PPO; E.C. 1.10.3.1) activity was measured according to the method of Raymond et al. (1993) in absorbance at 430 nm. The reaction mixture contained 1900 µl 50 mM sodium phosphate buffer (pH = 6.8), 500 µl pyrogallol 0.02 mM and 100 µl enzyme extract.

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DPPH activity

The measurement of the DPPH radical scavenging activity was performed according to the methodology described by Brand-Williams et al. (1995). The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of DPPH radical solution 0.5 mM in ethanol. The changes in color (from deep violet to light yellow) were read at 517 nm after 100 min of reaction. The mixture of ethanol (3.3 mL) and sample (0.5 mL) serve as a blank. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL).

Determination of SVglys content and compositions

The SVglys content and compositions of stevia leaves were determined according to the procedures used by other researchers (Ceunen and Geuns, 2013; Karimi et al., 2015). 0.1 g of powdered leaves (dried at 65 °C for 48 h in hot air avon) was transferred to 15 mL tubes, 3 mL distilled water were added and kept in a water bath for 30 min at 80 °C. The resultant solution was firstly centrifuged at 12,000 × g for 5 min and the supernatant recovered. Then, 3 mL distilled water was added to the pellet and then centrifuged. This process was repeated three times and the supernatant from each process was pooled. The pooled supernatant was centrifuged again (12,000 × g for 5 min) and the new supernatant was transferred to new tubes. The volume of the final supernatant was exactly diluted to 10 mL using distilled water and filtered using 0.45 m nylon filter attached to a syringe. Then, a C18 cartridge was used for SVglys purification. The C18 cartridge was firstly washed with 3 mL methanol and then conditioned with 3 mL of distilled water. Thereafter, 0.5 mL of the filtered supernatant was loaded into the cartridge and then the cartridge was washed with acetonitrile/water mixture (20:80, v/v). Finally, SVglys were eluted from the C18 cartridge with 1 mL of acetonitrile/water (80:20, v/v) and kept in 1.5 mL tubes at -20 °C until further analysis.

For the chromatographic SVglys analysis, two reverse-phase C18 columns were connected in series and a UV-vis detector set at 202 nm was used. A solvent gradient of acetonitrile and water, as mobile phases, were created with a flow rate of 0.5 mL min^{-1}. The acetonitrile ratio was increased into the solvent gradient in 50, 65, 80, 80 and 50% during 0-10, 10-18, 18-22, 22-24 and 24-30 min, respectively. In order to carry out SVglys assay, 40 µL of the purified extract was injected into the HPLC pump. Among SVglys compositions, Reb A, Stev, Reb F, Reb C and Dulc A were detected. For quantification purposes, pure Stev and Reb A (purity > 99%) were used as external standards. Then, Reb F, Reb C, and Dulc A were quantified by their molecular weight ratio to Reb A, because it has been shown that all SVglys have similar molar extinction coefficients (Geuns, 2010). The HPLC peak area was calculated by Chromstar 7.0 software and the results of SVglys content were expressed as a percentage of leaf dry weight (W/W), using the calibration curves obtained from the relationship between external standards (ppm) and their relative HPLC peak area.

Statistical analysis

All data was analysis with SAS 9.2 software and means comparisons was performed by Duncan multiple ranges in 5% of probability.

Results

Root characteristics

The effect of salinity was significant on RFW, RDW, RV, RL, RA, root diameter, RSD, RMD and DRMD (*Table 3*). With the increasing amount of sodium chloride showed a significant decrease in all root traits measured (*Table 4*). The highest mean traits of RFW, RDW, RV, RL, RA, root diameter, RSD, RMD, and DRMD were in the NaCl 0 mM, however, the traits of RDW, RL and DRMD in the NaCl 30 mM did not show significant decrease compared to the control (non-stress) (*Table 4*). The salinity of NaCl 60 mM compared to the control (NaCl 0 mM) causes decreased approximately 50% in the mean of root characteristics (mentioned above). Salinity level of NaCl 150 mM compared to the control decreased 80.16, 73.24, 72.10, 73.23 and 72.73% at RFW, RDW, RV, RL and RA, respectively (*Table 3*). In *Figure 1*, the structure of root morphology shown in different levels of sodium chloride.

Table 3. *Analysis of variance for effect of salinity (NaCl) on root characteristics of Stevia*

Sources of variance	Mean square (MS)									
	df	**RFW**	**RDW**	**RV**	**RL**	**RA**	**Root diameter**	**RSD**	**RMD**	**DRMD**	**RF**
Salinity (NaCl)	5	737.2**	10.4**	703.0**	8.29**	894.1**	0.21*	960.1**	0.038**	0.00055**	0.001 ns
Experimental error	18	20.69	0.30	20.50	0.24	12.01	0.08	18.61	0.001	0.00001	0.001
CV (%)	-	21.15	17.26	20.12	17.26	12.34	9.33	15.64	21.15	17.26	22.16

ns, * and ** non-significant, significant at 5% and 1% respectively
(RFW: Root Fresh Weight, RDW: Root Dry Weight, RV: Root Volume, RL: Root Length, RA: Root Area, RSD: Root Surface Area Density, RMD: Root Mass Density, DRMD: Dry Root Mass Density, RF: Root Fineness (Root Length/ Root Fresh Mass)

Table 4. *Mean comparison of salinity levels (NaCl) on root parameters of stevia*

Salinity (mM)	**RFW (g/plant)**	**RDW (g/plant)**	**RV (cm^3)**	**RL (m)**	**RA (cm^2)**	**Root diameter (cm)**	**RSD (m^2 m^{-3})**	**RMD (g m^{-3})**	**DRMD (g m^{-3})**
$NaCl_0$	44.57 a	5.42 a	47.50 a	4.82 a	53.47 a	3.43 a	51.98 a	0.323 a	0.039 a
$NaCl_{30}$	28.74 b	4.81 a	26.50 b	4.28 a	37.61 b	2.92 b	38.87 b	0.208 b	0.034 a
$NaCl_{60}$	20.94 c	2.98 b	19.75 c	2.65 b	25.54 c	3.17 ab	26.38 c	0.151 c	0.021 b
$NaCl_{90}$	16.83 c	2.90 b	14.25 c	2.58 b	21.29 c	2.89 b	23.27 c	0.122 c	0.021 b
$NaCl_{120}$	9.07 d	1.66 c	13.75 c	1.48 c	15.95 d	2.78 b	12.97 d	0.065 d	0.012 c
$NaCl_{150}$	8.84 d	1.45 c	13.25 c	1.29 c	14.58 d	2.96 b	11.96 d	0.064 d	0.010 c

Means in each column followed by similar letter (s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test
(RFW: Root Fresh Weight, RDW: Root Dry Weight, RV: Root Volume, RL: Root Length, RA: Root Area, RSD: Root Surface Area Density, RMD: Root Mass Density, DRMD: Dry Root Mass Density, RF: Root Length/ Root Fresh Mass)

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***Figure 1.** The effect of NaCl concentration on the root morphology*

Physiological traits

The effect of NaCl was significant on the protein content, activity of CAT, POD, PPO and DPPH ($P \leq 0.01$) (*Table 5*). The increase of NaCl concentration caused significantly enhuncment protein content so that the highest protein content was in the highest levels of sodium chloride (120 and 150 mM) and the lowest protein percentage was control level of salinity (*Table 6*).

***Table 5.** Analysis of variance for effect of salinity (NaCl) on biochemistry traits of stevia*

Sources of variance	Mean square (MS)					
	df	Protein	CAT	POD	PPO	DPPH
Salinity (NaCl)	5	31.45**	38.33**	2.85**	29.64**	7.94**
Experimental error	18	1.05	0.17	0.01	0.79	0.064
CV (%)	-	10.11	11.95	9.84	25.15	10.74

ns, * and ** non-significant, significant at 5% and 1% respectively

***Table 6**. Mean comparison of salinity levels (NaCl) on biochemistry traits of stevia*

Salinity (mM)	Protein (%)	CAT (U/gFW)	POD (U/gFW)	PPO (U/gFW)	DPPH (U/gFW)
$NaCl_0$	5.84 e	0.021 e	2.64 a	8.02 a	0.61 e
$NaCl_{30}$	8.27 d	0.123 e	2.03 b	5.24 b	1.30 d
$NaCl_{60}$	9.88 c	2.34 d	1.71 c	3.53 c	2.03 c
$NaCl_{90}$	11.17 bc	5.18 c	0.89 d	2.36 cd	2.36 c
$NaCl_{120}$	12.46 ab	6.26 b	0.73 d	1.42 de	3.67 b
$NaCl_{150}$	13.43 a	7.04 a	0.50 e	0.67 e	4.19 a

Means in each column followed by similar letter (s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test

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The highest and lowest CAT activity were in NaCl 150 mM and control levels respectively, in other words, increasing sodium chloride increased CAT activity (*Table 6*).

The POD activity showed a significant decrease in the increasing amount of sodium chloride so that the highest activity of this enzyme was in control level and lowest activity was at the highest level of salinity (NaCl 150 mM) (*Table 6*).

The activity of PPO and POD showed a significant decrease by the increasing amount of sodium chloride (on the contrary CAT activity), so that in the NaCl 0 mM and NaCl 150 mM observed the highest and lowest activity of this enzymes respectively (*Table 6*).

The total antioxidant activity DPPH significantly increased by the increasing sodium chloride. In other words, salinity stress was significantly increased the antioxidant capacity of the plant, so that the lowest of activity was in control level and the highest activity was NaCl 150 mM (*Table 6*).

SVglys production

The salinity caused a significant variation in SVglys compositions and SVglys content of stevia (*Table 7*). In the NaCl 30 mM obtained the highest value of total SVglys yield and SVglys content (0.51 g/plant and 12.47% of the leaf dry weight, respectively) (*Table 8*). However, regarding SVglys yield of stevia, there was no significant difference between control, NaCl 30, and NaCl 60 mM treatment.

The effect of NaCl treatment was significant on SVglys compositions (Such as Stev, Reb A, Reb B, Reb C and Dulc A). The highest value of Stev, Reb A, Reb B, Reb C and Dulc A were achieved in NaCl 30 mM level. The Svglys compositions increased by NaCl 30 mM treatment and thereafter decreased when the stress became more severe. Reb A/Stev ratio (sweetness quality) was significantly affected by NaCl treatment with the highest value in NaCl 90 and NaCl 120 mM treatments (*Table 8*).

Discussion

Salinity is one of the factors reducing growth and yield of many crops around the world and also, affect on growth and composition of secondary metabolites in medicinal plants (Aghaei Joubani, 2015). The NaCl stress caused a decrease in all root traits measured (Such as RFW, RDW, RV, RL, RA, root diameter, RSD, RMD, and DRMD). By increasing salinity levels, root morphology were bulky, shorter and slower growth (*Figure 1*). Reported that salinity stress decrease RL, RF, and RDW in quinoa plant (Panuccio et al., 2014). Root is an organ which could affect resource conversion, use efficiency and transduction of production sources. So structural changes in root morphology caused by salinity stress could alter root structure and affected physiological process occurred in the root (water absorbance and nutrient availability) (Malamy, 2005; Panuccio et al., 2014).

Safwat et al. (2016) reported that salinity decreased root number and length in stevia. The RFW and RDW in *Periploca sepium* Bunge were reduced strongly with increasing salinity levels (Sun et al., 2011). Researchers mentioned the reduction of photosynthesis, degradation of cell membranes, reduction water availability to roots and accumulation of sodium ions as the main factors reduce plant growth under salt stress (Sharifi et al., 2007).

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Table 7. *Analysis of variance for SVglys compositions of stevia under NaCl treatments (0, 30, 60, 90, 120 and 150 mM)*

Sources of variance	Mean square (MS)								
	df	**Stev**	**Reb A**	**Reb B**	**Reb C**	**Dulc A**	**Total SVglys**	**Reb A/Stev ratio**	**SVglys yield**
Salinity (NaCl)	5	18.06**	1.33**	0.041**	0.0061**	0.036**	34.25**	0.0151**	0.104**
Error	18	0.026	0.023	0.0004	0.00009	0.0038	0.043	0.0018	0.009
CV (%)	-	3.16	7.53	7.92	6.20	10.23	2.53	10.58	27.14

ns, * and ** non-significant, significant at 5% and 1% respectively
(SVglys: Steviol glycosides; Stev: Stevioside; Reb A: RebaudiosideA; Reb C: Rebaudioside C; Dulc A: Dulcoside A)

Table 8. *Mean comparison of salinity levels (NaCl) on SVglys compositions of stevia*

Salinity (mM)	Stev (%)	Reb A (%)	Reb B (%)	Reb C (%)	Dulc A (%)	Total Svglys (percent of leaf dry matter)	Reb A/Stev ratio	SVglys yield (g/plant)
$NaCl_0$	5.74 c	2.03 c	0.26 c	0.16 c	0.61 bc	8.83 c	0.35 c	0.46 ab
$NaCl_{30}$	8.25 a	2.82 a	0.43 a	0.21 a	0.75 a	12.48 a	0.34 c	0.51a
$NaCl_{60}$	6.83 b	2.54 b	0.34 b	0.18 b	0.66 b	10.57 b	0.37 bc	0.47 ab
$NaCl_{90}$	3.88 d	1.84 c	0.20 d	0.13 d	0.57 bcd	6.65 d	0.47 a	0.33 b
$NaCl_{120}$	3.28 e	1.56 d	0.18 d	0.12 de	0.53 cd	5.70 e	0.47 a	0.16 c
$NaCl_{150}$	2.99 f	1.30 e	0.17 d	0.10 e	0.48 d	5.07 f	0.43 ab	0.15 c

Means in each column followed by similar letter (s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test
(SVglys: Steviol glycosides; Stev: Stevioside; Reb A: RebaudiosideA; Reb C: Rebaudioside C; Dulc A: Dulcoside A)

The salinity stress an important subject is the generation of excessive ROS which caused membrane destruction, cell toxicity, and cell death (Chookhampaeng et al., 2008). According to researchers opinion, salinity stress converts superoxide radical (O_2) to hydrogen peroxide (H_2O_2) within the cell, this conversion prevents Calvin cycle activity and the process carbohydrates in plants. Therefore increase in activity of antioxidant enzymes such as CAT and SOD was from adverse effects of hydrogen peroxide formed which impact on the carbohydrates production in the chloroplasts. High levels of antioxidant activity in plants under stress are not the only mechanism of salinity tolerance, but also this mechanism can be compatible with components suppliers such as proline and carbohydrates to increase crop tolerance (Abo-Kassem, 2007).

Also, in this experiment, salinity increased CAT activity and total antioxidant activity DPPH in stevia (*Table 6*). CAT is an important antioxidant enzyme that converts H_2O_2 to water in the peroxisomes. In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration. Higher activity of CAT and Ascorbate peroxidase decreased H_2O_2 rate in cell and increased the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2directly inhibits CO_2 fixation (Bhutta, 2011). The activity

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of the enzyme of CAT is higher than that of POD, PPO, and DPPH at NaCl 150 mM, which suggests that CAT provides a better defense mechanism against NaCl stress-inducted oxidative damage in stevia. The results are similar to Sun et al. (2011); Yeonghoo et al. (2004).

Stevia plants have gained importance as sweeteners because of their Stev and RebA contents. The results indicated that NaCl 30 mM (low salinity level) compared to the non-salinity (control) caused increases of SVglys compositions and SVglys yield. Similar results were also obtained by Zheng et al. (2013), who found that lower salinity concentration in soil could alter the composition of SVglys by distinctly improving Reb A content. However, higher NaCl levels (90, 120 and 150 mM) causes prominent decrease of SVglys composition (Such as Stev, Reb A, Reb B, Reb C and Dulc A) and SVglys yield. Zheng et al. (2013) also reported similar results. It seems that when plants lived under high salinity stress conditions, energy was first allocated to the process of maintaining metabolic homeostasis, such as synthesis of simple osmolytes and enhancing activities of antioxidant enzymes (Abrol et al., 2012) rather than the synthesis of complex secondary metabolites. The concentrations of various secondary plant products are strongly dependent on the growing conditions and have an impact on the metabolic pathways responsible for the accumulation of the related natural products (Ramakrishna and Ravishamkar, 2011).

Conclusion

The NaCl stress caused changes in physiological traits and reduces of root characteristics (caused decrease approximately 50% in the mean traits of root characteristics). The increaseing antioxidant defense system (DPPH, CAT, POD and PPO) also responded to this stress. The level of NaCl 30 mM (low salinity) compared to the non-salinity caused increase of SVglys compositions and SVglys yield. It seems that increasing the SVglys rate in low levels of salinity stress can be one of the tolerance mechanisms in the plant, which requires further research.

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DRYING AND PELLET CHARACTERIZATION OF SAND-SEPARATED DAIRY MANURE FROM STEPHENVILLE, TEXAS, USA

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Abstract. Concentrated animal feeding operations often result in tremendous amounts of animal manure that can become a major problem to both the business and the environment. This study aims to propose an alternative use of manure for local energy generation. Dairy manure with final moisture contents ranging from 0% to 15% at 5%-interval were prepared using a bench-type convection dryer inside a SHERER Environmental Chamber. The dried manure was sieved and then was pelletized using an extruder connected to an MTS Test System. The drying and physical characteristics of sand-separated manure pellets were investigated as a potential energy fuel. The average drying rate within the 20-h drying period was measured to be at 0.025 kg moisture removed per kg of dried sample-hour. The manure pellets have 716 kg/m3 ± 71.6 kg/m3 bulk density with total carbon content of about 24.25% ± 3.27%. Removal of sand from the raw manure sample increased the heating value from 3.29 MJ kg^{-1} to 10.20 MJ kg^{-1}. Sand-free manure has better elemental and fuel characteristics compared with sand-mixed as received manure from dairy farms. The resulting pellets can withstand 1.12 kilogram-force (kg-f) to 1.80 kg-f at 5-10% MC, which can be a candidate bioenergy fuel in terms of its over-all elemental and durability properties.
Keywords: *bioenergy, biomass fuel, heating value, pellet durability, waste management*

Introduction

Concentrated animal feeding operations have merged into fewer, but larger operations in the U.S. animal industry. The significant increase of the number of animals per facility posed a challenge to manage enormous quantities of manure (Cantrell et al., 2007). Historically, animal manure serves as an organic fertilizer primarily because of its nutrient content. Unfortunately, inappropriate and excessive application of the animal manure can escalate concentrations of nitrogen and phosphorous compounds leading to eutrophication; contaminate groundwater sources; spread pathogens and contribute to greenhouse gas and odor pollution (Gerba and Smith, 2005).

One of the practical solutions to address the environmental and logistical problems of manure management is to reduce the water content through convective drying. It also improves handling and storage conditions, making it less susceptible to bacteriological degradation – a precursor process of noxious and offensive gases (Aboltins and Kic, 2014). The drying process reduces the moisture content (MC) down to 1-15 percent, wet basis (wb). Dried manure can generally attain high heating value of 17 MJ kg^{-1} on a dry ash-free basis which can be comparable to as-received low grade coal (Mukhtar et al., 2008).

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Dairy manure is a potential cheap source of biomass energy to support local farm operations. After drying, manure can be processed into pellets that would require a low moisture content of about 8% wb, typical for biomass raw materials (Mani et al., 2006). Pelletizing can improve the bulk density of biomass materials by about 5 to 7 times, and helps solve typical logistics and storage problems faced by industries that mainly use solid fuels (Tumuluru et al., 2016; Biomass Energy Resource Center, 2007).

The process of pellet making usually involves molding machines like disk pelleter (dry method) and the extruder (wet method). The raw material MC greatly affects the strength and processing time of pellets for both methods (Hara, 2000). For wood pellets, increasing temperature results in stronger pellets, whereas higher MC results into weaker pellets (Gardner et al., 2009). In several studies conducted for grasses, the MC and compressive strengths (Mani et al., 2006; Tabil and Sokhansanj, 1996) affected the quality of pellets. The present study utilized the extruder type machine where the raw material undergoes compression into an installed pellet die.

There were a number of studies for manure pellets but primarily focused on agricultural applications. An operating moisture content of 4% to 15% of mixed manure is typical for the production of pellets. Manure pellets are considered as potential fertilizer for vegetable production in urban areas and soil ameliorant for increased crop production (Allen and Farrah, 2005; Zafari and Kianmehr, 2012). The 5% MC pellet manure mix provided the best resistance to fragmentation when used as organic fertilizer in the field (Romano et al., 2014). Poultry litter pellets combined with bedding material was prepared within a moisture range of 6% to 22% for storage and handling (McMullen et al., 2005).

Animal waste management and income options of farmers are improved and diversified by producing dried manure pellets (EPA, 2004). Fuel pellets from dairy manure is a potential value-adding commodity for farmers, especially for those in developing countries. However, there are only few studies investigating energy pellets from swine and cattle farms. This study aims to investigate the drying and pellet characteristics of sand-separated dried dairy manure and its potential as an energy fuel.

Materials and experimental methods

Manure collection and handling

Raw manure mixed with sand came from the Southwest Regional Dairy Center in Stephenville, Texas, USA. Dairy often use sand bedding in order to promote comfort, easier management and less frequent cleaning. Selected sand materials are normally non-abrasive and fine. Dairy facilities should maintain their sand beds at full capacity at least once a week. The mixture of animal excretions and sand typically contains an initial moisture content wet basis (MCwb) of 65% to 80%. The collected raw manure was transported and air-dried at the Bioenergy Testing Laboratory at Texas AandM University to reduce the moisture content up to 52-55% MCwb.

Material preparation

A bench-type fabricated convection dryer reduced the moisture contents of air-dried manure to the final desired levels, suitable for biomass pelleting. Using convective drying, four batches of dried manure samples with final moisture contents of 0, 5, 10,

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and 15% were prepared and used for pelletizing. A blower heater with a 1.26 kW rating supplied the heated air at about 14 L s^{-1}.

The dried manure undergone size reduction and sand separation. A Thomas-Wiley laboratory mill ground the sample into smaller particles. An ASTM E-11 sieve # 45 separated and recovered the sand particles, which are normally at the range of 0.25 mm to 2 mm. The recovered dried manure particles had an equivalent diameter of equal to or less than 355 µm. Each run utilized about 30 g of dried sand-separated manure for pelleting.

The sand-separated manure was loaded on the dryer and placed on a Toledo Digital Weighing Scale with an accuracy of ±10%. The changes in MCs were measured based on the decrease of manure weight. A one-hour interval reading was programmed using a Campbell CR 3000 data logger to monitor and record the temperature and moisture content. The convective drying was performed inside a SHERER Environmental Chamber to control the ambient conditions, particularly room temperature within 40-41°C range.

Pellet production

The dried sand-separated manure was loaded inside a fabricated stainless steel mold and extruded at a compression ratio of 4:1 to produce manure pellets. Each run lasted for about 40 s. Three replicates were made for every pellet at different final moisture contents of 0, 5, 10, and 15% prepared from convective drying.

Characterization

The bulk density of the dried manure sample (bone dry) was estimated after sieving. The masses and volumes of both the dried manure and the separated sand were recorded. The ratio of the total mass and volume provided an estimate of the bulk density of the dried manure sample. A Micromeritics AccuPyc 1330 was used to measure particle density. The AccuPyc is an instrument that uses a non-destructive technique of inert gas displacement to measure the volume of a known mass of solid sample. The processing time lasts for about 3 min and is known to produce results having 0.03% accuracy.

The weight percent composition of each elemental carbon, hydrogen, nitrogen, oxygen and sulfur (C, H, N, O and S) in the dried sand-free manure sample were analyzed using ultimate analysis according to ASTM D 3176 standard, typical for coal analysis. The sample was sieved using ASTM Sieve #45 to produce fine manure particles. Less than 1 g of each replicate was used to prepare samples for ultimate analysis.

The heating value of the dried sand-free manure was measured in accordance with ASTM E 711 standard for fuel testing. A Parr bomb calorimeter 6200 was used with an installed Parr 1108 oxygen bomb to produce reliable and repeatable results at 0.1% precision. Each test was completed within 10 min.

Drying rates

Changes in moisture content levels were monitored for every one-hour period. The periodic change in weight of the sample was assumed equal to the moisture loss. The changes in MC were monitored from the manual read out of the Toledo weighing scale. Drying operation continued for about two days until there were no changes recorded in

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the weight of the sample for four consecutive hours. A drying curve was constructed by plotting the moisture content versus time in order to estimate the drying rate of dairy manure without sand.

Durability tests and analysis

About 10 g of the dried sand-free manure was loaded into the pellet mold and compressed for 5 min to produce pellets. The pellet compressive strength was tested using an MTS 810 Material Testing system. Three pellets made for every final moisture content and tested for the durability to withstand increasing compression force. The MTS instrument induces compression with an increasing rate of around 0.05% of the initial force applied. A single-factor analysis of variance (ANOVA) was conducted to evaluate any statistical difference between the different final moisture contents of the pellets in relation to their compressive strengths. A normal quantile plot was generated to assess the normal distribution of the parameters within the 95% confidence region. The ordered differences between means were tested using t-test. Positive values show pair of means that are significantly different. The values $P < 0.05$ were considered statistically significant. JMP interactive statistical software by the SAS Institute Inc. was used for the statistical analysis.

Results and discussion

Particle density

Bedding stalls often use sand in typical dairy farm operations. However, the resulting sand-laden dairy manure (SLDN) becomes a constraint in maximizing manure-handling goals (Gooch and Wedel, 2002). Sand is usually removed to increase handling capacity, avoid equipment wear, clogging of transport channels and pre-treatment prior to further processing, such as for bioenergy production (Grimberg, 2008).

Table 1 shows the manure-to-sand ratio is 1:4 on a bone dry basis. The estimated apparent bulk density of the dried dairy manure was 716 kg m^{-3}. The density results are typical of biomass pellets but higher compared to pellets prepared from sawdust (606 kg m^{-3}), logging leftovers (552 kg m^{-3}), corn stover (550 kg m^{-3}) and switchgrass (445 kg m^{-3}) (Ciolkosz, 2016).

Table 1. *Bulk density and ratio of the manure with sand bedding by volume and weight at bone dry conditions*

	Volume of sample (cm^3)	**Weight of sample (g)**	**Bulk density (g cm^{-3})**	**Ratio by volume with total (%)**	**Ratio by weight with total (%)**
Manure	12400	4640	0.374	38	18
Sand	20500	21160	1.032	62	82
Mixture	32900	25800	0.716	100	100

Elemental analysis

Table 2 shows the results of the elemental analysis of sand-separated dairy manure. Sand-separated manure proved to be 4 to 5 times higher in terms of elemental composition compared to a related study conducted by Mukhtar, Goodrich, and

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Capareda (2008). Available carbon is the most important constituent when dealing with biomass fuels. The majority of the contribution to the overall heating value comes from the carbon content. Sand-separated manure has 24.25% of carbon by weight. The removal of inorganic sand increased the carbon composition of manure. SLDN has a C/N ratio of 19.67, which is higher compared with the 14.71 ratio of sand-separated manure. In biogas production, a balanced C/N ratio of feedstock favors an improved methane production system (Wu et al., 2010). An optimal C/N ratio is about 20-30 (Yao and Miller, 2010; Wang et al., 2014). There should be of no need for sand separation if dairy manure will be used for composting or anaerobic digestion.

Table 2. *Elemental analysis for sand-separated and sand-laden dairy manures*

Composition	**Experimental sand-separated manure mean ± SD**	**Dairy manure with sand-bedding* mean ± SD**
Available Carbon %	24.25 ± 3.27	5.9 ± 0.70
Hydrogen %	2.73 ± 0.32	0.7 ± 0.10
Nitrogen %	1.67 ± 0.20	0.3 ± 0.00
Oxygen %	0.38 ± 0.30	0.24 ± 1.00
Sulfur %	0.38± 0.00	0.1 ± 0.00

*Mukhtar et al., 2008

Results for dairy manure composition and constituents vary widely across different conditions and areas in the United States (Wu, 2013; Wu et al., 2012). Variations can be mainly accounted to animal diet and nutrition, manure handling and storage techniques. Different animals produce different manure compositions (ASAE D384.2, 2005; Mukhtar, 2007).

Heating value

The High Heating Value (HHV) of the SLDM was 3.29 MJ kg^{-1}. Upon removal of sand, the HHV increased up to 10.20 MJ kg^{-1}. On another study, sand-separated dairy manure HHV was 9.1 MJ kg^{-1} (Nam et al., 2015). Dairy manure from University of Nebraska farms used for fluidized-bed gasification had a HHV of 11.6 MJ kg^{-1} (Wu et al., 2012). Based on these results, it is recommended to separate sand from the manure to attain better quality of biomass energy fuel. When compared to other biomass fuel, wood pellets have HHV of 17 to 23 MJ kg^{-1} (Leaver, 2001); poultry litter pellets 15.3 MJ kg^{-1} and coal 20-30 MJ kg^{-1} (Kuligowski, 2011).

Pellet drying rate and durability test

The drying characteristic of the dairy manure is shown in *Figure 1*. Convective drying was performed inside a SHERER Environmental Chamber. The average drying rate within the 20-h drying period was measured to be at 0.025 kg moisture removed per kg of dried sample-hour. Decrease of the drying rate curve was observed beyond 20 h of drying time. The pellets were prepared based on different final moisture content with resulting drying times: 15% MC (26.02 h); 10% MC (29.53 h); 5% MC (33.05 h) and 0% MC (38.56 h).

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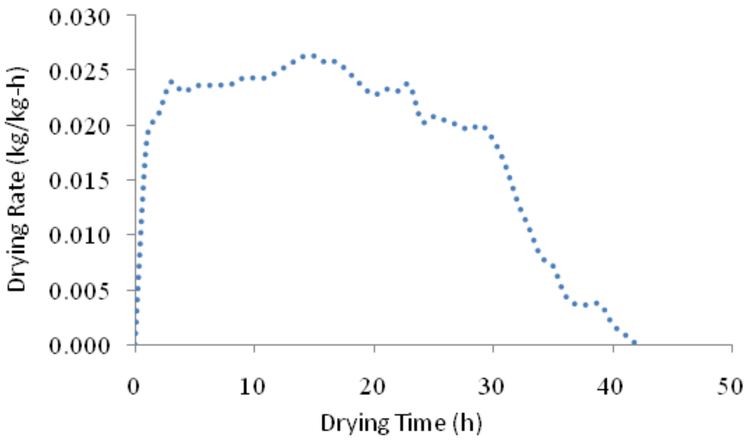

***Figure 1.** Drying curve for the preparation of dried dairy manure pellets*

Studies conducted on biomass pellets for energy fuel show that the optimum moisture content of 10% to 15% can increase durability from 62% to 84% (Kaliyan and Morey, 2006). For wood and agricultural residues, the optimal range for MC is between 10% to 20% (O'Dogherty and Wheeler, 1984). These past results for energy pellets were used as the basis for this study.

The strength of the pellets is inversely proportional to the moisture content as shown in *Figure 2*. The pellets at 0% MC level reached the breaking point after it was compressed for about 0.60 cm. Both the 5% and 10% MC levels pellets reached 1.04 cm compression before breakage. The 15% MC level pellets had the highest compression displacement of 1.06 cm but with the lowest force tolerance of only 0.94 kgf. The 5% MC level pellets can tolerate up to 1.90 kgf while the 10% MC level out at 1.66 kgf.

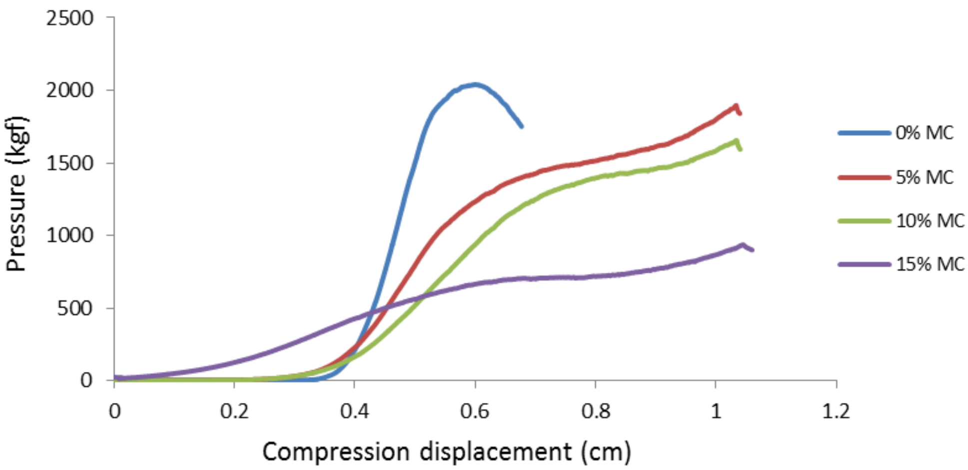

***Figure 2.** Compressive strength test for the pelletized manure at different moisture contents*

The general trend of the MC level and compressive force interaction showed that increasing the moisture content decreased the pellet strength. On a micro level, the solid-solid interaction between manure particles is one factor that provides the ability to withstand pressure. The increase in moisture content increases the spacing between particles, thereby increasing the tendency for shearing (Zafari and Kianmehr, 2012).

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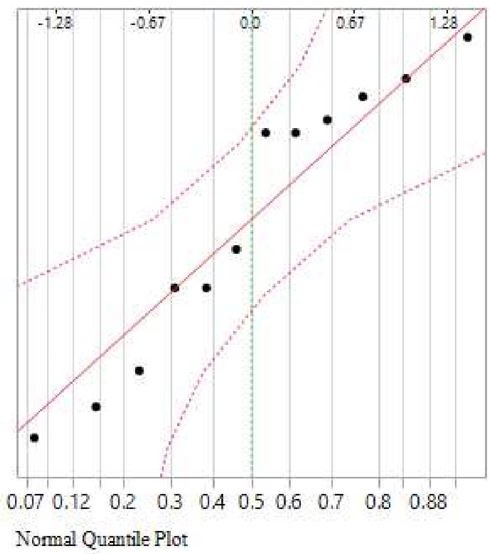
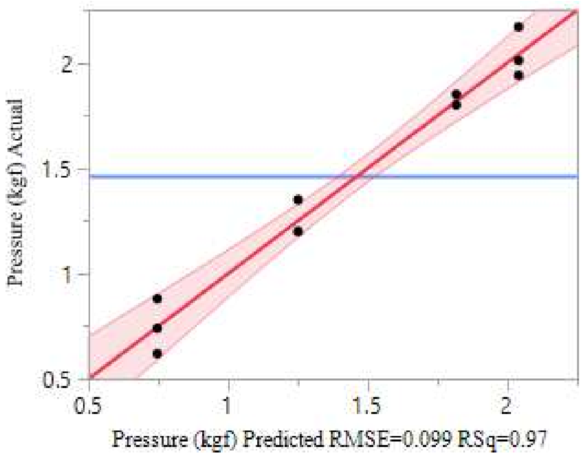
The shearing effect may have reduced the bonding forces between manure particles during pressure application.

Livestock manure moisture content for pelletizing was proposed to be optimal between 20% to 40% (Zafari and Kianmehr, 2012). However, the result was based on a sand-mixed manure system. The sand was observed to be finer particles compared to that of manure using #45 mesh sieves. Since most of the fine particles were removed, there is a tendency for durability to decrease (Wilson, 2010). Observations on the pellets with higher MC seems to have the properties of polymer where it was broken easily but can still hold its form for a longer period as compared to the lower MC pellets that were stronger but once broken it will immediately collapse and disintegrate.

A one-factor three-level ANOVA was used for statistical analysis. The response parameter measured was the pressure tolerance (kgf) of the pellets as indicated by the displacement of the MTS instrument. *Figure 3a* shows that the resulting pressure readings are within the normal distribution region at 95% significance level. The predicted pressure values versus the actual values (*Figure 3b*) of the experiment are within the confidence region, resulting into a fit model with regression value of 0.975.



(a)



(b)

Figure 3. *(a) Normal distribution and (b) predicted values for the pressure and moisture content interaction*

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The test of the fit model significance showed that at 5% level of significance (*Table 3*), the moisture content levels were found to statistically affect the strength of pellets (p-value < 0.0001). Upon using ordered difference between resulting means, all moisture content levels were found to be significantly different. Only the 0% and 5% moisture levels were found to be statistically similar (*Table 4*). Moisture content of 15% falls out of the confidence levels range of compressive force of (1.12 kgf, 1.80 kgf), which shows that pellets should be prepared below 15% MC level in order to withstand the given compressive force range. Dusting was observed to be minimal at 5% and 10% MC level after compression tests. Within the resulting compression forces, the manure pellets are good candidates as biomass fuel thermal processing systems that produces power or energy. The mechanical properties of the prepared manure pellets can tolerate the mechanical handling and avoid dusting concerns commonly experienced by boiler systems in the power industry (Baxter, 2011).

Table 3. *One-way ANOVA of pressure (kgf) applied at varying MCs (%)*

Source	DF	Sum of squares	Mean square	F ratio	Prob > F
MC%	3	3.05	1.016	103.81	< 0.0001*
Error	8	0.08	0.010		
C. Total	11	3.13			

*Significant different at $\alpha = 0.05$

Table 4. *Ordered difference between MC % levels*

+ Level	- Level	Difference	p-Value
0	15	1.29	< 0.0001*
5	15	1.07	< 0.0001*
0	10	0.79	< 0.0001*
5	10	0.57	0.0005*
10	15	0.50	0.0011*
0	5	0.22	0.0928

*Significant different at $\alpha = 0.05$

Conclusion

Physical characteristics of sand-separated dairy manure pellets were investigated as a potential solid fuel in the form of pellets. Raw manure from dairy farms in Stephenville, TX was convectively dried and sand-separated prior to pelletizing. The average drying rate within the 20-h drying period was measured to be at 0.025 kg moisture removed per kg of dried sample-hour. Decrease of the drying rate curve was observed beyond 20 h of drying time. Dried sand-free manure has better elemental and fuel characteristics compared with the SLDM. The dried dairy manure HHV of 10.20 MJ kg^{-1} is already comparable with existing biomass fuels. The resulting pellets can withstand 1.12 kgf to 1.80 kgf at 5-10% MC, which can be a candidate biomass fuel in terms of its elemental and durability properties.

One-way of effectively managing manure wastes is to prepare pellets after convective drying. This will either provide better income options for livestock farm owners or use the pellets for local heat or energy generation. However, to reach the

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optimum MC level for pellets, dryers should be made available to livestock owners rather than solely rely on air-drying. In terms of available technology, there are areas that can still be improved. Manure high initial MC and abrasive qualities are two main challenges that often result into high operating and equipment maintenance cost. Higher-moisture tolerant pelletizers may be developed to even potentially avoid the need for pre-drying.

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THE IMPACT OF THE SOIL CONDITIONER UGMAX ON SELECTED QUALITATIVE CHARACTERISTICS OF POTATO TUBERS

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Abstract. A field experiment was carried out in 2008-2010 on soil of a very good rye complex. The soil reaction ranged from slightly acidic to acidic (pH in 1 n KCl was 4.81-5.91), and the soil was very suitable for potato cultivation. The experiment was arranged as a two-factorial split-plot design with three replicates. The trial included the following two factors: I – two edible potato cultivars (Satina and Tajfun), II – five UGmax application methods (1. control without UGmax, 2. UGmax applied prior to tuber planting at the rate of 1.0 dm^3 ha^{-1}, 3. UGmax applied prior to tuber planting at the rate of 0.5 dm^3 ha^{-1} followed by two foliar applications at the rate of 0.25 dm^3 ha^{-1}, 4. UGmax applied prior to tuber planting at the rate of 1.0 dm^3 ha^{-1} followed by two foliar applications at the rate of 0.5 dm^3 ha^{-1}, 5. two foliar applications of UGmax at the rate of 0.5 dm^3 ha^{-1}). Random tuber samples from 10 plants were collected in each plot, and used to conduct chemical analysis and assess tuber consumption-related characteristics. The soil conditioner UGmax beneficially affected tuber content of starch and vitamin C, and slightly enhanced after-cooking darkening compared with control tubers. Weather conditions in the study years significantly affected dry matter, starch, and vitamin C content as well as raw flesh darkening and after-cooking darkening of tubers. Under the weather conditions in 2010, when precipitation was abundant, the contents of the components studied were the lowest but raw flesh darkening and after-cooking darkening were more intense due to a higher accumulation of reducing sugars.
Keywords: *table potato, dry matter, starch, vitamin C, consumption-related characteristics*

Introduction

Intensification of crop production (cultivation in monoculture, reduced soil tillage, declining soil content of humus, an application of plant protection chemicals, use of heavy machinery) and livestock production (declining farmyard manure supply due to animal facility modernisation) results in a microorganism- and soil fauna-impoverished environment, as well as exacerbating physical, chemical and microbiological properties of the soil, which in turn affects both plant yields and yield quality (Melero et al., 2006; Trawczyński and Bogdanowicz, 2007; Mrówczyński and Roth, 2009; Liu et al., 2010). The negative effect of the aforementioned factors becomes even more pronounced due to unfavourable weather conditions during the growing season, and agrotechnological

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mistakes. Thus, farmers are more and more interested in various types of commercially available biostimulants, extracts, bacterial vaccines, EM (effective microorganisms) products, biofertilisers and conditioners which enhance soil humus and organic carbon, increase soil pH, improve plant resistance to diseases and other stress conditions, contribute to better nutrient uptake from the soil, and increase plant yields (Emitazi et. al., 2004; Boligłowa and Gleń, 2008; Sulewska et al., 2009; Piotrowska et al., 2012; Zarzecka et al., 2014).

Organic matter content is a basic indicator of soil quality and fertility, and a key factor in proper plant growth, development and yield performance (Haynes, 2005; Lal, 2011; Krasowicz et al., 2011). The soil conditioner UGmax, one of products applied in plant cultivation, is a natural liquid concentrate containing beneficial microorganisms such as lactic acid bacteria, photosynthetic bacteria, *Azotobacter, Pseudomonas,* yeast, actinomycetes as well as some macroelements and microelements (Trawczyński and Bogdanowicz, 2007). Results of research into the effect of this product are sometimes equivocal, however. Several workers have not confirmed a positive influence of UGmax (Kaczmarek et al., 2008; Dinesh et al., 2010; Martyniuk, 2011); others have reported a beneficial effect of this conditioner (Sulewska et al., 2009; Ratajczak et al., 2016). Piotrowska et al. (2010, 2012) found that Ugmax application increased soil pH, organic carbon (C_{org}) content, total nitrogen content, and soil enzymic activity compared with control. In contrast, a study by Dębska et al. (2016) established an increase in soil organic carbon content and organic matter, including humins and humic acids. Many authors applied UGmax and observed a tendency for crop yields to increase, or a beneficial influence on crop yield chemical composition and increased plant resistance to diseases. Sulewska et al. (2009) reported that the yield of maize cultivated for grain and silage increased by 0.71 and 5.6 $t \cdot ha^{-1}$, respectively, compared with control. Kotwica et al. (2013) noticed an increase in winter wheat biomass and grain yield whereas Górski et al. (2015) pointed to enhanced sugar content in sugar beet roots and higher root yield. Kołodziejczyk et al. (2013) reported an increase in spring wheat grain yield and Keutgen et al. (2014) found that the quality of carrot storage roots improved due to increased total carotenoids and lower ascorbic acid losses during processing. In their study on potato, Trawczyński and Bogdanowicz (2007) recorded total tuber yield that increased by 7.1%, Jabłoński (2012) reported that the total yield and marketable yield were higher by 12.2 and 15.1%, respectively, and Frąckowiak-Pawlak (2011), who conducted a six-year study, found a 30% increase in tuber yield. Kołodziejczyk (2014a) pointed to a favourable effect of UGmax on tuber yield and yield components, and in another study, the author noted a positive effect of the product on tuber fresh and dry matter yields as well as plant dry matter yield. Kowalska (2016) reported an increase in table potato tuber yield, a higher share of these tubers in the total yield, and reduction of *Phytophthora infestans* symptoms on the plant. Zarzecka et al. (2011) demonstrated that an application of UGmax contributed to an increase in tuber yield and a decline in the share of tubers with *Streptomyces scabies* and *Rizoctonia solani* symptoms. In other study with UGmax, Zarzecka et al. (2014) recorded increased nitrogen and magnesium amounts in potato tubers compared with control. UGmax is a well-established product which is popular with farmers as it improves soil properties, boosts crop yields and is cost-effective (Trawczyński and Bogdanowicz, 2007; Piotrowska et al., 2012). There are few studies examining the effect of the soil conditioner UGmax on tuber chemical composition and consumption-related characteristics. Moreover, the reports that exist are unequivocal. Thus, more research

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into UGmax application in potato cultivation is fully justifiable. Potatoes are a major staple food and, as a result, are one of the most important crop plants in the world, Europe and Poland (Lisińska et al., 2009; Abolgasem, 2014; Wegener et al., 2015). Potato consumption in Poland is quite high and has amounted to 100-121 kg per capita in the last decade (Dzwonkowski et al., 2016). Hence, the objective of the study reported here was to determine the effect of the soil conditioner UGmax on selected qualitative characteristics of table potato tubers.

Materials and methods

The experimental site

A three-year field trial was conducted at the Zawady Experimental Farm (52°20′ N; 22°30′) on grey brown podzolic soil representing the very good rye class of agricultural land suitability (class IVa). The soil ranged from slightly acidic to acidic (4.81-5.91, pH in 1 n KCl), had high to very high available phosphorus content, low to high potassium content, and low to average magnesium content. The experiment was a split-plot arrangement with three replicates. Factor I included two medium-early table potato cultivars: Satina and Tajfun − high-yielding varieties characterised by consumption characteristics that are significant for customers, which makes them popular on the market and hence in production in east-central Poland. Moreover, in the study years, they were included in the List of Recommended Cultivars for Mazovian Voivodeship based on research of Post-Registration Variety Testing. Factor II consisted of five application methods of the soil conditioner UGmax presented in *Table 1*.

Table 1. *The use of UGmax in the experiment*

Treatments	Rates ($dm^3 \cdot ha^{-1}$)	Application dates of UGmax		
		Before tuber planting	10-15 cm potato plant height	Flowering
1. Control– without UGmax	-	-	-	-
2. UGmax – before planting	1.0	1.0	-	-
3. UGmax – before planting and double foliar application	1.0	0.5	0.25	0.25
4. UGmax – before planting and double foliar application	2.0	1.0	0.5	0.5
5. UGmax – double foliar treatment	1.0	-	0.5	0.5

The conditioner was dissolved in 300 $dm^3 \cdot$water per ha^{-1}. The product contained the following macroelements and microelements: 1200 N, 220 P, 2905 K, 100 Mg, 200 Na, 0.3 Mn, and the following microorganisms: lactic acid bacteria, photosynthetic bacteria, *Azotobacter, Pseudomonas,* yeast and actinomycetes. In autumn, farmyard manure was applied at a rate of 25.0 $t \cdot ha^{-1}$ in addition to mineral fertilisers used at the following rates: 44.0 $kg \cdot ha^{-1}$ P (in the form of 46% triple superphosphate), 124.5 $kg \cdot ha^{-1}$ K (in the form of 60% potassium salt) and 100 kg N per 1 ha (in the form of 34% ammonium saltpeter), nitrogen applied in spring. Potatoes were planted at 67.5 x 37 cm spacing in mid-April and harvested in September. To control weeds, a mixture of the herbicides Command 480 SC 0.2 $dm^3 \cdot ha^{-1}$ + Afalon Dyspersyjny 450 SC 1.0 $dm^3 \cdot ha^{-1}$ was applied

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5-7 days prior to potato plant emergence. Potato blight was controlled using Ridomil Gold MZ 68 WG and Dithane 455 SC and Colorado potato beetle was controlled by means of Apacz 50 WG and Actara 25 WG. Prior to harvest, random samples of tubers were collected from each plot and used for chemical analysis and assessment of consumption-related characteristics of the tubers.

Chemical analysis methods

Vitamin C content was determined by means of Tillman's method as modified by Pijanowski (Rutkowska, 1981). The dry matter content of potatoes was determined by drying to constant weight at 105°C, and starch concentration using a Reimann hydrostatic balance. The assessment of consumption-related (organoleptic) tuber characteristics was performed 8-10 days after harvest by a team which consisted of four people. The darkening of raw flesh was evaluated subjectively on ten tubers collected from each treatment, 10 min after cutting, and darkening of cooked flesh 10 min after cooking, using a Danish colour scale on which 1 – most intense darkening, and 9 – unchanged colour (Roztropowicz, 1999). All the analyses were performed at the Chemical Laboratory of the Department of Agrotechnology of Siedlce University of Natural Sciences and Humanities.

Meteorological conditions

The weather conditions in the study years varied (*Table 2*). In 2008, air temperature was similar to the long-term mean whereas precipitation was by 96.2 mm higher but unevenly distributed throughout the growing season. The year was conducive to potato plant growth, development and yielding. In 2009, the average temperature was higher than the mean calculated across 1987-2000 and precipitation was unevenly distributed. The year 2010 was warmer than the previous years, and wet because of heavy precipitation which exceeded the average long-term sum by 184.5 mm.

Table 2. *Air temperature and rainfall during potato growing seasons according to the Zawady Meteorological Station*

Months	Air temperature (°C)				Rainfall (mm)			
	Long-term mean	Monthly means			Long-term mean	Monthly sums		
	1987-2000	2008	2009	2010	1987-2000	2008	2009	2010
April	7.8	9.1	10.3	8.9	38.6	28.2	8.1	10.7
May	12.5	12.7	12.9	14	44.1	85.6	68.9	93.2
June	17.2	17.4	15.7	17.4	52.4	49	145.2	62.6
July	19.2	18.4	19.4	21.6	49.8	69.8	26.4	77
August	18.5	18.5	17.7	19.8	43	75.4	80.9	106.3
September	13.1	12.2	14.6	11.8	47.3	63.4	24.9	109.9
April-September	14.7 mean	14.7	15.1	15.6	275.2 sum	371.4	354.4	459.7

Statistical analysis

Results of the study were analysed by ANOVA. Significance of sources of variation was checked with the Fisher-Snedecor test and the significance of differences between

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means was tested using the multiple comparison Tukey's test at the significance level of P = 0.05. Statistical calculations were performed in Excel using the authors' own algorithm based on the split-plot mathematical model.

Results

The dry matter content of potato cultivated in the experiment reported here ranged from 184.5 to 238.9 $g \cdot kg^{-1}$ and was significantly affected by cultivars and weather conditions in the study years (*Tables 3, 6*). Significantly higher dry matter amounts were accumulated by Tajfun vs Satina, and the most favourable years for dry matter accumulation were 2009 and 2008. In these study years, temperatures throughout the months of the greatest yield production (July, August and September) which are the most conducive to dry matter accumulation were the closest to the long-term mean. The research reported here demonstrated that UGmax application, regardless of the treatment, did not significantly affect the tuber content of dry matter. However, there was observed a tendency for the amount of this component to increase compared with control.

Table 3. *The dry matter content and starch in potato tubers ($g \cdot kg^{-1}$)*

Treatments	Dry matter			Starch		
	Cultivars		Mean	Cultivars		Mean
	Satina	Tajfun		Satina	Tajfun	
1	184.5	231.1	207.8	127	156.1	141.6
2	190	236.7	213.4	128.1	157	142.6
3	190.5	233.9	212.2	127.4	156.6	142
4	194.2	238.9	216.6	130	159.5	144.8
5	192.8	232.8	212.8	128.2	158.9	143.6
Mean	190.4	234.7	212.6	128.2	157.6	142.9
Mean for treatments 2-5	191.9	235.6	213.8	128.4	158	143.2
$LSD_{0.05}$ for:						
Cultivars			3.1			1.8
Treatments			n.s.			3
Interaction cultivars x treatments			n.s.			n.s.

n.s. – non-significant differences

Starch content in tubers depended on the cultivar, UGmax application methods and weather conditions during the experimental period, and ranged from 127.0 to 159.5 $g \cdot kg^{-1}$ (*Tables 3, 6*). Tajfun rather than Satina accumulated more starch. UGmax application positively influenced starch accumulation, the most starch being accumulated by potatoes which had been sprayed with the conditioner three times at the total rate of 2.0 $dm^3 \cdot ha^{-1}$.

Analysis of an accumulation of this basic carbohydrate in individual growing seasons revealed that the most starch was accumulated in 2009, the accumulation being significantly lower in the remaining study years.

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Vitamin C content in potato tubers ranged from 195.6 to 234.8 mg·kg^{-1} fresh matter and was affected by UGmax application and weather conditions in the study years (*Tables 4, 6*). The cultivars studied had a similar vitamin C content. In the present study, vitamin C accumulation was significantly higher in tubers harvested in plots where UGmax had been applied three times (prior to tuber planting, when plants were 10-15 cm high, and at the beginning of bloom time) compared with control. They found a significant effect of atmospheric conditions in the study years on vitamin C content. The highest concentration of this component was determined in 2008 when the air temperature was similar to the long-term mean and precipitation was evenly distributed compared with 2009 and 2010.

Table 4. *The vitamin C content in potato tubers (mg·kg^{-1} fresh matter)*

Treatments	Cultivars		Mean
	Satina	Tajfun	
1	209.6	211.7	210.6
2	212.4	215.7	214.1
3	214.7	217	215.9
4	217.7	218.6	218.2
5	211.8	214.8	213.3
Mean	213.2	215.5	214.4
Mean for 2-5 objects	214.2	216.5	215.4
$LSD_{0.05}$ – for:			
Cultivars			n.s.
Treatments			3.9
Interaction cultivars x treatments			n.s.

n.s. – non-significant differences

The most important characteristics of table potato consumption-related/organoleptic/sensory value include: raw tuber flesh darkening, after-cooking darkening, flavour and culinary type. In the study reported here, raw tuber flesh darkening, assessed 10 min after tubers were cut through, was significantly affected by cultivars and weather conditions in the study years (*Tables 5, 6*). Satina darkened less than Tajfun, the darkening for both cultivars being low as it averaged 8.89 and 8.87, respectively, on a 1-9 scale. Analysis of the effect of weather conditions on raw tuber flesh darkening demonstrated that it was the highest in 2010, when precipitation was high, and the lowest in the 2008 growing season.

Organoleptic testing of cooked tubers demonstrated a significant effect of UGmax application methods and weather conditions throughout the study years on this characteristic (*Tables 5, 6*). An application of the conditioner contributed to an increase in after-cooking darkening of tubers harvested in the plots where UGmax had been applied two and three times (treatment 3, 4 and 5). The change in colour was inconsiderable as after-cooking darkening did not exceed 0.1 point on a 1-9 scale. Under the climatic conditions which prevailed in 2010, after cooking darkening of tubers was significantly higher compared with the remaining study years, which indicates that excessive precipitation during the growing season has a negative effect of this attribute.

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Table 5. *Darkening of raw flesh and cooked flesh of potato tubers after 10 min (scale 1-9)*

Treatments	Raw flesh darkening			After-cooking darkening		
	Cultivars		Mean	Cultivars		Mean
	Satina	Tajfun		Satina	Tajfun	
1	8.91	8.89	8.9	8.98	8.94	8.96
2	8.91	8.89	8.9	8.92	8.9	8.92
3	8.88	8.87	8.87	8.89	8.87	8.88
4	8.88	8.88	8.88	8.89	8.88	8.89
5	8.89	8.85	8.86	8.87	8.87	8.87
Mean	8.89	8.87	8.88	8.91	8.89	8.9
Mean for treatments 2-5	8.89	8.87	8.88	8.89	8.88	8.89
$LSD_{0.05}$ – for:						
Cultivars			0.02			n.s.
Treatments			n.s.			0.05
Interaction cultivars x treatments			n.s.			n.s.

n.s. – non-significant differences

Table 6. *Effect of study years on the content of dry matter, starch and vitamin C, and flesh darkening of potato tubers*

Years	Dry matter ($g \cdot kg^{-1}$)	Starch ($g \cdot kg^{-1}$)	Vitamin C ($mg \cdot kg^{-1}$f.m.)	Flesh darkening (scale 1-9)	
				Raw	Cooked
2008	220.5	142.6	234.8	9	8.99
2009	221.2	151.8	212.8	8.87	8.94
2010	196	134.3	195.6	8.78	8.78
Mean	212.6	142.9	214.4	8.88	8.9
$LSD_{0.05}$	0.5	0.3	4.4	0.03	0.06

Discussion

Potato nutritional value is mainly related to tuber chemical composition, in particular dry matter content, starch content, total sugars, reducing sugars, protein content, vitamin C content, minerals and harmful substances (Lisińska et al., 2009; Leszczyński, 2012; Zarzecka et al., 2013; Sawicka et al., 2015). The majority of traits which are significant in potato production are dependent on the genotype, agrotechnological practices and meteorological conditions, in particular precipitation and air temperature during the growing season (Sawicka, 2000; Mazurczyk and Lis, 2001; Leszczyński, 2012; Kołodziejczyk, 2014a; Zarzecka et al., 2014).

In the study reported here, dry matter content in potato tubers was significantly affected by the cultivar and weather conditions. Of the cultivars examined, cv. Tajfun accumulated more dry matter than Satina. Moreover, 2009 and 2008, when temperatures in the months of yield formation were similar to the long-term mean, were the most conducive to the accumulation of this component. A similar effect of cultivars and weather conditions during the growing season on dry matter content was reported by Sawicka and Mikos-Bielak (2008) and Sawicka and Pszczółkowski (2005). Also

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Mazurczyk and Lis (2001), Trawczyński and Prokop (2016) found that potato tubers accumulated the most dry matter and starch under favourable hydrothermal conditions.

The research reported here demonstrated that UGmax application, regardless of the treatment, did not significantly affect the tuber content of dry matter. However, there was observed a tendency for the amount of this component to increase compared with control. An insignificant effect of UGmax was also noted by Kołodziejczyk (2016) whereas Trawczyński and Bogdanowicz (2007) reported a statistically significant increase in dry matter content following an application of the conditioner.

Starch is the main component of potato tuber dry matter, and starch content in cultivars registered in Poland ranges from 11.0 to 18.3%. In the study discussed here, the tuber content of starch was significantly affected by cultivars, methods of UGmax application and climatic conditions in the study years. Similarly to dry matter, starch content was higher in Tajfun than Satina. Soil conditioner application significantly improved potato quality as it contributed to an increase in the tuber content of starch. An increase in starch content following UGmax application when accompanied by full mineral fertilisation, compared with control, was also recorded by Szewczuk et al. (2016) and Kołodziejczyk (2016). In turn, Trawczyński and Bogdanowicz (2007) demonstrated no changes in starch content due to an application of the conditioner. Analysis of starch content in individual growing seasons demonstrated that more starch was accumulated in tubers in 2009 when air temperatures were similar to the long-term mean. Starch accumulation was the lowest in 2010 when precipitation was high during the growing season. Puła and Skowera (2004) demonstrated that high precipitation was a factor which significantly reduced starch accumulation in tubers. Many authors have reported that starch content in potato tubers is affected by the genetic factor and weather conditions (Sawicka and Mikos-Bielak, 2008; Lisińska et al., 2009; Abolgasem, 2014; Kołodziejczyk, 2016; Trawczyński and Prokop, 2016).

In order to function properly, the human body needs vitamins in addition to carbohydrates, proteins, fats and minerals. Potato tubers are high in vitamin C. The vitamin is one of major anti-oxidants which also participates in many processes and metabolic conversions. Moreover, as it is not synthesised by the human body so the vitamin has to be provided in the diet (Lisińska et al., 2009; Leszczyński, 2012).

In the present study, vitamin C content was significantly affected by an application of UGmax and weather conditions over the experimental period. In contrast, the cultivars studied differed insignificantly in terms of the characteristic in question. In turn, Sawicka et al. (2014) demonstrated that vitamin C content was more affected by genotype than the environment. An application of UGmax positively affected an accumulation of vitamin C in potato tubers. An increase in vitamin C content following UGmax application was reported by Wichrowska et al. (2015) who examined potato tubers, and by Keutgen et al. (2014) who studied carrot storage roots. Chemical analyses conducted in the study reported here revealed the highest vitamin C content in tubers harvested under the optimum weather conditions of 2008, compared with the remaining years. Similar results were obtained by Mazurczyk and Lis (2001) and Trawczyński and Prokop (2016).

The following attributes determine the sensory quality of table potato tubers: raw tuber flesh darkening, after-cooking darkening, flavour and culinary type (Lisińska et al., 2009; Leszczyński, 2012). In the study reported here, raw flesh darkening was significantly affected by the cultivar and weather conditions whereas after-cooking darkening was significantly influenced by UGmax application and climatic conditions

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in the study years. Differences in raw flesh darkening as affected by cultivar properties were also reported by Sawicka (2000). Raw tuber flesh of potatoes cultivated in 2010, characterised by high precipitation, was the most prone to darkening compared with 2008 and 2009. Also Sawicka (2000) noticed that sunny and dry weather was associated with slight darkening of raw tuber flesh. In contrast, Kołodziejczyk (2016) reported no effect of hydrothermal conditions on raw flesh darkening. Moreover, the author found no influence of UGmax on raw tuber flesh darkening, which agrees with the findings reported here.

After-cooking darkening is a very important attribute for consumers (Leszczyński, 2012). Organoleptic assessment of cooked tubers revealed that UGmax increased after-cooking darkening. Differences in the assessment of flesh darkening ranged from 0.02 to 0.09 points on a scale of 1-9, the control being the most favourable in this respect. The available literature lacks reports on the effect of UGmax on after-cooking darkening of potato tubers. Potatoes grown in 2008, characterised by hot summer, had the lowest after-cooking darkening, it being the highest in the wet 2010. Too much rain increased flesh darkening due to an increased accumulation of reducing sugars in potato tubers. A similar finding was reported by Grudzińska and Zgórska (2008) who noted that a decline in the potato tuber content of starch was followed by an increase reducing sugars, the changes being the result of starch transformation into simple sugars.

Conclusions

The study demonstrated that the soil conditioner UGmax applied to potatoes had a positive effect on starch content and vitamin C content in their tubers, and slightly increased after cooking darkening compared with control tubers where no UGmax had been applied. The cultivar factor significantly affected the attributes tested. Weather conditions during the period of *Solanum tuberosum* growth and development had a significant effect on dry matter, starch and vitamin C content in tubers as well as raw flesh darkening and after-cooking darkening. In 2010, the concentration of the components examined was lower but potato tuber flesh darkening was more intense due to excessive precipitation, because of an increased accumulation of reducing sugars whose concentration increases as starch content declines.

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CORRELATION BETWEEN THE ENVIRONMENTAL KNOWLEDGE, ENVIRONMENTAL ATTITUDE, AND BEHAVIORAL INTENTION OF TOURISTS FOR ECOTOURISM IN CHINA

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Abstract. Due to the popularity of recreation and entertainment activities, the concept of ecotourism is getting popular in recent years. The research targets in this study include tourists to the Wuyi Mountain Duanyuan Ecological Tourism Zone, China. The convenience sampling approach is used in this study and a total of 500 questionnaire copies are dispatched. There are 372 valid questionnaire copies returned and the rate of response is 74%. The results of this study indicate that 1.there is a positive correlation between the environmental knowledge and the environmental attitude, 2.there is a positive correlation between the environmental attitude and the environmental behavior, and 3.there is a positive correlation between the environmental knowledge and the environmental behavior. Finally, recommendations are proposed based on the research results and expected to be beneficial for culturing common people's environmental literacy, correct environmental attitude, environmental and ecological caring, and fulfillment of environmental behaviors.
Keywords: *consumer movement, eco-management movement, environmental value, ecology, environmental belief*

Introduction

Since the industrial revolution in the eighteenth century, with the prosperity of economy, technological advances, and the endless extraction of resources, people have consumed a vast amount of natural resources and further damaged the natural environment. The generation of more contaminations in air, water, and soil therefore leads to the accelerate extinction of species and the imbalance of the ecosystem. In recent years, with the fast development of economy, people are getting wealthier and the level of living standard is enhanced. However, various types of pollution are generated in succession. In recent years, the environmental problems are getting worse. Due to the popularity of the concept of recreation and entertainments and because of abundant humanism ancient monuments, eco-environmental planning and recreational facilities in the country, there are always heavy traffic and crowds of tourists during holidays. Although this phenomenon has activated the tourism industry, the large amount of garbage and air pollution also results in hassles. The motivation of this study is to understand tourists' environmental knowledge, environmental attitude, and environmental behavior. Nowadays, the environmental protection problems around the globe include greenhouse effect, acid rain, water resource depletion, garbage pollution, air pollution, and wildlife

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reduction. The ecosystem has been damaged and this leads to ecological imbalance and the deterioration of the living environment as well as the direct influence on people's lifeblood of sustainable survival. Moreover, with the fast expansion of human activities, the resources on the Earth have been consumed rapidly and an excessive amount of pollutions and wastes have been generated. The resources on the Earth are limited and we have to value the sustainable development of the environment and avoid excessive developments and utilizations which lead to the deterioration of our living environment. The important and inherent path to changes is to enhance our knowledge via education and change every individual's attitude and behavior. This allows every person to own abundant environmental knowledge, adequate environmental attitude, and correct environmental behaviors. Therefore, we appreciate the emphasis by the international society on the environmental knowledge and expect to change every individual's attitude and behavior via education. This is because the eventual goal of the environment education is to improve people's environmental behaviors. The best timing for improving behaviors is to culture them from the childhood. During this stage, people have a better learning capability with a greater level of flexibility. Via the process of conceptual perceptions and the clarification of value during this stage, people can learn to establish the environmental awareness and sensitivity, environmental concept and knowledge, environmental value and attitude, environmental action skills, and environmental action experiences. It is therefore necessary and urgent to culture people's environmental literacy, correct the environmental attitude, environmental and ecological caring, and the fulfillment of environmental behaviors.

Literature review

The study of the correlation between environmental knowledge and environmental attitude

Van Birgelen et al. (2011) indicated that a significant correlation existed between a schoolchild's environmental knowledge of oceans and he/her attitude. Lu and Shon (2012) studied high school students who took ten days of environmental science education and discovered that the students had a higher level of environmental knowledge and held a more optimistic and positive attitude toward the environment. The studies of other scholars (Lee, 2011a) also indicated that, after a student taking the course of training of the environment for four to eight weeks, there was a positive correlation between the environmental knowledge and his/her environmental attitude. It is obvious that the education behavior is positively beneficial for enhancing environmental knowledge and the environmental attitude. The study by Van Birgelen et al. (2011) indicated that there was a significantly positive correlation between a high school teacher's environmental sensitivity and the environmental knowledge. For the correlation between perception and the affective domain, Kim et al. (2014) proposed that an elementary school teacher's environmental knowledge was positively correlated to the perception of environmental problems in Taiwan, environmental awareness, cosmic belief, and social values. Araghi et al. (2014) selected students of a public vocational training organization as the research targets and discovered that there was a significant correlation between a student's environmental knowledge and his/her environmental attitude. As a result, this study proposes several hypotheses as follows.

Hypothesis 1: The environmental knowledge is positively correlated to the environmental attitude.

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The study of the correlation between environmental attitude and environmental behavior

Scheelhaase et al. (2010) also indicated that factors of (1) Environmental sensitivity, (2) Personal control belief, (3) Group control belief, and (4) Attitude toward pollution had a significantly positively correlation to environmental behavior. Abdollahzadehgan et al. (2013) used garbage recycling as an example for investigating the correlation between environmental behavior and various types of factors. In his study, the environmental attitude was found to be independent of these factors. In another word, people who had positive and optimistic environmental attitude would not necessarily fulfill the behavior of garbage recycling actively. Sultan (2013) conducted an investigation on 1225 nature and environment education centers and indicated that the development of an individual's attitude was beneficial for developing responsible behaviors toward the environment. Davison et al. (2014) carried out the study of factors relevant to responsible environmental behaviors. They also found that an individual with a more positive and optimistic attitude toward environmental issues could contribute to his/her responsible behaviors. Chen (2013) also conducted an investigation of environmental attitudes and behaviors on Taipei City and Kaohsiung City citizens. It was found that the degree of correlation between environmental attitude and behavior was low to moderate. Vlahakis et al. (2013) selected six-grade students of elementary schools in Taoyuan County as the research targets and found that the environmental attitude was correlated to the choice of environmental action strategies. Zsóka et al. (2013) selected students of the National Taiwan University as the targets and revealed the research results that these students presented a significant correlation and consistency between their overall attitude toward eco-friendly and their real eco-friendly behaviors that had been observed. As a result, another hypothesis is proposed in this study as follows.

Hypothesis 2: Environmental attitude is positively correlated to the environmental behavior.

The study of the correlation between environmental knowledge and environmental behavior

Van Der Linden (2014) conducted a meta-analysis of studies relevant to the aspect of environmental behaviors since 1971. It was found that the environmental behavior was positively correlated to an individual's degree of understanding for environmental problems. Shabnam (2013) also proposed that there were significantly positive correlations between the knowledge of environmental action strategies and environmental behaviors. In other words, the knowledge of environmental action strategy had a significant influence on environmental behaviors. The results obtained by Ziegler et al. (2012) from telephone surveys also indicated that the people with more sufficient preservation knowledge tended to undertake more tasks such as garbage recycling and were willing to devote themselves to environment protection tasks. Enfield and Mathew (2012) used garbage recycling as an example for investigating the correlation between environmental behaviors and various types of factors. They proposed that there was a close correlation between a frequent supplement of information and guidance and the fulfillment of a behavior. The study by Ko and Dennis (2011) indicated that environmental knowledge was correlated to environmental action. As a result, another hypothesis is proposed in this study as follows.

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Hypothesis 3: Environmental knowledge is positively correlated to environmental behavior.

Environmental knowledge

Environmental knowledge refers to an individual's understanding of the things and objects in the environment (Lee, 2011a) and the scope of its coverage is very extensive. Davison et al. (2014) classified the environment education into thirteen categories which included environmental management, environmental management technique, environmental problem, geo-ecology, economics, social and cultural environment, adaptation and evolution, natural resource, culture, politics, family, individual, and mentality. Any knowledge relevant to this territory was included in the scope of the environmental knowledge. Kim et al. (2014) proposed eleven principal ideas of the teaching materials for environmental education, including the general concepts, air pollution, noise pollution, urban problem, balance of nature, forest and wood preservation, human resource preservation, land resource maintenance, wise utilization of minerals, hydraulic resource preservation, and wildlife preservation. Araghi et al. (2014) proposed that the environmental knowledge was an interdisciplinary subject that was a type of knowledge derived from the nature, social science, and anthropology. It sometimes was also related to ethics including value and the assignment of authority. The level of its coverage was very extensive and people's basic necessities of life could not sustain without the environment. For this reason, any knowledge that is relevant to people's life and the environment could be called the environmental knowledge (Mantzicopoulos and Patrick, 2011).

According to the study by Chang (2011), the constituent elements for evaluating the environmental knowledge can be classified as follows.

- Ecology: Environmental conservation and the diversity of creatures.
- Environmental science: Air pollution and prevention, water resource preservation and water pollution prevention, soil contamination prevention and soil and water conservation, noise pollution and prevention, garbage and business wastes pollution prevention, environmental sanitation and food hygiene, and sustainable energy.
- Environmental issue: Environmental issue, nuclear power generation and radiation safety, population hygiene, air pollution and prevention, water resource preservation and water pollution prevention, and garbage and business wastes pollution prevention.

Environmental attitude

Lian et al. (2014) proposed that an attitude was an individual's inner intention toward a target object and was the origin and preparation for a behavior. An attitude is a type of complex psychological process which includes the tendency of perception, emotion, and behaviors and is provided with enduring and consistent characteristics. Enfield and Mathew (2012) proposed that the *environmental attitude* indicated the combination of the beliefs toward the special conditions of the environment, the entire environment, and the people or objects directly relevant to the environment. These combinations comprised an overall assessment which could be assent or objection, and like or aversion. When an individual is provided with this emotion, he/she will generate a strong sense of caring for the environment, actively participate in environmental

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protection, and generate the motivation of improvements (Mitchener and Jackson, 2012). Abdollahzadehgan et al. (2013) proposed that an environmental attitude was an individual's level of dedication and support for the things and objects in the environment. Sultan (2013) proposed that an attitude was a type of enduring and consistent tendency of an individual for people, things, and the surrounding world. It could be conjectured from explicit behaviors but its connotation was not limited to purely explicit behaviors. Ko and Dennis (2011) proposed that an *environmental attitude* meant a schoolchild's value for the overall environment and the opinion of people's duty and role for residing in the environment. Furthermore, depending on the integration of individual perceptions, they generated the emotional tendency to agree or disagree, like or dislike certain things or objects that were relevant to the environment. For example, these included the self-awareness of the duty for the environment or the degree of their concern for environmental problems. Van Birgelen et al. (2011) proposed that an environmental attitude meant an individual's opinion or tendency for the belief and value of environmental issues and whether they agreed with or opposed to and preferred or were averse to an action. An environmental attitude can therefore be delimited as an individual's characteristics that are formed in the long run. They will continuously be concerned with environmental issues and eventually take actions in environment protection. In this case, the environmental attitude includes the environmental value and the environmental belief (Van Birgelen et al., 2011). At the same time, it can also demonstrate an individual's opinion of the duty and role of human beings in the environment.

The environmental attitude in this study is combined with the environmental knowledge and the main purpose is to investigate the attitude held by the research targets for the environmental problems. Based on the study by Chen (2013), the environmental attitude is therefore classified into two constituent elements, including environmental value and environmental belief, so as to understand environmental attitude and tendency.

Behavioral intention

The individual behaviors studied in psychology indicate that individual activities can be observed or measured by instruments in a narrow sense of explanation. From a generalized definition, they include not only the explicit activities that can be directly observed or measured, but also the internal psychological processes that can be indirectly predicted. The internal psychological processes include the conscious process and unconsciousness process (British Airways, 2012). A behavior is a type of generalization term and its connotation includes all activities of an individual (Lee, 2011b). Alatawi et al. (2012) defined an environmental behavior as *the behavior that was presented by an individual or group so as to resolve a certain environmental problem*. In another word, it meant the path taken by an individual or a group to prevent or resolve environmental problems or issues (Chen et al., 2011). The most important key factor is whether an individual can actively participate in. An environmental behavior is namely the behavior presented by a person, an individual, or a group to resolve a certain environmental problem. After an environmental citizen is equipped with the environmental knowledge, environmental attitude, and environmental skills, he/she has to take actions and participate in resolving various types of environmental problems. The levels of participating in such type of actions may have different names such as environmental action, citizen participation, or responsible environmental behaviors. However, all of them emphasize the importance of people's active participation and actions to resolve or prevent environmental problems (Hadzigeorgiou et al., 2011). In this study, the behavioral intention is classified into two constituent elements, including eco-management movement and consumer movement proposed by Joe et al. (2013).

Methodology

Research targets and sampled data

Tourists to the Wuyi Mountain Duanyuan Ecological Tourism Zone, China, are selected in this study as the research targets. The convenience sampling approach is used in this study. A total of 500 questionnaire copies are dispatched. The number of valid questionnaire copies returned is 372 and the rate of response for this questionnaire is 74%. After questionnaire copies are returned, the statistical software SPSS is used for carrying out data analysis. Statistical methods including the factor analysis and reliability analysis, regression analysis, and structural equation models are used for examining each of the hypotheses. The Wuyi Mountain Duanyuan Ecological Tourism Zone is in Fujian, China. The most distinguishing feature of the Wuyi Mountain Duanyuan Ecological Tourism Zone is its coastline. It has been the most favorite tourist attraction for Chinese people. Due to millions of years of crust motions, the lands and the ocean have been integrated into each other and this brings up the unique landscapes in this area. Chen et al. (2011) stated the world under the sea is even more fabulous and colorful. Various types of fish species and colorful corals have become the representing distinguishing feature. From the ecological aspect, the tropical region climate has cultivated vital tropical and seashore plants. There are numerous migratory birds transiting every year in autumn and winter and this makes it become a notable bird-watching attraction (Lian et al., 2014). In addition, several prehistoric monuments and cultural heritages of the indigenous people are also discovered in this area and these have become the invaluable humanism assets.

Analysis method

The regression analysis is used in this study for understanding the correlation between environmental knowledge and environmental attitude. Furthermore, the analysis of structural equation models is used for understanding the correlation between the tourists' environmental attitude and their behavioral intention.

Results

Reliability and validity analysis

After the factor analysis in this study, three factors are extracted from the environmental knowledge. The first factor is "ecology" (eigenvalue =2.671, α=0.83). The second factor is "environmental science" (eigenvalue =2.247, α=0.80). The third factor is "environmental issue" (eigenvalue =1.951, α=0.82). The cumulative explained common variance of these three factors reaches 79.524%.

After the factor analysis in this study, two factors are extracted from environmental attitude. The first factor is "environmental value" (eigenvalue =3.162, α=0.88). The second factor is "environmental belief" (eigenvalue =2.976, α=0.85). The cumulative

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explained common variance of these two factors reaches 81.195%.

After the factor analysis in this study, three factors are extracted from the behavioral intention. The first factor is “eco-management movement” (eigenvalue =2.533, α=0.86). The second factor is “consumer movement” (eigenvalue =1.884, α=0.89). The cumulative explained common variance of these two factors reaches 82.438%.

The analysis of the correlation between environmental knowledge and environmental attitude

The analysis results are shown in *Table 1*. After verifying Hypothesis H1, the analysis results reveal that, ecology (t=2.134 **), environmental science (t=1.734 *), and environmental issue (t=2.238 **) have a significant influence on environmental value. On the other hand, ecology (t=1.942 *), environmental science (t=1.856 *), and environmental issue (t=1.914 *) have a significant influence on environmental belief. Hypothesis H1 is therefore valid.

Table 1. *Analysis of the constituent elements of environmental knowledge versus environmental attitude*

Dependent variable →	**Environmental attitude**			
Independent variable ↓	Environmental value		Environmental belief	
Environmental knowledge	Beta	t	Beta	t
Ecology	0.202	2.134 **	0.188	1.942 *
Environmental science	0.168	1.734 *	0.175	1.856 *
Environmental issue	0.213	2.238 **	0.182	1.914 *
F Value	24.528		37.462	
Significance	0.000 ***		0.000 ***	
R2	0.256		0.334	
R2 after adjustment	0.022		0.030	

Remark: * p Value <0.05, ** p Value <0.01, *** p Value <0.001.
Data Sources: Summarized by this study.

The study of the correlation between environmental knowledge and behavioral intention and environmental attitude

(1) The analysis of the correlation between environmental knowledge and behavioral intention

The analysis results are shown in *Table 2*. After verifying Hypothesis H3, the analysis results reveal that, ecology (t=2.216 **), environmental science (t=1.857 *), and environmental issue (t=1.946 *) have a significant influence on the eco-management movement. On the other hand, ecology (t=2.361 **), environmental science (t=1.762 *), and environmental issue (t=2.188 **) have a significant influence on the consumer movement. Consequently, Hypothesis H3 is valid.

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(2) The analysis of the correlation between environmental attitude and behavioral intention

The analysis results are shown in *Table 2*. After verifying Hypothesis H2, the analysis results reveal that, environmental value (t=2.325 **) and environmental belief (t=2.264 **) have a significant influence on the eco-management movement. On the other hand, environmental value (t=2.463 **) and environmental belief (t=2.587 **) have a significant influence on consumer movement. Accordingly, Hypothesis H2 is valid.

Table 2. *Analysis of the constituent elements of environmental knowledge versus environmental attitude*

Dependent variable →	**Behavioral intention**							
Independent variable ↓	Eco-management movement		Consumer movement		Eco-management movement		Consumer movement	
Environmental knowledge	Beta	t	Beta	t				
Ecology	0.211	2.216 **	0.223	2.361 **				
Environmental science	0.171	1.857 *	0.163	1.762 *				
Environmental issue	0.182	1.946 *	0.206	2.188 **				
Environmental attitude								
Environmental value					0.221	2.325 **	0.237	2.463 **
Environmental belief					0.214	2.264 **	0.241	2.587 **
F Value	16.438		19.524		22.381		25.163	
Significance	0.000 ***		0.000 ***		0.000 ***		0.000 ***	
R2	0.165		0.173		0.213		0.238	
R2 after adjustment	0.014		0.016		0.019		0.021	

Remark: * p Value <0.05, ** p Value <0.01, *** p Value <0.001.
Data Sources: Summarized by this study.

LISREL model evaluation indicators

The data obtained in this study are summarized in *Table 3* and *Table 4*. The simple goodness-of-fit, internal goodness-of-fit, and global goodness-of-fit of this model are described respectively as follows.

The results of the full model analysis in *Table 3* reveal that, from the simple goodness-of-fit aspect, the three constituent factors (ecology, environmental science, and environmental issue) of environmental knowledge reach the significant level ($t>1.96$, $p<0.05$) in the explanation of environmental knowledge. The two constituent factors (environmental value and environmental belief) of environmental attitude reach

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the significant level ($t>1.96$, $p<0.05$) in the explanation of the environmental attitude. The two constituent factors of behavioral intention reach the significant level ($t>1.96$, $p<0.05$) in the explanation of behavioral intention. It is known from this that the global model of this study is provided with good simple goodness-of-fit.

From the internal goodness-of-fit aspect, there is a positive significant correlation between environmental knowledge and environmental attitude (0.843, $p<0.01$). There is also a positively significant correlation between environmental attitude and behavioral intention (0.866, $p<0.01$). Environmental knowledge and behavioral intention (0.873, $p<0.01$) also provide a positive significant correlation, representing that Hypotheses 1, 2, and 3 are all supported.

Table 3. *Analysis results of the global linear structural model*

Evaluation items	**Parameter / assessment criteria**		**Results**	**t Value**
Simple goodness-of-fit	Environmental knowledge	Ecology	0.716	8.52 **
		Environmental science	0.721	9.66 **
		Environmental issue	0.711	7.84 **
	Environmental attitude	Environmental value	0.753	10.62 **
		Environmental belief	0.762	11.85 **
	Behavioral intention	Eco-management movement	0.811	12.36 **
		Consumer movement	0.823	13.55 **
Internal goodness-of-fit	Environmental knowledge → environmental attitude		0.843	33.46 **
	Environmental attitude→ behavioral intention		0.866	38.72 **
	Environmental knowledge → behavioral intention		0.873	43.15 **
Global goodness-of-fit	X2/Df		1.822	
	GFI		0.973	
	AGFI		0.917	
	RMR		0.004	

Remark: * p Value <0.05, ** p Value <0.01, *** p Value <0.001.

From the model's global goodness-of-fit aspect, the global model's goodness-of-fit value $\chi 2$/Df is 1.822, which is smaller than the criterion of 3. The RMR value is 0.004, which indicates the resulting criteria of $\chi 2$/DF and RMR (Root mean square residual) are adequate. Moreover, since the chi-square value is very sensitive to the sample size, it is not adequate to directly use this for determining the suitable condition. However, the GFI (Goodness of fit index) value of the global model goodness-of-fit standard is 0.973 and the AGFI (Adjusted goodness of fit index) is 0.917 which are larger than the criteria of 0.9. (When the GFI, AGFI values are closer to 1, it indicates the model's goodness-of-fit is better.) As a result, this model is provided with a better goodness-of-fit indicator.

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Table 4. *Verification of hypotheses*

Research hypotheses	Correlation	Empirical results	P Value	Result
Hypothesis 1	+	0.843	P<0.01	Valid
Hypothesis 2	+	0.866	P<0.01	Valid
Hypothesis 3	+	0.873	P<0.01	Valid

Discussions

The results of this study reveal that people with a higher score of environment knowledge present a more optimistic environmental attitude and vice versa. These two factors present a significantly positive correlation. People with more optimistic environmental attitudes present positive environmental behaviors and vice versa. These two factors present a significantly positive correlation. People with more abundant environment knowledge display more positive environmental behaviors and vice versa. These two factors present a significantly positive correlation. It is thus clear that environmental issues are an important topic that people in the 21th century have to face seriously. The relationship between people and the environment is getting worse. Unless people change their behaviors and show a respectful attitude towards the environment, the end of the world and people can be expected. To resolve the environmental problems, it is necessary to trigger people's environmental awareness and therefore this highlights the importance of the education of environmental knowledge. While the government is developing the tourism industry, it is recommended that the relevant environmental protection organizations can plan their strategies for the above-mentioned pollutions. The required manpower and expenditures should be included during budget planning so as to present landscapes and the living environment with high quality to tourists and citizens.

Conclusions

Based on the above research results obtained in this study, some conclusions are proposed as follows for future researches and practicers.

1. Organizing eco-friendly activities: It is recommended to organize eco-friendly activities such as beach cleanup, mountain cleanup, resource recycling, etc. On one hand, these activities enhance people's environmental value. On the other hand, via firsthand experiences, people can be cultured to have more optimistic environmental attitudes and responsible environmental behaviors.
2. Application of selection tools: People need to keep enriching their environmental knowledge. It is recommended to well use the Internet, newspaper and magazine media, advocating DVDs, and posters. The resources provided by the local government's environmental protection bureau can also be used for enriching people's knowledge and capability obtained from the environmental education. This can further affect the acquisition of people's environment knowledge and it is advised to indeed fulfill environmental education into the activities of daily living.

3. Supplying related information and training: It is recommended to fulfill and integrate the environment education knowledge into school courses. Depending on the different grades of schoolchildren, different breadth and depth should be available. During the activities of daily living, it is necessary to continuously emphasize the importance of the environmental attitudes toward the improvement of our environment, such as turning off lights when leaving and using both sides of paper and then have it recycled. It is expected to enhance schoolchildren's environmental attitude. Particularly, people emphasize eco-friendly awareness and sustainable developments nowadays, schoolchildren are required to possess optimistic environmental attitudes so as to present more positive and optimistic behaviors in their life.

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INTERACTION OF *PSEUDOMONAS FLUORESCENCE* BACTERIA AND PHOSPHORUS ON THE QUANTITATIVE AND THE QUALITATIVE YIELD OF RAPESEED (*BRASSICA NAPUS* L.) CULTIVARS

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Abstract. Canola (*Brassica napus* L.) is a very important crop. Hence, the effects of chemical and biological fertilizer on canola oil production and nutrient uptake, under calcareous conditions, were evaluated in a field experiment. The experiment was carried out in factorial in a randomized complete blocks design with three replications in Babolsar, Iran in 2010 and 2011. The bacterial inoculation factor comprised the following five levels: *Pseudomonas fluorescence* I (PSf_1), *Pseudomonas fluorescence* II (PSf_2), *Pseudomonas putida* I (PSp_1), *Pseudomonas putida* II (PSp_2), and control treatment (PS_0) The triple superphosphate fertilizer factor comprised the consumption of triple superphosphate fertilizer based on the soil test (P_1), the consumption of triple superphosphate fertilizer based on 25 % less than the soil test (P_2), the consumption of triple superphosphate fertilizer based on 50 % less than the soil test (P_3), and control treatment (P_0) as well as the cultivars Hayula 401 and Sarigol. Results showed that the use of growth-stimulating bacteria had increased the seed yield, biologic yield, 1,000-seed weight and shoot, and grain phosphorus concentratio of canola cultivars. The highest seed yield belonged to the *Pseudomonas putida* II bacteria, with an average of 3,587 kg ha^{-1} and 17.4 % edge in comparison to the control group. The highest seed yield, with an average of 3,711 kg ha^{-1}, which showed a 30 % increase in comparison to the control treatment, was obtained in the treatment of 25 % less phosphorus than the soil test. In the most investigated qualities, Hayula 401 excelled Sarigol, and the consumption of biologic fertilizers, as the complements of chemical fertilizers, increased the yield and yield components, and improved the phosphorus content of the leaf and the grain.
Keywords: *biofertilizer, inoculation, canola, nutrient uptake, oil, phosphorus-solubilizing bacteria, sustainable agriculture*

Introduction

Rapeseed (*Brassica napus* L.) is one of the most important oilseeds in the world and the most important crop species of the *Brassica* type (Rosas et al., 2006). After soybean and oil palm, canola is the third highest source of oil production (Salimpour et al.,

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2012). Despite this product's importance in the world as rich source of oil and protein, its yield in the Mazandaran Province is affected by special situations of water logging and phosphorus fixation in calcareous soil. As a result, nitrogen and phosphorus are two elements that limit canola yield in Mazandaran Province, Iran. Phosphorus is the second important key element after nitrogen as a mineral nutrient in terms of quantitative plant requirement. Although abundant in soils, in both organic and inorganic forms, the availability of phosphorus is restricted as it occurs mostly in insoluble forms (Sharma et al., 2013). Local farmers normally use 100 to 150 kg of triple superphosphate fertilizer or ammonium phosphate; only 20–25 % of phosphorus is consumed by the plant due to phosphorus fixation in the soil (Sharma et al., 2013), which, using much chemical input, contributes to high energy consumption and greenhouse gas emissions (Pazouki et al., 2017). According to the reports presented in a short period of time, two-thirds of the used phosphate fertilizers get inaccessibly fixated in the soil (Afzal et al., 2005). Some of the most important biological fertilizers include phosphate-solvent bacteria. The release of phosphorus from the insoluble and the fixated forms by phosphate-solvent bacteria is relevant to the amount of accessible phosphorus in the soil and its transfer to the plant (Khan and Joergesen, 2009). Microorganisms increase the accessibility of plants to phosphorus by mineralizing organic phosphorus in soil through solving deposited phosphate (Chen et al., 2006; Kang et al., 2002; Pradhan and Sukla, 2005). According to reports, phosphate-solvent bacteria can constitute up to 88 % of all phosphate-solvent microorganisms (Falah, 2006). The use of phosphate-solvent microorganisms can increase crop yield up to 70 % (Verma, 1993). According to studies, phosphate-solvent bacteria, along with other plant-growth-promoting rhizobacteria, decreased the use of phosphate fertilizers up to 50 % without any significant decrease in crop production (Jilani et al., 2007; Yazdani et al., 2009). This is a good perspective toward the sustainable production of crops. The fixation or the mineralization of soil-solvable and inorganic phosphate chemical fertilizers constitutes an important factor for the fertilizer's inaccessibility to the plant (Dey, 1988). The *Pseudomonas* type of bacteria is especially important because of its expanded distribution in soil, and its ability to colonize the rhizosphere of many plants and to produce a great range of metabolites. One of the most important growth-promoting characteristics of plants is their ability to solve phosphate, which has been reported by researchers (Rashid et al., 2004; Ramezanpour, 2009).

Other study results showed that seed and straw yields, and the nitrogen uptake of canola were significantly increased by bacterization. Besides nitrogen, the uptake of other nutrients (Fe and Mn) was also increased by these microorganisms. In general, pyrite amendment along with bacterization improved yields and nutrient uptake in canola and wheat over other treatments without pyrite addition (Joseph et al., 2014). Both bacterial and fungal strains exhibiting P solubilizing activity are detected by the formation of a clear halo (a sign of solubilization) around their colonies. Production of a halo on a solid agar medium should not be considered the sole test for P solubilization. When colonies grow without a halo after several replacements of the medium, an additional test in liquid media to assay P dissolution should be performed and the few isolates that are obtained after such rigorous selection should be further tested for the abundant production of organic acids; isolates complying with these criteria should be tested on a model plant as the ultimate test for potential P solubilization (Bashan et al., 2013). Madani et al. (2011) revealed that statistical analyzes showed that PSB3 treatment was the best treatment for seed yield increase. The highest rate of seed yield

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(9.9 t/ha) was recorded in dual fertilizing applied both in sowing time and after the overwintering stage of the rosette. The interaction effects of phosphorus-solubilizing bacteria and ammonium phosphate fertilizer application did not have a significant effect on plant height, biomass yield, the number of silique per plant, seed oil percentage, and seed yield. The interaction effects of phosphorus-solubilizing bacteria and the application of ammonium phosphate fertilizer were significant for phosphorus content in plant tissues.

The effect of the mentioned bacteria in increasing the function of plants, such as rice and canola, has been reported (Ramezanpour, 2010; Yasari and Patwardhan, 2007). Different results of studies have shown that the use of strains of *P. fluorescence* and *P. putida* have lengthened the roots of the shoot in canola and tomato, and have also increased the function in wheat, rice, and sugar beet (Egamberdiyevaa et al., 2003; Dobbelaere et al., 2002; Rodriguez and Fraga, 1999). Yasari et al. (2008) reported that the inoculation of *Azotobacter* and *Spirillum* had increased the amount of canola oilseed. The purpose of this research was to investigate the effects of using different species of *P. fluorescent* as the solvent bacteria of the soil's insoluble phosphate on the function and phosphorus content of the leaves and the seeds of the canola cultivars Hayula and Sarigol. The results of previous studies were performed mostly under greenhouse conditions or the bacteria's individual effects on crop yield (Salimpour et al., 2012). This research investigated the effects of the inoculation of different types with different amounts of solving power and different amounts of triple superphosphate fertilizer on canola in the farm.

Materials and methods

This farm experiment was performed in factorial in the form of randomized complete blocks in Babolsar between 2010 and 2011. The used *Pseudomonas* bacteria were supplied and chosen from the superior *Phosphorus solvent strains* in the previous studies of the fourth writer (Ramezanpour, 2009; 2011).

The main bacterial factor consists of five bacterial levels: *Pseudomonas fluorescence* I (PSf_1), *Pseudomonas fluorescence* II (PSf_2), *Pseudomonas putida* I (PSp_1), *Pseudomonas putida* II (PSp_2), and control treatment without bacteria (PS_0). The fertilizer factor consists of four levels of triple superphosphate fertilizer; these include consuming fertilizer based in soil test (P_1), 25 % less than the soil test (P_2), 50 % less than the soil test (P_3), and the level of control treatment (P_4), and the cultivars Hayula 401 and Sarigol. It was performed in three replications on a farm in Babolsar, Mazandaran Province, Iran. The triple superphosphate fertilizer was consumed in four levels of 200, 150, 100, and 0 kg ha^{-1}. According to the physical and the chemical analyzes of the soil laboratory, the clay loam soil has a pH of 7.4, total nitrogen of 0.12 %, organic material of 1.46 %, accessible phosphorus of 6.5 mg kg^{-1}, and accessible potassium of 190 mg kg^{-1}. The urea fertilizers were added in a quantity of 200 kg ha^{-1} and the potassium sulfate was added in a quantity of 150 kg ha^{-1} to the soil in the following three steps: before planting, at the end of tillering, and during the flowering stages. The area of each plot was 15 m^2, including nine lines of planting, with a distance of 30 cm and a length of 5 m. The distance between the plants was determined to be 3–5 cm. The seeds were inoculated for 24 h in a temperature range of 24 ± 2 °C with inoculation liquids; they were placed to be dried in fresh air for two hours. In order to supply the inoculation liquid, bacteria were cultivated for 48 h in

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nutrient Agarin at 24 °C. The population of the isolate was almost 108 mm of suspension. To inoculate the seeds, 7 mm of inoculation liquid was used for every 100 seeds. The seeds were poured in sterilized containers and immersed in inoculation liquid for 24 h. The seeds used in control treatment, like in bacterial treatment, bloomed in sterilized water and were then cultivated in experimental plots specific to each treatment. The data collected from this experiment includes phosphorus concentration in the shoot and the seed, seed yield, biological yield, 1,000-seed weight, the number of pods per plant, the number of seeds per pod, and the length of the pod during harvest. The measurement of the leaf and the seed phosphorus concentration was performed by the Olson method. The results of this experiment were statistically analyzed by the MSTATC software and the data mean was compared with Duncan's multiple range tests.

Results and discussion

Quantitative trait

The results in *Table 1* showed that in bacteria and phosphorous treatment, plant height, the height of the first pod, the number of pods per plant, the number of grains per pod, 1,000-seed weight, seed yield, biological yield, and harvest index were significant at the 5 % probability level. In addition, the cultivar effect and the interaction of year with cultivar were significant at the 5 % probability level for all the traits that have been described (*Table 1*). Interaction of bacteria × cultivar, year × phosphorous, bacteria × phosphorous, cultivar × phosphorous, and triple interaction had a significant effect on all the traits except pod length at the 5 % probability level; the number of grains per pod had no significant effect on the interaction of cultivar × phosphorous (*Table 1*).

Table 1. *Mean square of quantities and qualities yield of rapeseed cultivars pseudomonas fluorescence bacteria and amounts of phosphorus*

S.O.V.	DF	Plant height	Height of first pod	Pod length	Number of pod per plant	Number of grain per pod	1000-seed weight	Seed yield	Biological yield	Harvest index
Year (Y)	1	758.78	419.50	24.31	5927.22	2674.00	3.23	225190.10	27434.82	392.91
Error	4	117.97	3359.17	0.38	13343.51	36.19	0.24	1776481.03	11587492.86	94.04
Bacteria (B)	4	84.45*	4755.11*	0.07	4317.55*	43.74*	0.45*	2129631.30*	19573442.22*	11.82*
Y×B	4	49.91*	2922.23*	0.12	2983.92*	53.14*	0.20*	728064.62*	7210592.70*	102.86*
Cultivar (C)	1	10494.83*	20938.41*	15.87*	23540.22*	685.13*	10.78*	1785892.54*	72994334.02*	645.83*
Y×C	1	6040.67*	11213.50*	8.35*	62072.02*	45.50*	3.78*	9664.70*	365820835.27*	954.49*
B×C	4	150.06*	2646.04*	0.12	7586.11*	25.78*	0.18*	38579.47*	6254873.23*	33.78*
Phosphorous (P)	3	891.86*	3455.81*	0.11	17906.06*	23.78*	1.32*	8818923.84*	86008864.86*	24.00*
Y×P	3	233.14*	3123.34*	0.14	8543.96*	55.65*	0.26*	110139.98*	873928.16*	9.20*
B×P	12	83.33*	2870.01*	0.27	4433.21*	46.37*	0.13*	290189.86*	3268713.8*	7.01*
C×P	3	169.17*	1384.87*	0.04	3907.91*	0.50	0.04*	35721.55*	1202114.63*	3.99*
B×C×P	12	97.80*	2544.31*	0.33	6740.89*	93.62*	0.07*	14076.18*	1049884.93*	1.78*
Error	187	92.15	3003.76	0.18	5419.48	1198.81	0.13	87187.54	4748429.00	19.71
CV. (%)	-	6.24	5.98	7.43	40.19	13.11	10.03	8.84	15.95	17.64

** and *, respectively, significant in 1 % and 5 % level

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The result of the main mean comparison showed that pod length, seed yield, and harvest index in the first year were, respectively, 11.45, 5.87, and 10.74 % more than the second year; the number of grains per pod and the 1,000-seed weight in the second year were, on the other hand, 41.77 and 6.94 %, respectively, more than the first year (*Table 2*).

Table 2. *Mean comparison of quantities and qualities yield of rapeseed cultivars pseudomonas fluorescence bacteria and amounts of phosphorus*

S.O.V.	Plant height (cm)	Height of first pod (cm)	Pod length (cm)	Number of pod per plant	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest index (%)
Year									
First year	155.72 a	90.21 a	6.13 a	188.15 a	15.97 b	3.46 b	3429.12 a	13647.20 a	26.40 a
Second year	152.16 a	92.86 a	5.50 b	178.21 a	22.64 a	3.70 a	3238.72 b	13668.59 a	23.84 b
Bacteria									
PSf_1	152.8 a	87.48 a	5.79 a	179.9 a	19.02 a	3.65 a	3362 b	13850 a	24.53 a
PSf_2	155.7 a	90.22 a	5.86 a	176.4 a	19.77 a	3.65 a	3481 ab	13850 a	25.69 a
PSp_1	154.9 a	109 a	5.85 a	177.8 a	18.78 a	3.48 b	3218 c	13500 ab	25.46 a
PSp_2	152.6 a	84.66 a	5.78 a	182.1 a	19.10 a	3.65 ab	3587 a	14410 a	25.39 a
PS_0	153.7 a	86.34 a	5.79 a	199.7 a	19.86 a	3.46 b	3055 d	12680 b	24.75 a
Cultivar									
Hayula 401	147.28 b	90.22 b	6.13 a	193.08 a	15.98 b	3.79 a	3426.50 a	13105 b	26.81 a
Sarigol	160.30 a	92.86 a	5.50 b	173.28 b	22.65 a	3.37 b	3254.25 b	14210 a	23.52 b
Phosphorous									
P_1	157.4 a	102.50 a	5.77 a	176.5 b	19.73 a	3.72 a	3570 b	14480 a	25.46 ab
P_2	155.3 a	90.04 a	5.82 a	207.2 a	19.50 a	3.67 a	3711 a	14720 a	25.83 a
P_3	154.6 a	88.68 a	5.87 a	182.3 ab	19.02 a	3.52 b	3229 b	13330 b	25.00 a
P_4	148.4 b	84.96 a	5.79 a	166.7 b	18.99 a	3.40 b	2852 d	12110 c	24.36 a

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

In the bacteria level, the greatest 1,000-grain weight was observed in PSf_1 and PSf_2, and the lowest 1,000-grain weight was obtained in PSp_1 and PS_0. The highest seed yield (3,587 kg ha^{-1}) was produced in PSp_2 and the lowest seed yield (3,055 kg ha^{-1}) was achieved in PS_0. The maximum biological yields were equal to 13,850, 13,850, and 14,410 kg ha^{-1}, which were produced for PSf_1, PSf_2, and PSp_2, respectively (*Table 2*). The result of cultivar comparison showed that plant height and the height of the first pod in Sarigol were 8.84, 2.93, and 41.74 % more than Hayula 401, which causes an increase of 8.43 % in the biological yield. However, pod length, number of pods per plant, and the 1,000-seed weight for Hayula 401 were 11.45, 11.42, and 12.46 % more than Sarigol, thus bringing about the highest seed yield (3,426.5 kg ha^{-1}) and the harvest index equal to 26.81 % (*Table 2*). The mean comparison of phosphorous treatment showed that the maximum plant height was observed in P_1, P_2, and P_3, but the largest number of pods per plant (207.2 pods) was observed in P_3, which caused the highest seed yield (3,711 kg ha^{-1}). The greatest 1,000-seed weight and biological yield were obtained in P_1 and P_2, respectively. The highest harvest index was achieved in P_2, P_3, and P_4 (*Table 2*).

Result of year × bacteria interaction showed that the highest plant heights (158.2 and 157.7 cm) were obtained in Y_1PSf_2 and Y_1PSp_2, respectively. The greatest height of the first pod (123.3 cm) was observed in Y_2PSp_1. The maximum number of grains per pod (23.40 pods) was obtained in Y_2PSf_2. The greatest seed yield (3,670 kg ha^{-1}) was produced in Y_2PSp_2 and the highest harvest index was observed in Y_1PSp_1 (*Table 3*). The year × cultivar result revealed that the greatest plant height (167.3 cm) and the

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greatest height of pod (106.4 cm) were obtained in Y_1C_2. The highest pod length (6.2 cm), seed yield (3,517 kg ha^{-1}), and harvest index (30.08 %) were produced in Y_1C_1. The greatest number of grains per pod (24.77 grains) and 1,000-seed weight (4.03 g) were achieved in Y_2C_1 (*Table 4*).

Table 3. *Interaction of year × bacteria on quantities and qualities yield of rapeseed cultivars*

Interaction	Plant height (cm)	Height of first pod (cm)	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Harvest index (%)
Y_1PSf_1	154.8 ab	88.57 b	16.55 c	3.53 c	528 ab	26.02 b
Y_1PSf_2	158.2 a	96.65 ab	16.15 c	3.50 c	557 ab	25.62 bc
Y_1PSp_1	157.7 a	94.60 ab	15.17 c	3.38 c	3462 b	29.11 a
Y_1PSp_2	152.9 ab	83.50 b	15.34 c	3.45 c	500 ab	25.66 bc
Y_1PS_0	154.9 ab	87.75 b	16.66 c	3.44 c	141 cd	25.79 bc
Y_2PSf_1	150.8 b	86.38 b	21.52 b	3.75 a	319 c	23.04 cd
Y_2PSf_2	153.2 ab	83.80 b	23.40 a	3.81 a	405 b	25.75 bc
Y_2PSp_1	152.0 ab	123.30 a	22.40 ab	3.58 bc	297 d	21.80 d
Y_2PSp_2	152.3 ab	85.82 b	22.87 ab	3.84 a	367 a	25.13 bc
Y_2PS_0	152.5 ab	84.94 b	23.05 ab	3.48 c	296 d	23.71 b-d

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

Table 4. *Interaction of year × cultivar on quantities and qualities yield of rapeseed cultivars*

Interaction	Plant height (cm)	Height of first pod (cm)	Pod length (cm)	Number of pod per plant	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest index (%)
Y_1C_1	144.1 c	74.04 b	6.20 a	182 a	17.23 c	3.55 b	3517 a	11900 d	30.08 a
Y_1C_2	167.3 a	106.40 a	6.06 ab	194.3 a	14.72 d	3.38 c	3358 b	15400 a	22.81 b
Y_2C_1	150.6 b	90.35 ab	5.94 b	204.2 a	24.77 a	4.03 a	3336 b	14320 b	23.53 b
Y_2C_2	153.8 b	95.36 a	5.05 c	152.2 b	20.52 b	3.36 c	3151 c	13020 c	24.24 b

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

The interaction of year × phosphorous result showed that the maximum height of pod (114.1 cm) was observed in Y_2P_1. The pod length and the harvest index in the first year for all the phosphorous treatment was higher than the second-year phosphorous treatment results (*Table 5*). The number of grains per pod in the second year for the phosphorous levels was more than the first year. The greatest seed yield (3,770 kg ha^{-1}) was produced in Y_1P_2 and the least seed yield (2,695 kg ha^{-1}) was achieved in Y_2P_4 (*Table 5*). The result of bacteria × cultivar interaction showed that the plant height of Sarigol in the bacteria level was more than Hayula 401 (*Table 6*). The greatest height of pod (112.6 cm) was observed in PSp_1C_1. The maximum pod length was achieved for Hayula 401 in PSf_1 and PSp_1. The highest number of pods per plant (211.1 pods) was obtained for Sarigol in PS_0. The maximum 1,000-seed weight (3.91 g) was obtained in PSp_2 for Hayula 401. The greatest seed yield (3,680 kg ha^{-1}) was produced for Hayula 401 in PSp_2 and the least seed yield (2,943 kg ha^{-1}) was obtained in PS_0 for Sarigol. The

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most and the least harvest index (27.79 and 21.71 %) was obtained for PSp_0 in Hayula 401 and Sarigol cultivars, respectively (*Table 6*).

***Table 5.** Interaction of year × phosphorous on quantities and qualities yield of rapeseed cultivars*

Interaction	Plant height (cm)	Height of first pod (cm)	Pod length (cm)	Number of pod per plant	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest index (%)
Y_1P_1	157.8 a	90.82 ab	6.02 a	183.3 b	17.06 b	3.59 b	3644 ab	14420 ab	26.49 a
Y_1P_2	157.7 a	91.46 ab	6.19 a	228 a	16.17 bc	3.48 bc	3770 a	14740 a	26.70 a
Y_1P_3	154.7 a	88.54 ab	6.13 a	181.8 b	14.99 c	3.14 bc	3328 c	13180 cd	26.51 a
Y_1P_4	152.8 a	90.03 ab	6.08 a	159.6 b	15.67 c	3.36 c	3008 d	12250 de	26.07 a
Y_2P_1	157.1 a	114.10 a	5.42 b	169.8 b	22.39 a	3.86 a	3495 b	14530 ab	24.44 ab
Y_2P_2	152 a	88.63 ab	5.45 b	186.5 b	22.84 a	3.86 a	3652 ab	14700 a	24.95 ab
Y_2P_3	154.5 a	88.83 ab	5.61 b	182.9 b	23.05 a	3.16 b	3131 d	13470 bc	23.49 b
Y_2P_4	144.1 b	79.88 b	5.50 b	173.7 b	22.32 a	3.43 bc	2695 e	11970 e	22.66 b

*Values within a column followed by the same letter are not significantly different at Duncan (P ≤ 0.05)

***Table 6.** Interaction of bacteria × cultivar on quantities and qualities yield of rapeseed cultivars*

Interaction	Plant height (cm)	Height of first pod (cm)	Pod length (cm)	Number of pod per plant	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest index (%)
PSf_1C_1	145.2 b	76.56 ab	6.10 a	196.08 b	20.58 a	3.77 ab	3468 bc	13760 ab	25.63 a-d
PSf_1C_2	160.4 a	98.40 ab	5.48 b	163.90 ab	17.49 b	3.52 cd	3256 d	13940 ab	23.43 de
PSf_2C_1	149.8 b	78.43 ab	6.09 a	197.50 ab	21.47 a	3.93 a	3521 ab	13010 bc	27.35 ab
PSf_2C_2	161.7 a	102 ab	5.62 b	155.3 b	18.08 b	3.37 de	3440 bc	14690 a	24.02 cde
PSp_1C_1	146.3 b	112.60 a	6.04 a	191.8 ab	21.01 a	3.68 bc	3299 cd	12940 bc	26.36 abc
PSp_1C_2	163.5 a	105.30 ab	5.67 b	163.8 ab	17.56 b	3.29 e	3138 d	14070 ab	24.55 bcd
PSp_2C_1	145.7 b	71.85 b	6.07 a	191.8 ab	21.34 a	3.91 a	3680 a	14100 ab	26.88 ab
PSp_2C_2	159.5 a	97.47 ab	5.49 b	172.3 ab	16.87 b	3.38 de	3494 b	14720 a	23.91 cde
PS_0C_1	149.7 b	71.48 b	6.05 a	188.3 ab	21.61 a	3.66 bc	3166 d	11720 c	27.79 a
PS_0C_2	157.7 a	101.20 ab	5.53 b	211.1 a	18.11 b	3.27 e	2943 e	13630 ab	21.71 e

*Values within a column followed by the same letter are not significantly different at Duncan (P ≤ 0.05)

The result of the bacteria × phosphorous interaction revealed that the highest plant heights of 159.3 and 160.8 cm were observed in PSp_1 for P_1 and P_3, respectively. The maximum number of pods per plant (269.4 pods) was achieved in PS_0P_1. The greatest 1,000-seed weight (3.89 g) was observed in PSf_2P_2. The greatest seed yield (4,202 kg ha^{-1}) and biological yield (16,190 kg ha^{-1}) were produced in PSp_2P_2. The least seed yield (2,648 kg ha^{-1}) was observed in PS_0P_4 (*Table 7*). The result of cultivar–phosphorous interaction demonstrated the maximum plant heights of 162.9 cm and 162.2 cm, which were observed for the Sarigol cultivar in P_1 and P_2, respectively. The greatest pod length and number of grains per pod were obtained in all the phosphorous treatment level for

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Hayula 401. The greatest seed yield (3,802 kg ha^{-1}) was produced in P_2 for Sarigol cultivar, but the least seed yield (2,801 kg ha^{-1}) was achieved for Sarigol in P_4 (*Table 8*).

Table 7. *Interaction of bacteria × phosphorous on quantities and qualities yield of rapeseed cultivars*

Interaction	Plant height (cm)	Number of pod per plant	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})
PSf_1P_1	155.6 abc	174.7 b	20.50 ab	3.86 ab	3495 d-g	14630 a-d
PSf_1P_2	155.7 abc	185.9 b	18.75 ab	3.74 abc	3807 bc	15170 abc
PSf_1P_3	153.7 a-d	182.9 b	18.85 ab	3.52 b-f	3239 ghi	13570 b-f
PSf_1P_4	146.3 d	176.3 b	18.03 a	3.44 c-f	2908 jk	12020 fg
PSf_2P_1	158.4 d	162.6 b	19.67 ab	3.64 a-e	3640 cde	14150 a-e
PSf_2P_2	156.3 abc	182.9 b	20.58 a	3.89 a	3995 ab	14900 abc
PSf_2P_3	154.5 a-d	192.6 b	19.33 ab	3.57 a-e	3368 f-i	13690 b-f
PSf_2P_4	153.6 a-d	167.4 b	19.50 ab	3.51 c-f	2920 jk	12670 d-g
PSp_1P_1	159.3 a	178 b	18.78 ab	3.69 a-d	3598 c-f	15210 ab
PSp_1P_2	153.4 a-d	194.7 b	18.67 ab	3.54 a-f	3333 f-i	14240 a-e
PSp_1P_3	160.8 a	179.4 b	18.78 ab	3.38 def	3120 ij	12750 d-g
PSp_1P_4	146 d	159.1 b	18.90 ab	3.32 ef	2823 kl	11810 fg
PSp_2P_1	159.9 ab	190.5 b	18.96 ab	3.67 a-d	3726 cd	14590 a-d
PSp_2P_2	153.8 a-d	203.2 b	19.73 ab	3.69 a-d	4202 a	16190 a
PSp_2P_3	152.7 a-d	172.5 b	18.73 ab	3.73 abc	3457 e-h	14240 a-e
PSp_2P_4	147.1 cd	162.1 b	18.73 ab	3.50 c-f	2962 jk	12620 d-g
PS_0P_1	157 ab	269.4 a	19.00 ab	3.75 abc	3389 e-i	13800 b-f
PS_0P_2	157.2 ab	184.2 b	20.73 a	3.49 c-f	3221 hi	13100 c-f
PS_0P_3	151.3 a-d	168.4 b	19.79 ab	3.40 c-f	2962 jk	12390 efg
PS_0P_4	149.2 bcd	59.29 c	19.53 ab	3.22 f	2648 l	11410 g

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

Table 8. *Interaction of cultivar × phosphorous on quantities and qualities yield of rapeseed cultivars*

Interaction	Plant height (cm)	Pod length (cm)	Number of pod per plant	Number of grain per pod	1000-seed weight (g)	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest index (%)
C_1P_1	151.9 cd	5.99 a	187 ab	21.39 a	3.95 a	3678 ab	14090 ab	26.87 a
C_1P_2	147.4 d	6.08 a	206.3 a	21.16 a	3.88 a	3802 a	14210 ab	27.26 a
C_1P_3	149.7 d	6.14 a	193.7 a	20.79 a	3.76 a	3323 c	12740 d	26.91 a
C_1P_4	140.1 e	6.08 a	185.3 ab	20.66 a	3.58 b	2903 e	11380 e	26.18 ab
C_2P_1	162.9 a	5.55 b	166.1 ab	18.06 b	3.50 b	3461 c	14860 ab	24.06 bc
C_2P_2	162.2 a	5.56 b	208.1 a	17.85 b	3.47 b	3620 b	15230 a	24.39 bc
C_2P_3	159.5 ab	5.61 b	170.9 c	17.35 b	3.28 c	3135 d	13920 bc	23.09 c
C_2P_4	156.6 bc	5.51 b	148 c	17.32 b	3.22 c	2801 e	12830 cd	22.55 c

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

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Investigating the plant height, we observed that phosphorus fertilizer influenced this trait, and the lowest plant height belonged to phosphorous control treatment and all the levels of phosphorus fertilizer consumption stood in the same group without a significant difference. Therefore, it can be understood that with an increase in phosphate consumption to 50 % of the soil test, the plant height increased, and despite canola's greater height in phosphorus fertilizer consumption that was equal to the soil test, it did not show a significant difference in comparison to the treatment of phosphorus consumption up to 50 % of the soil test. It seems that the extra consumed phosphorus did not influence the canola's height. These results are consistent with the results of Malakuti and Sepehr (2002), who state that the excessive use of phosphorus will not eventually lead to the increase of crop growth, but it will gradually fixate in soil, and in the situation of water shortage and dryness, it will cause drought stress. The results of this investigation are inconsistent with the results of Asghar et al. (2004), who stated that the inoculation of the seeds of canola cultivars (*Brassica Juncea*) with different rhizosphere bacteria brought about a greater height of the plant (up to 5.56 %) in comparison to the non-inoculated seeds; this inconsistency can be a result of the differences in the climate and the soil of the experimental region or the different cultivated cultivars. In addition, it can be determined that canola cultivars are different in the height of the first pod from the ground surface and this factor is mostly controlled by the plant's genetic factor. Investigating the characteristic related to the height of the first capsule from the ground surface reveals that the type of the cultivars influenced the characteristic of the height of the first pod from the ground surface, and Sarigol, with 100.88 cm, had the biggest height and Hayula 401, with 82.19 cm, had the lowest height of the first capsule from the ground surface. Results showed that different cultivars differ in the height of the first capsule from ground, and the reason behind this difference can be that the cultivar Sarigol grows higher than the cultivar Hayula 401; therefore, its first capsule is also brings about a taller height.

In terms of pod length result, it can be understood that pod length is a characteristic mostly controlled by genetic factors and less affected by environmental factors. Karper and Andri (1991) showed that the number of blooming branches increases due to the application of phosphorus; moreover, phosphorus application in the flowering period, and increases the total dry weight and the number and the dry weight of the capsule. According to the mean comparison, the consumption of phosphorus fertilizer was 25 % lower than the soil test, with an average of 207.2 pods had the most pod obtained in phosphorous control treatment with 166.7 pods, which had the fewest pods per plant. This way, these demonstrated a significant difference with the treatment of no phosphorus fertilizer consumption, and with its genetic advantage, the cultivar Hayula 401 produced more capsules than the cultivar Sarigol. Bacteria levels could not have a significant effect because of the absorption of the needed phosphorus by the cultivars of canola.

In fact, the pod length of Hayula 401 was 20 % more than that of Sarigol, which can be due to the genetic differences present between these two types of canola; the mentioned characteristic was less influenced by non-genetic factors. Pod length in canola is mostly controlled by genetic structure and increase of capsule length, which leads to the increased number of seeds in capsule. This was consistent with our findings and the findings of Kim et al. (1996), who stated that the difference in pod length could be due to climatic situations as well. The number of seeds per pods seems to be extremely dependent on pod length; therefore, the longer the pods, the more seeds a pod

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can contain. Hayula 401 exceeded Sarigol both in the characteristic of capsule length and the characteristic of seed number. However, other researchers stated that their findings were consistent with the results of Asghar et al. (2004), who had said the inoculation of the seeds of some canola cultivars (*Brassica Juncea*) with different rhizosphere bacteria increases the weight of a thousand seeds (up to 33.9 %) compared to the non-inoculated seeds. Investigating the effects of the application method of the phosphorus-solvent bacteria and its relationship with the consumption of the chemical fertilizer ammonium phosphate in canola's autumn cultivation, Madani et al. (2010) stated that they observed significant changes in the weight of a thousand seeds due to the use of different amounts of ammonium phosphate.

Phosphorus consumption equal with the soil test and 25 % less than the soil test are categorized into Group "a" and the treatments of phosphorus fertilizer consumption 50 % less than the soil test and the control group are categorized into Group "b". Putenam et al. (1992) stated that the 1,000-seed weight is a constituent of the seed yield, which has an important role in stating the power and the potential of production, and is influenced by genetic and environmental factors. 1,000-seed weight is an index of a plant's power for the proportionality of reservoir request in the periods of seed filling, which can be increased by eliminating different stresses such as irrigation limitation. The results obtained from this experiment show that capsule length and the 1,000-seed weight in the cultivar Hayula were greater than the corresponding features in the cultivar Sarigol.

Phosphorus consumption 25 % less than the soil test showed the greatest yields, and phosphorous control treatment revealed the least yield in the unit of area. The application of the bacteria *Pseudomonas* IV (*Putida* 168) and the advantage of 17 % compared to the control treatment and categorization in Group "a" had the most seed yield per hectare; it stood in the same group as the bacteria *Pseudomonas* II (*Fluorescence* 93); the least seed yield was observed in the control treatment. The most seed yield belonged to the bacteria *Pseudomonas* IV (*Putida* 168) in the second year and the least seed yield belonged to the treatment without *Pseudomonas* bacteria consumption. Overall, the effect of bacteria on seed yield was more apparent in the first year than in the second year. The most seed yield belonged to *Pseudomonas* IV (*Putida* 168) and phosphorus fertilizer 25 % less than the soil test, and the least yield was obtained in the treatment of no bacteria and phosphorus fertilizer consumption. Furthermore, the results show that the bacteria *Pseudomonas* IV4 and II (*Putida* 168 and 93) cause more increase in yield than *Pseudomonas* I and IV (*Fluorescence* 41 and 99). Seed yield in the treatment of phosphorus fertilizer consumption 25 % less than the soil test was 30 % more than the treatment of no phosphorus fertilizer consumption, thus indicating an increased yield under the influence of phosphorus. With an increased amount of phosphorus fertilizer against the soil test, not only did not the seed yield per plant increase but we also observed decreased seed yield in canola. The excessive increase of an element in soil seems to disturb the absorption of other elements and decrease the crop yield. As canola's need for fertilizer compared to phosphorus was met with the treatment of phosphorus fertilizer 25 % less than the soil test in the experiment, thereby increasing the fertilizer level decreased the efficiency of canola root in absorbing other elements, and therefore, the yield declined. Hamilton et al. (1993) stated that an increase in the amount of phosphorus in soil would decrease the yield; this was due to the high proportion of phosphorus to zinc and phosphorus to iron, and the accumulation of molybdenum and cadmium in the plant tissues. Moreover, they stated

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that canola reacts to application of phosphorus fertilizer when the soil phosphorus is less than 100 kg ha^{-1}, which is consistent with the results of the present study. Furthermore, in other studies, it has been reported that hormone-producing and phosphate-solvent microorganisms increase phosphorus absorption and crop yield in soils deficient in phosphorus. Ramezanpour (2009), in his experiments on rice, corn, and other grains reported increased yield owing to the application of phosphate-solvent bacteria. Biological yield became significant at the statistical level of one percent at the levels of phosphorus fertilizer, bacteria, and cultivar. Lack of phosphorus fertilizer consumption produced a smaller biological yield than the treatments of fertilizer consumption; therefore, the consumption of phosphorus fertilizer 25 % less than the soil test had the most biological yield and phosphorous control treatment had the least biological yield. As the application of phosphorus fertilizer influences the straw yield, it also influences the biological yield.

Qualitative traits

Qualitative traits, which include oil content, oil yield, shoot phosphorous concentration and absorption, and grain phosphorous concentration and absorption, showed significant differences in 5 % in bacteria, phosphorous, and cultivar treatment (*Table 9*). Except oil yield and grain phosphorous absorption, all the traits were significant at all interaction treatments. Oil content was significant at all double-interaction of treatment (*Table 9*).

Table 9. *Mean square of quantities and qualities yield of rapeseed cultivars pseudomonas fluorescence bacteria and amounts of phosphorus*

S.O.V.	DF	Oil content	Oil yield	Shoot P concentration	Shoot P absorption	Grain P concentration	Grain P absorption
Year (Y)	1	7.99	477365.35	0.0001	10.45	0.001	63.43
Error	4	4.67	283545.11	0.001	24.87	0.0001	41.35
Bacteria (B)	4	4.71*	348784.71*	0.31*	4175.44*	0.095*	277.04*
Y×B	4	5.82*	119407.73*	0.001*	55.01*	0.001*	14.66
Cultivar (C)	1	37.13*	526817.91*	0.006*	282.17*	0.007*	82.20*
Y×C	1	1.91*	6780.56*	0.0001*	2944.41*	0.0001*	0.78
B×C	4	1.23*	11255.20*	0.0001*	45.98*	0.001*	1.35
Phosphorous (P)	3	29.56*	1685639.59*	0.03*	1210.40*	0.076*	534.94*
Y×P	3	0.27	12503.14*	0.0001*	11.07*	0.001*	4.45
B×P	12	1.04*	41591.37*	0.001*	58.79*	0.004*	20.88*
C×P	3	4.77*	24287.02*	0.0001*	14.31*	0.0001*	2.69
B×C×P	12	1.17*	1978.27	0.0010*	10.49*	0.001*	0.67
Error	187	1.52	15487.44	0.0001	35.85	0.001	3.93
CV. (%)	-	3.18	9.58	4.22	21.31	6.75	11.79

** and *, respectively, significant in 1 % and 5 % level

Based on the main mean comparison of qualitative traits, the results indicated that in first year, all the qualitative traits were greater than second year (*Table 10*). In the year × bacteria interaction, the greatest oil content (36.76 %) was obtained in first year for

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PSf_1. Oil yield in this interaction was varied because of the different seed yield variations. The greatest shoot phosphorous content and absorption was observed in both years for PSp_2. Grain phosphorous content and absorption was highest in the first year for PSp_2 (*Table 11*).

Table 10. *Mean comparison of quantities and qualities yield of rapeseed cultivars pseudomonas fluorescence bacteria and amounts of phosphorus*

S.O.V.	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
Year						
First year	39.00 a	1343.33 a	0.50 a	17.32 a	0.27 a	26.40 a
Second year	38.64 a	1254.13 b	0.50 a	16.29 b	0.27 a	23.84 b
Bacteria						
PSf_1	39.17 a	1320 b	0.2440 c	26.08 c	0.4969 b	16.79 bc
PSf_2	38.91 a	1354 ab	0.3181 b	33.01 b	0.4988 b	17.46 b
PSp_1	38.97 a	1257 c	0.1946 e	20.17 a	0.5063 b	16.35 c
PSp_2	38.68 ab	1389 a	0.3835 a	41.67 a	0.5600 a	20.07 a
PS_0	38.35 a	1173 d	0.2035 c	19.57 d	0.4344 c	13.37 d
Cultivar						
Hayula 401	39.21 a	1343.50 a	0.2736 a	27.90 a	0.5046 a	17.32 a
Sarigol	38.43 a	1254 b	0.2639 b	28.31 a	0.5022 a	16.30 a
Phosphorous						
P_1	39.75 a	1420 a	0.2837 b	30.84 a	0.5070 b	18.11 b
P_2	38.95 b	1447 a	0.2897 a	32.26 a	0.5370 a	19.92 a
P_3	38.40 c	1238 b	0.2635 c	27.10 b	0.5018 b	16.28 c
P_4	38.17 c	1089 c	0.2382 d	22.20 c	0.4512 c	12.92 d

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

Table 11. *Interaction of year × bacteria on quantities and qualities yield of rapeseed cultivars*

Interaction	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
Y_1PSf_1	39.76 a	1406 a	0.2521 c	26.30 c	0.4958 c	17.60 b
Y_1PSf_2	38.62 bcd	1374 a	0.3175 b	34.37 b	0.4975 c	17.78 b
Y_1PSp_1	39.25 ab	1361 a	0.1988 f	19.37 d	0.5067 c	17.60 b
Y_1PSp_2	39.07 a-d	1371 a	0.3812 a	40.20 a	0.5717 a	19.14 a
Y_1PS_0	38.30 d	1204 bc	0.1996 f	19.22 d	0.4363 d	13.79 d
Y_2PSf_1	38.59 bcd	1233 b	0.2358 d	25.87 c	0.4979 c	15.99 c
Y_2PSf_2	39.19 abc	1334 a	0.3187 b	31.65 b	0.5000 c	17.13 b
Y_2PSp_1	38.70 bcd	1154 c	0.1904 g	20.98 d	0.5058 c	15.10 c
Y_2PSp_2	38.30 d	1407 a	0.3858 a	43.14 a	0.5483 b	20.30 a
Y_2PS_0	38.40 cd	1142 c	0.2075 e	19.91 d	0.4325 d	12.95 d

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

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Based on year × cultivar interaction, the most oil content and oil yield (39.31 % and 1,385 kg ha^{-1}) and grain phosphorous content and absorption (17.85 % and 17.85 kg ha^{-1}) was observed in the first year for Hayula 401 cultivar. The highest shoot phosphorous content and absorption were produced in both years for both cultivars (*Table 12*).

Table 12. *Interaction of year × cultivar on quantities and qualities yield of rapeseed cultivars*

Interaction	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
Y_1C_1	39.31 a	1385 a	23.31 c	23.31 c	17.85 a	17.85 a
Y_1C_2	38.70 b	1302 b	32.48 a	32.48 a	16.79 b	16.79 b
Y_2C_1	39.12 ab	1306 b	30.73 a	30.73 a	16.94 b	16.94 b
Y_2C_2	38.15 c	1202 c	25.89 b	25.89 b	15.65 c	15.65 c

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

The result of year × phosphorous interaction showed that the most oil content (39.99 %) was achieved in first year for P_1. The least oil content 37.98 % was obtained in second year for P_4. The highest oil yields, 1,459 and 1,479 kg ha^{-1}, were produced in the first year in P_1 and P_2. The maximum shoot phosphorous content and absorption were obtained in both years for P_2. The greatest grain phosphorous content (0.54 %) was achieved in both years for P_2. The highest grain phosphorous absorption (19.72 kg ha^{-1}) was observed in the second year for P_2 (*Table 13*).

Table 13. *Interaction of year × phosphorous on quantities and qualities yield of rapeseed cultivars*

Interaction	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
Y_1P_1	39.99 a	1459 a	0.2857 ab	30.65 ab	0.5073 b	18.52 b
Y_1P_2	39.17 bc	1479 a	0.2897 a	32.13 a	0.5360 a	12.01 f
Y_1P_3	38.46 de	1281 c	0.2647 c	26.33 c	0.5063 b	16.88 c
Y_1P_4	38.36 de	1154 d	0.2397 d	22.47 d	0.4567 c	13.76 e
Y_2P_1	39.51 ab	1382 b	0.2817 b	31.03 ab	0.5067 b	17.70 bc
Y_2P_2	38.73 cd	1415 ab	0.2897 a	32.39 a	0.5380 a	19.72 a
Y_2P_3	38.32 de	1196 d	0.2623 c	27.88 bc	0.4973 b	15.68 d
Y_2P_4	37.98 e	1024 e	0.2370 d	21.94 d	0.4457 c	12.08 f

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

Bacteria × cultivar interaction results showed that the maximum oil content (39.63 %) was obtained for Hayula 401 in PSf_1 (*Table 14*). The most oil yield (1,446 kg ha^{-1}) was achieved for Hayula 401 in PSp_2. The highest shoot phosphorous concentration (0.39 %) was obtained for Hayula 401 in PSp_2. The greatest grain phosphorous content (0.51 and 0.57 %) was obtained for Hayula 401 in PSp_1 and PSp_2 (*Table 14*).

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Table 14. *Interaction of bacteria × cultivar on quantities and qualities yield of rapeseed cultivars*

Interaction	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
PSf_1C_1	39.63 a	1379 ab	0.2488 e	26.33 d	0.5025 c	17.55 c
PSf_1C_2	37.82 bc	1261 c	0.2392 f	25.84 c	0.4913 c	16.04 de
PSf_2C_1	39.26 ab	1380 ab	0.3229 c	30.78 b	0.5037 c	17.81 c
PSf_2C_2	38.55 bcd	1329 bc	0.3133 d	35.24 e	0.4938 c	17.10 cd
PSp_1C_1	39.12 ab	1293 cd	0.1975 h	19.16 e	0.5092 a	16.87 cde
PSp_1C_2	38.83 bc	1222 d	0.1917 i	21.19 a	0.5033 b	15.83 e
PSp_2C_1	39.27 ab	1446 a	0.3912 a	41.06 a	0.5708 a	20.81 a
PSp_2C_2	38.10 cd	1332 bc	0.3758 b	42.28 a	0.5492 b	19.33 b
PS_0C_1	38.78 bc	1230 d	0.2075 g	17.76 e	0.4367 d	13.92 f
PS_0C_2	37.72 d	1117 e	0.1996 h	21.37 e	0.4321 d	12.82 f

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

Based on bacteria × phosphorous interaction, the result revealed that the maximum oil content (40.38 %) was obtained in PSf_1P_1 (*Table 15*).

Table 15. *Interaction of bacteria × phosphorous on quantities and qualities yield of rapeseed cultivars*

Interaction	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
PSf_1P_1	40.38 a	1414 cd	0.2533 i	28.13 efg	0.4983 ghi	17.41 ef
PSf_1P_2	39.63 abc	1508 bc	0.2733 h	31.05 def	0.5350 cde	20.33 bcd
PSf_1P_3	38.57 cd	1249 efg	0.2458 i	26.45 fgh	0.5050 fgh	16.37 fgh
PSf_1P_4	38.13 d	1108 hij	0.2033 k	8.52 ijk	0.4492 kl	13.06 ij
PSf_2P_1	39.69 abc	1445 c	0.3317 f	34.78 cd	0.4858 hij	17.65 ef
PSf_2P_2	38.93 cd	1560 ab	0.3493 e	37.99 bc	0.5417 cd	21.64 b
PSf_2P_3	38.82 cd	1297 ef	0.3117 g	32.15 de	0.5075 e-h	17.12 efg
PSf_2P_4	38.18 d	1114 hij	0.2800 h	27.12 e-h	0.4600 jk	13.42 ij
PSp_1P_1	40.08 ab	1445 c	0.2211 j	25.79 fgh	0.5217 d-g	18.79 de
PSp_1P_2	39.15 bcd	1306 def	0.2050 k	22.35 hi	0.5325 c-f	17.74 ef
PSp_1P_3	38.55 cd	1202 fgh	0.1883 l	17.86 ijk	0.4983 ghi	15.51 gh
PSp_1P_4	38.12 d	1076 ij	0.1633 m	14.70 k	0.4725 ijk	13.35 ij
PSp_2P_1	39.67 abc	1478 bc	0.3900 b	42.32 b	0.5567 bc	20.72 bc
PSp_2P_2	38.60 cd	1620 a	0.4142 a	49.55 a	0.6208 a	25.23 a
PSp_2P_3	38.05 d	1317 de	0.3717 c	40.19 b	0.5700 b	19.69 cd
PSp_2P_4	38.42 d	1141 ghi	0.3583 d	34.62 cd	0.4925 hl	14.63 hi
PS_0P_1	38.94 cd	1321 de	0.2217 j	22.99 g-u	0.4725 ijk	15.98 fgh
PS_0P_2	38.44 d	1240 efg	0.2067 k	20.34 ij	0.4550 kl	14.62 hi
PS_0P_3	38.03 d	1127 hi	0.2000 k	18.88 ijk	0.4283 l	12.70 j
PS_0P_4	38 d	1006 j	0.1858 l	16.07 jk	0.3817 m	10.15 k

*Values within a column followed by the same letter are not significantly different at Duncan ($P \leq 0.05$)

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The maximum oil yield (1,620 kg ha^{-1}), shoot phosphorous content (0.41 %), shoot phosphorous absorption (49.55 kg ha^{-1}), grain phosphorous content (0.62 %), and grain phosphorous absorption (25.23 kg ha^{-1}) were achieved in PSp_2P_2 (*Table 15*). The result of cultivar × phosphorous interaction showed that the maximum oil content (40.42 %) was obtained in P_1 for Hayula 401 (*Table 16*). The greatest oil yield (1,487 and 1,502 kg ha^{-1}) was observed in P_1 and P_2 for Hayula 401. The highest shoot and grain phosphorous content (0.3 and 0.54 %), and grain phosphorous absorption (20.56 kg ha^{-1}) were produced for Hayula 401 in P_2 (*Table 16*).

Table 16. *Interaction of cultivar × phosphorous on quantities and qualities yield of rapeseed cultivars*

Interaction	Oil content (%)	Oil yield (kg ha^{-1})	Shoot P concentration (%)	Shoot P absorption (kg ha^{-1})	Grain P concentration (%)	Grain P absorption (kg ha^{-1})
C_1P_1	40.42 a	1487 a	0.2883 b	29.94 ab	0.5117 b	18.85 b
C_1P_2	39.49 b	1502 a	0.2970 a	31.57 a	0.5443 a	20.56 a
C_1P_3	38.75 cd	1284 c	0.2687 d	26.15 cd	0.5087 b	16.97 c
C_1P_4	38.19 d	1109 e	0.2403 f	20.41 e	0.4537 c	13.19 e
C_2P_1	39.08 bc	1354 b	0.2790 c	31.74 a	0.5023 b	17.37 c
C_2P_2	38.41 d	1391 b	0.2823 c	32.94 a	0.5297 a	19.28 b
C_2P_3	38.06 d	1193 d	0.2583 e	28.06 a	0.4950 b	15.59 d
C_2P_4	38.15 d	1070 e	0.2360 e	24 d	0.4487 c	12.65 e

*Values within a column followed by same the letter are not significantly different at Duncan ($P \leq 0.05$)

The highest seed oil percentage was obtained in the treatment of phosphorus fertilizer equal to the soil test and the lowest was obtained in the treatment of the phosphorous control treatment. Supplying the plant with its needed nutritional sources increases its protein and oil percentage; practicing the phosphorus fertilizer also increased the oil content in this experiment. Furthermore, since phosphorus enhances shooting and flowering, it has a role in increasing the formation of more capsules, and eventually, the formation of more seeds. As a result, with an increase in seeds, the oil content also increases. Since better nutrition leads to better photosynthesis during canola's growth, and since supplying the needed phosphorus also fills the pod and seed, it affects the percentage and the yield of canola oil. With regard to the oil yield, phosphorus levels became significant. The consumptions of phosphorus fertilizer equal to the soil test and 25 % less than the soil test did not demonstrate a significant difference with each other in the oil yield and the least oil yield was obtained in the phosphorous control treatment. Different levels of phosphorus led to significant changes in the seed phosphorus concentrations; therefore, the most seed phosphorus was obtained in the treatment of phosphorus fertilizer 0.25 % less than the soil test and the least seed phosphorus was obtained in the treatment of no phosphorus fertilizer consumption. The bacteria *Pseudomonas* IV in the presence of phosphorus fertilizer 0.25 % was less than the soil test was the most and the least belonged to the treatment of no bacteria consumption and no bacteria. The phosphorus content of the seeds and shoots became significantly different at the levels of cultivar, bacteria, and phosphorus. The bacteria *Pseudomonas* IV led to the greatest phosphorus percentage in the seeds and the shoots, and the treatment of zero bacteria consumption had the least amount of

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phosphors content. Other researchers showed that the seed yields and the nitrogen uptake of canola were significantly increased by bacterization. Besides nitrogen, the uptake of other nutrients was also increased by these microorganisms. In general, pyrite amendment along with bacterization improved yields and nutrient uptake in canola and wheat over other treatments without pyrite addition (Joseph et al., 2014). Both bacterial and fungal strains exhibiting P-solubilizing activity were detected by the formation of a clear halo around their colonies. Production of a halo on a solid agar medium should not be considered the sole test for P-solubilization. When the colonies grow without a halo after several replacements of the medium, an additional test in the liquid media to assay P-dissolution should be performed, and a few isolates that are obtained after such a rigorous selection should be further tested for the abundant production of organic acids; the isolates complying with these criteria should be tested on a model plant as the ultimate test for potential P-solubilization (Bashan et al., 2013). Madani et al. (2011) revealed that the highest rate of seed yield (9.9 t/ha) was recorded in dual fertilizing applied both in sowing time and after the overwintering stage of the rosette. The interaction effects of phosphorus-solubilizing bacteria and the ammonium phosphate fertilizer application did not have a significant effect on plant height, biomass yield, the number of silique per plant, seed oil percentage, and seed yield. Interaction effects of phosphorus-solubilizing bacteria and ammonium phosphate fertilizer application were significant for phosphorus content in plant tissues.

The effect of the mentioned bacteria in increasing the function of plants, such as rice and canola, has been reported (Ramezanpour, 2010; Yasari and Patwardhan, 2007). Yasari et al. (2008) reported that inoculation of *Azotobacter* and *Spirillum* had increased the amount of canola oilseed. The purpose of this research was to investigate the effects of using different species of *P. fluorescent* as the solvent bacteria of the soil's insoluble phosphate on the function and the phosphorus content of the leaves and the seeds of the canola cultivars Hayula and Sarigol.

Conclusion

The application of phosphorus-solubilizing bacteria alone as a basic phosphorus fertilizer could not add to the phosphate compounds in plant parts. An increase in phosphorus content in plant parts by using chemical phosphorus fertilizers alongside phosphorus-solubilizing bacteria could cause major effect availability of soil phosphorus for rapeseed. According to the results which were obtained from this experiment, it can be stated that the bacterial inoculation of plants with the bacteria *Pseudomonas Putida* improved growth and it was better than the bacteria *Pseudomonas Putida*. Moreover, with the consumption of the bacteria, we can decrease the phosphate fertilizer consumption by 25 %; in this research, the cultivar Hayula 401 was better than the cultivar Sarigol with regard to yield and yield constituents.

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CORRELATION ANALYSIS OF EAST ASIAN SUMMER MONSOON AND SUBSURFACE OCEAN TEMPERATURE FROM THE PACIFIC OCEAN TO THE INDIAN OCEAN IN THE TROPICS

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Abstract. In order to offer some meaningful information on prediction of East Asian summer monsoon and ENSO, this paper mainly calculates the relativity between the East Asian summer monsoon and tropical upper sea temperature and makes a composite analysis to discuss the interactions of them. It turns out that: The best correlation between the Tropical Central Indian Ocean, the West Pacific Ocean to the East Indian Ocean and EASM index is at 120m. The positive and negative correlation zones move from west to east over time from the Pacific Ocean to the Indian Ocean in the tropics. If the sea temperature field in autumn and winter in the previous year shows the phenomenon of La Nina-like, the west wind from the East Indian Ocean to the West Pacific Ocean will strengthen from December to April. From March to April, there would be easterly wind anomaly air current on the East Pacific Ocean, and if Walker Circulation strengthens, it may occur strong monsoon in summer, and is easy to turn into the phenomenon of El Nino-like in autumn and winter. About their interactional physical process, should further strengthen the mechanism and model study in the future.
Keywords: *El Nino, Walker circulation, composite analysis, upper sea temperature, correlation zone*

Introduction

The land-sea thermal difference changes along with the change of season caused by the different distribution of sea and land on the surface of the earth is the main cause of monsoon. The variations of monsoon play an important role in seasonal and inter-annual variations of the world's tropical atmospheric circulation (Qian, 2000; Wang, 2010). The large amounts of water vapor in monsoon region from tropical oceans that brought by East Asian summer monsoon (EASM) has vital influence on the

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distribution of rainfall, the move of the zone of precipitation, drought and flood in most part of China. Therefore, the research on the relationship between the thermal condition of tropical oceans and EASM is of great significance. In the tropical oceans, ENSO is a strong signal of air-sea interaction (Wang et al., 2000; Xie et al., 2010; Li et al., 2013), which will cause serious climate anomaly in many areas around the world.

Many studies (Zhang et al., 1999; Li and Shou, 2000; Zong et al., 2010; Chen et al., 2013; Xu et al., 2016) have shown that ENSO has important influence on the East Asian summer monsoon and our climate. It is pointed out that the anomaly of sea surface temperature (SST) is the strongest and most important factor for the East Asian summer monsoon forecast, and the difference of the El Nino warming period affects the strength and outbreak time of EASM, thus affecting the amount of summer precipitation in East Asia. As the interaction of the Western Pacific Warm Pool (WPWP) in tropics was dramatic, studies (Huang and Sun, 1994a; Jin and Chen, 2002; Chen and Huang, 2008; Huang et al., 2016) proposed that the WPWP played a key role not only in the tropical Pacific ENSO cycle, but also in the intraseasonal and interannual variation of EASM. Meanwhile, the tropical Pacific ENSO cycle also affected large-scale circulation and convection activities over WPWP and EASM circulation.

The above researches are based on SST in tropics. However, because of the fact that the distribution of SST depended on the complex process of three-dimensional ocean circulation, the research on subsurface is equally important. As subsurface ocean temperature (SOT) has been less affected by external factors, in other words, it has high stability, which gradually aroused attentions of meteorologists in recent years. According to the research of Li and Mu (1999) and Mu and Li (2000), SOT in WPWP continued to rise before the onset of El Nino event. The positive anomaly of SOT in the warm pool region, especially the spread of it to the equatorial central and eastern pacific directly caused the onset of El Nino event. It was an important reason for El Nino event. The research on SOT in recent years further pointed out that the anomaly of SOT in WPWP and ENSO cycle were interacted with each other (Chen et al., 2010; Li and Li, 2014; Shan et al., 2016).

Thus can be seen that the influence that SST in the tropical ocean on EASM and the relationship between SOT in WPWP on the equator and ENSO have been taken seriously, But the relationship between subsurface temperature change and EASM is seldom considered. Zhang et al. (2001a; 2001b; 2002) found that the temperature anomaly distribution of SOT from the tropical Pacific Ocean to the Indian Ocean played an important part in onset's strength and time of South China Sea summer monsoon, and revealed the correlation of them. Nevertheless, the relationship between EASM and SOT from the Pacific Ocean to the Indian Ocean in the tropics remains unknown. Therefore, this paper strives to seek the distribution modes of sea temperature between strong and weak monsoon and reveals the interaction of them according to the analysis of EASM intensity index and SOT from the Pacific Ocean to the Indian Ocean in the tropics.

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Materials and Methods

A set of data about the monthly anomaly of the sea temperature in the world's upper ocean sorted out by Scripps Joint Environmental Data Analysis Center (JEDAC) in the United States was obtained from Nanjing Atmospheric Data Service Center. The horizontal resolution of which was 5°×2° with a coverage of 30°E～180° ～0° ～30° E, 60° S～60° N and the locations of the monthly anomaly in vertical were 0, 20, 40, 60, 80, 120, 160, 200, 240, 300, 400 m from January 1955 to December 1998. This paper focused on the date of 30°S～40°N in the Pacific-Indian Ocean with a total of 528 time levels.

NCEP/NCAR reanalysis data of monthly average wind field at 850hPa from January 1955 to December 1998 with a horizontal resolution of 2.5° ×2.5°.

The computing method of East Asia Summer Monsoon Index (EASMI) defined by Zhang et al. (2003) was to calculate the average anomaly annual temperature of zonal wind in the monsoon trough of East Asia tropical monsoon (10～20°N, 100～150°E) and East Asia subtropical region (25～35°N, 100～150°E) at 850hPa from June to August. That was $I_{EASM} = U_{850hPa}(10～20°N, 100～150° E) - U_{850hPa}(25～35°N, 100～150°E)$. The higher the value, the stronger the summer monsoon and vice versa. This paper selected the data from 1955 to 1998. After standardizing, the paper reckoned that if the intensity index of EASM is greater than 1.0, it is a strong summer monsoon year, while the intensity index of EASM is less than -1.0, it is a weak summer monsoon year. The interannual variability was shown in *Figure 1*. The main methods used were synthetic analysis method and correlation coefficient method.

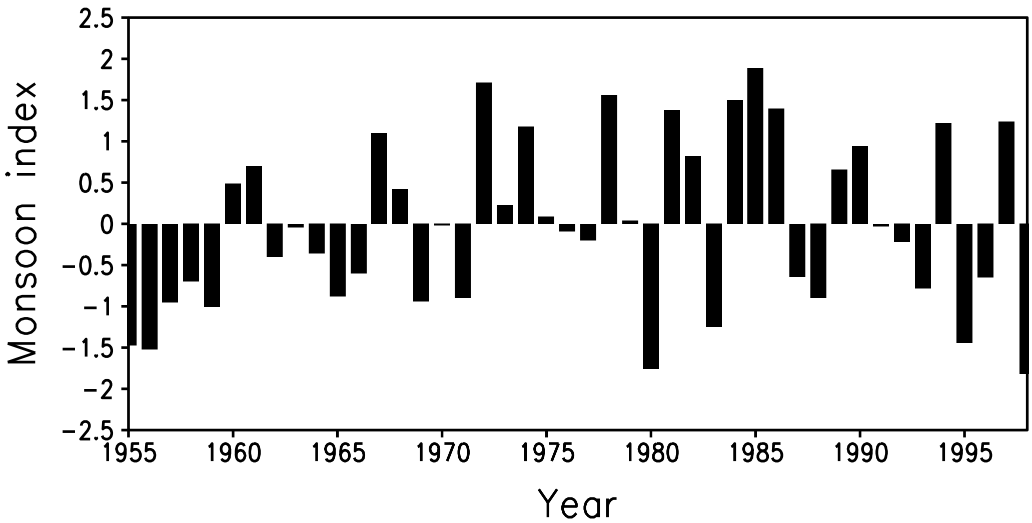

Figure 1. *Interannual variability of the East Asia summer monsoon index from 1955 to 1998*

Correlation Analysis of the EASMI and Sea Temperature

The formation of winter and summer monsoon circulations is related to the sea and land thermal regime in winter and summer. In summer, the land is a heat source that

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controlled by heat low and the sea is a cold source that controlled by cold high. According to the principle of gradient wind, the wind blows off anticyclone towards depression and thus formed the summer monsoon that blows off the sea towards the land. It can be seen that the sea thermal regime has significance influence on the variation of EASM circulation. Therefore, the paper firstly analyzed the relationship between EASM index and sea temperature.

After calculating EASM intensify index anomaly and sea temperature anomaly from the Pacific Ocean to the Indian Ocean in the tropics from 0m to 400m in monthly and analyzing the correlation filed in each level, this paper found out that the correlation distribution of the Indian Ocean, the West Pacific to the East Indian Ocean and the East Pacific region on the equator and the monsoon index changes over time.

Results

The average regional correlation field (*Fig. 2*) is given below, which shows the sea temperature that ahead of monsoon for 7 months and lagged behind monsoon for 4 months. The a～c are the correlation distribution of sea temperature and monsoon index in the Indian Ocean (15°S～15°N, 60～80° E), the West Pacific to the East Indian Ocean (15°S～15°N, 100～170°E) and the East Pacific (10°S～10°N, 170～80°W) region on the equator in December in the previous year; d～f is in November in the same year.

It could be seen that the value of the Indian Ocean Region (*Fig. 2a*) in December in the previous year demonstrated a negative correlation and the absolute value of the correlation distribution gradually decreased from 0m to 60m, while from 60m to 120m, this value gradually increased and the whole level showed a trend of shock weakening. The West Pacific to the East Indian Ocean Region (*Fig. 2b*) demonstrated a positive correlation and the correlation distribution gradually increased from 0m to 120m, while from 120m to 400m, this value gradually decreased and the whole level looked like a line “V”, it reached the crest value at 120m. The East Pacific region (*Fig. 2c*) demonstrated a negative correlation and the absolute value of the correlation distribution in this whole level showed a trend of weakening, it reached the crest value at 40m. This paper found that the sea temperature that ahead of monsoon for 7 months in tropics was a mode of “- + -” from West to East and at 120m, the Central Indian Ocean, the West Pacific to the East Indian Ocean Region on the equator and monsoon index showed a good relativity. In November, the Central Indian Ocean Region (*Fig. 2d*) demonstrated a positive correlation and reached the crest value at 120m. The West Pacific to the East Indian Ocean Region (*Fig. 2e*) demonstrated a negative correlation, and the absolute value of the correlation distribution in the whole level was the same as the period that the sea temperature ahead of monsoon for 7 months. That was the correlation distribution gradually increased from 0m to 120m, while from 120m to 400m, this value gradually decreased and the whole level looked like a line “V” and reached the crest value at 120m. The East Pacific region (*Fig. 2f*) demonstrated a positive correlation and the absolute value of the correlation distribution in the whole level was the same as the period that the sea temperature ahead of monsoon for 7 months, too. It showed a trend of

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weakening and reached the crest value at 40m. In summary, the sea temperature that lagged behind monsoon for 4 months in tropics was a mode of “+ - +”from West to East and the Central Indian Ocean, the West Pacific to the East Indian Ocean on the equator at 120m and monsoon index showed a best relativity (Zhang et al., 2001b, 2002).

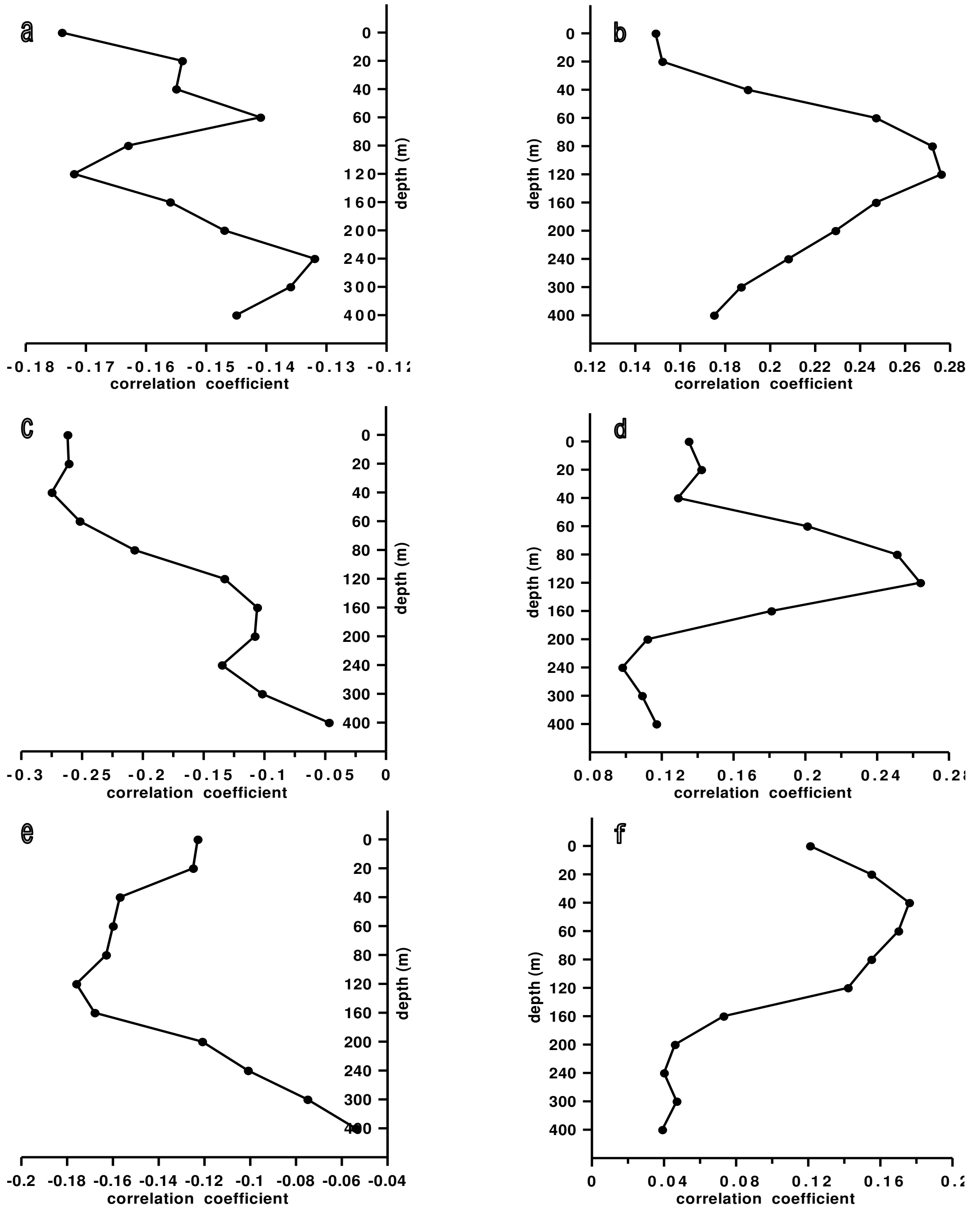

Figure 2.*Correlation coefficient of area average between East Asia summer monsoon index and sea temperature in 0～400m layer*

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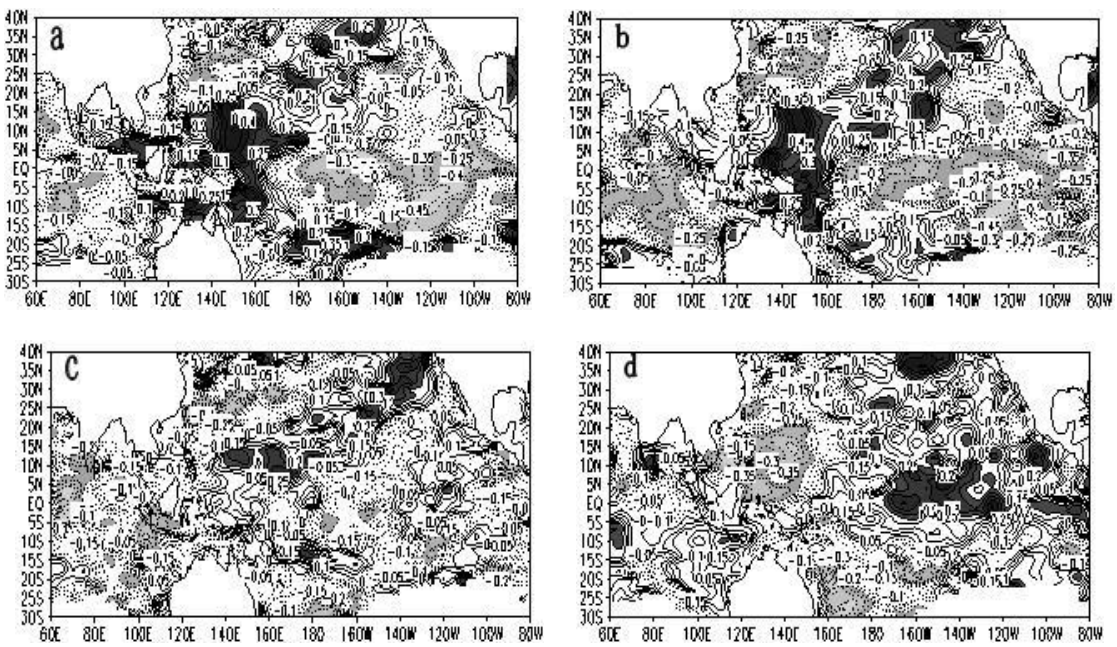
Above all, the mode of the Central Indian Ocean, the West Pacific to the East Indian Ocean and the East Pacific Ocean region on the equator and monsoon index associated with the transition of "- + -" to "+ - +" over time. The best relativity of the Central Indian Ocean, the West Pacific to the East Indian Ocean on the equator and EASM index occurred at 120m, while that of the East Pacific region on the equator and EASM index occurred at 40m. Which showed that the signal strength of EASM is the strongest in tropical sea subsurface. This result was consistent with Reference (Huang and Sun, 1994b). The reason for this phenomemon was that the depth of thermocline of the Indian Ocean in tropics is about 70m to 100m, and the thermocline of the Pacific Ocean in tropics was gradually uplifting from West to East. The depth of which was 140m in the West Pacific in tropics, while in the East Pacific on the equator, it was less than 50m (Mcphaden, 1999; Cai et al., 2005; Li et al., 2008). That was to say, the variation of anomaly at 120m reflected the changes of the sea temperature in thermocline in most regions. After analyzing the correlation filed in each level, this paper found that the distribution pattern of the correlation filed from 0m to 40m is close to each other, and that of the correlation filed in each level from 60m to 400m is alike. This paper selected 40m and 120m which has the best relativity among those levels to do our research. *Figure 3* was the distribution pattern of correlation field of these two kinds of sea temperature and EASM index. a～d were the distribution pattern of correlation field that the sea temperature ahead of monsoon for 7 months, 3 months, the corresponding period and lagged behind monsoon for 4 months at 40m; e～h were the distribution pattern of correlation field that the sea temperature ahead of monsoon for 7 months, 3 months, the corresponding period and lagged behind monsoon for 4 months at 120m. After testing by 0.10 confidential level, this paper found that the correlation field of the shadow region that up to 0.40 is exceed the significance level of the shadow region that up to 0.01.



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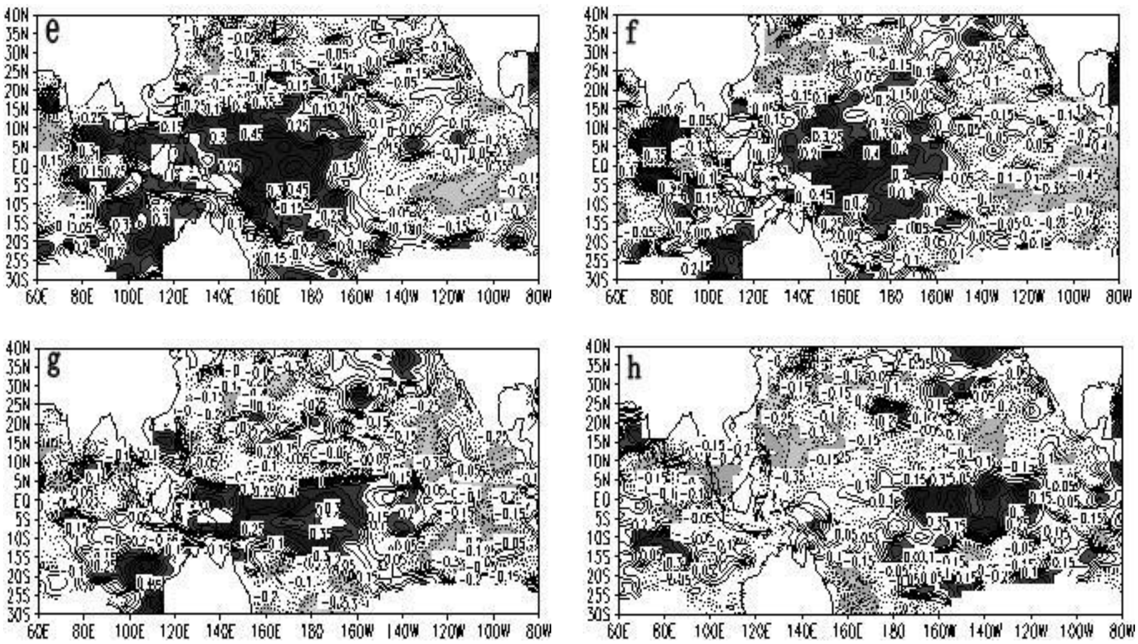

Figure 3. *The distributions of the correlation coefficients between the East Asia summer monsoon index and the subsurface ocean temperature*

According to *Figure 3*, the manifestation of correlation field at 40m and 120m from the Pacific Ocean to the Indian Ocean in tropics was different. When the sea temperature ahead of monsoon, the East Indian Ocean, Arabian Sea and North-western Australia's Waters on the equator at 40m demonstrated a negative correlation, while at 120m, it demonstrated a positive correlation. When the sea temperature and monsoon in the corresponding period, Western Australia's Waters, near the island of Sri Lanka and the Central Pacific Ocean on the equator at 40m demonstrated a negative correlation, while at 120m, it demonstrated a positive correlation. That's to say, the strength of EASM has different influences on different levels on tropical sea subsurface (Chen et al., 2011; Xu et al., 2016). But the distribution of these two kinds of correlation field in the East Pacific was about the same.

The positive and negative correlation zone moved over time from West to East. When the sea temperature ahead of monsoon for 7 month, the Central East Indian Ocean was the negative correlation center at 40m, it was linked with the negative zone in Northern South China Sea and the Waters of South Japan and moved to the West Pacific Ocean when the sea temperature lagged behind monsoon for 4 months. When the sea temperature ahead of monsoon for 7 months, the West Pacific Ocean 15°S～15°N is the significant positive correlation field and it moved to East Pacific when the sea temperature lagged behind monsoon for 4 months and narrowed to the area of 5°S～10°N. The positive correlation zone of the West Pacific at 120m moved to southeast monthly. When the sea temperature ahead of monsoon, the positive correlation field at 40m moved eastward to near the date line and reached near 160°W at 120m. We can conclude that the positive

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correlation field at 120m was ahead of that was at 40m and it was overlapped with the positive correlation field at 40m when the sea temperature lagged behind monsoon for 4 months. At that time, the correlation field in above and below of the Indian Ocean was basically the same. In combination with *Figure 2* whose distribution pattern of correlation field changed over time, this paper concluded that the positive and negative correlation zone's moving from West to East made the correlation field that the sea temperature ahead of monsoon for 7 months and lagged behind monsoon for 4 months associated with the transition of "- + -" to "+ - +".

In combination with the related analysis of the correlation field that the sea temperature ahead of monsoon for 7 months and lagged behind monsoon for 4 months in *Figure 2* and *Figure 3*, this paper concluded that the positive (negative) sea temperature of the West Pacific Ocean to the East Indian Ocean was anomaly in autumn and winter of the previous year; the negative (positive) anomaly on the Central Indian Ocean and the East Pacific Ocean can easily lead to strong (weak) summer monsoon in the next year. Conversely, strong (weak) summer monsoon could accelerate the change the temperature of the sea on the East Indian Ocean and the West Pacific (from cold to warm) and the Central Indian Ocean and the East Pacific Ocean (from warm to cold) (Yuan and Yang, 2012; Yuan et al., 2012). It was evident at 120m, which meant that the sea temperature in this level and EASM have close relationship.

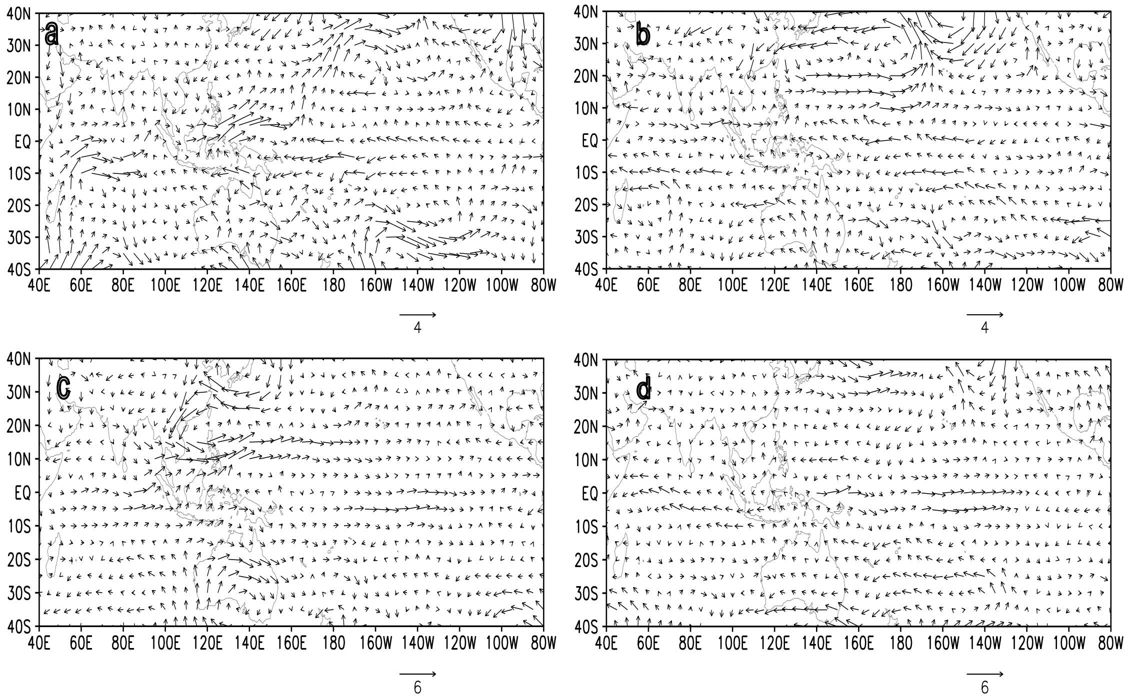
The Interaction of SOT from the Pacific Ocean to the Indian Ocean in the Tropics and EASM Circulation

To testify the above conclusion and clearly discern the circulation pattern in the year of strong EASM and weak EASM and the abnormalities of SOT. Then this paper made a composite analysis of anomaly wind field at 850hPa and sea temperature anomaly at 120m in the year of strong EASM and weak EASM to investigate the interaction of SOT and EASM circulation.

To reflect the annual variation of EASM objectively and quantitatively, it was necessary to represent the variation of monsoon with index. This paper applied the EASM index that defined by Zhang et al. (2003) (*Fig. 1*) and made a composite analysis of the year of strong EASM (in 1972, 1978, 1984, 1985, 1986) and the year of weak EASM (in 1956, 1980, 1983, 1995, 1998). *Figure 4* was the composite graph of wind anomaly in the year of strong summer monsoon and weak summer monsoon at 850hPa and *Figure 5* was the corresponding composite graph of sea temperature anomaly at 120m.

In autumn and winter of the previous year, the year of strong summer monsoon, the Indian Ocean on the equator was the westerly anomaly and the Indian Ocean on the equator was the easterly anomaly (*Fig. 4a*). The vertical velocity anomaly over the East Pacific Region on the equator were descending motions while over the West Pacific Region on the equator, there were ascending motions (*Fig. 6a*). It showed that Walker circulation on the Pacific Ocean Region on the equator was enhanced; the West Pacific Ocean to the East Indian Ocean region was strong positive sea temperature anomaly and it demonstrated the phenomenon of La Nina-like (*Fig. 5a*). In Spring, the westerly wind anomaly on the Indian Ocean was weakening and moved to northeast to Malay peninsula and turned into west wind anomaly air current; the westerly wind anomaly over the West Pacific Ocean stretched toward the northeast and formed strong west wind anomaly air current; the easterly wind anomaly on the East Pacific Ocean on the equator was weakening and there were negative sea temperature anomaly on the East Indian Ocean, Northern South China Sea, Taiwan and Eastern Luzon Island's Waters, the anomaly center that was equal or greater than 1°C in warm pool region moved from west of the date line to east to near 140°W (*Fig. 5b*).

In summer, there was a closed cyclonic anomaly circulation in the East Asia to Pacific Ocean in tropics (*Fig. 4c*). If there was a cyclonic anomaly circulation in the East Asian tropical monsoon region, it showed that EASM circulation was strengthen (Zhang and Wang, 2006); if there was a west wind anomaly air current on the East Pacific Ocean on the equator and the east wind was weakening, the whole East Indian Ocean on the equator at 120m showed a phenomenon of negative sea temperature anomaly. The anomaly center that was equal or greater than 1°C further strengthened (*Fig. 5c*). In autumn and winter, there was an east wind anomaly air current on the Indian Ocean on the equator and the west wind was weakening, which was called as weak east wind (*Fig. 4d*). The vertical velocity anomaly over the East Pacific Ocean region on the equator were ascending motions, while there were descending motions over the West Pacific Region on the equator (*Fig. 6b*). It showed that Walker circulation was weakened; the most area of the Central Indian Ocean to the Southeast Pacific Ocean at 120m was positive sea temperature anomaly and the WPWP was strong negative sea temperature anomaly, the phenomenon of El Nino-like was formed (*Fig. 5d*) (Kug et al., 2009; Chen et al., 2011).



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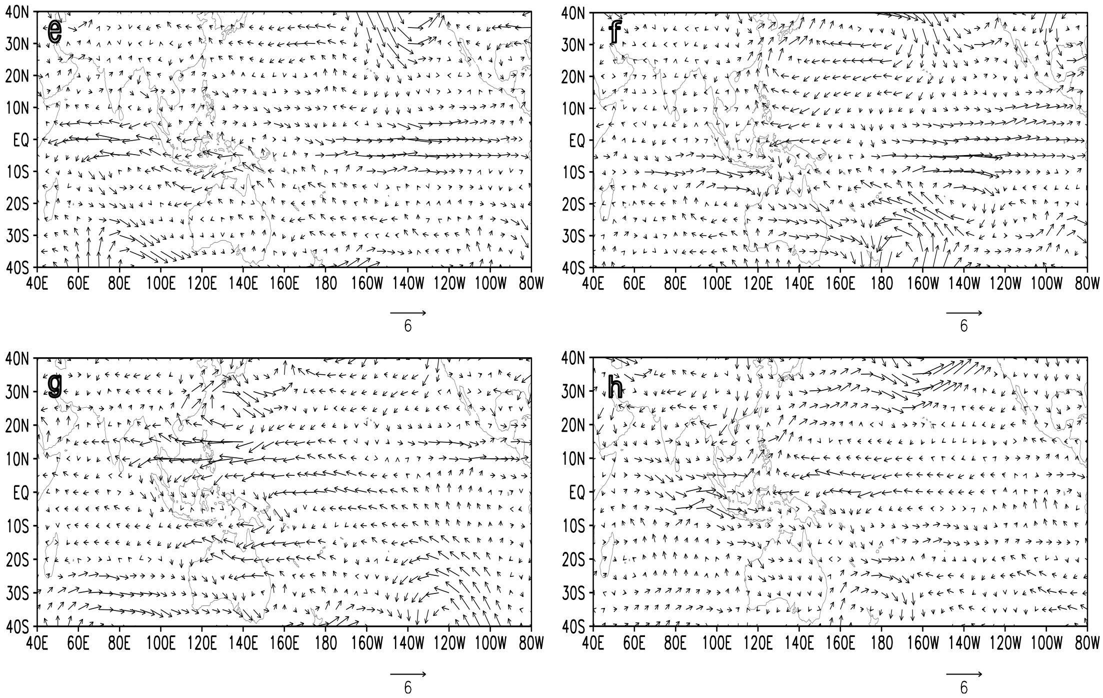

Figure 4. *Composite anomalous wind at 850 hPa: (a～d) strong East Asia summer monsoon case: (e～h) weak East Asia summer monsoon case (from front to back:last December, this April, July and November. unit: m·s^{-1})*

The characteristic of circulation pattern of the year of weak summer monsoon to low-level troposphere was in reverse. In autumn and winter of the previous year, there was an east wind anomaly air current on the Indian Ocean on the equator and the "Maritime Continent" in Indonesia and the east wind was weakening (*Fig. 4e*), Walker circulation was weakened (*Fig. 6c*); the West Pacific Ocean to the East Indian Ocean on the equator was a strong negative sea temperature anomaly. The south of 10°N and the east of 170°W was strong positive anomaly and showed the phenomenon of El Nino-like (*Fig. 5e*). In Spring, there was a west wind anomaly air current as east anomaly left the East Indian Ocean on the equator and there was a easterly wind anomaly over the West Pacific Ocean Region. At the same time, the negative anomaly of the sea temperature on the East Indian Ocean was weakened, and there was a positive anomaly Taiwan and Eastern Luzon Island's Waters that was equal or greater than 0.4°C. In Summer, there was a closed anticyclone anomaly circulation in the East Asia to Pacific Ocean Region in tropics (*Fig. 4g*). If there was a anticyclone anomaly circulation in EASM Region, the EASM circulation was weakened (Zhang and Wang, 2006), and there was an east wind anomaly air current on the Pacific Ocean Region on the equator; the positive sea temperature anomaly on the West Pacific Ocean to the East Indian Ocean was formed and the negative center that was less than or equal to -0.4°C moved to the south of 10°N and the east of 170°E (*Fig. 5g*). In autumn and winter, there was still an east wind anomaly air current on the Pacific Ocean region on the equator, Walker circulation was further strengthened (*Fig. 6d*); there was a strong positive sea temperature on the West Pacific

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Ocean Region on the equator; the most of the Central Indian Ocean and the East Pacific Ocean region was a negative sea temperature anomaly, the phenomenon of La Nina-like was formed (*Fig. 5h*) (Kug et al., 2009; Chen et al., 2011).

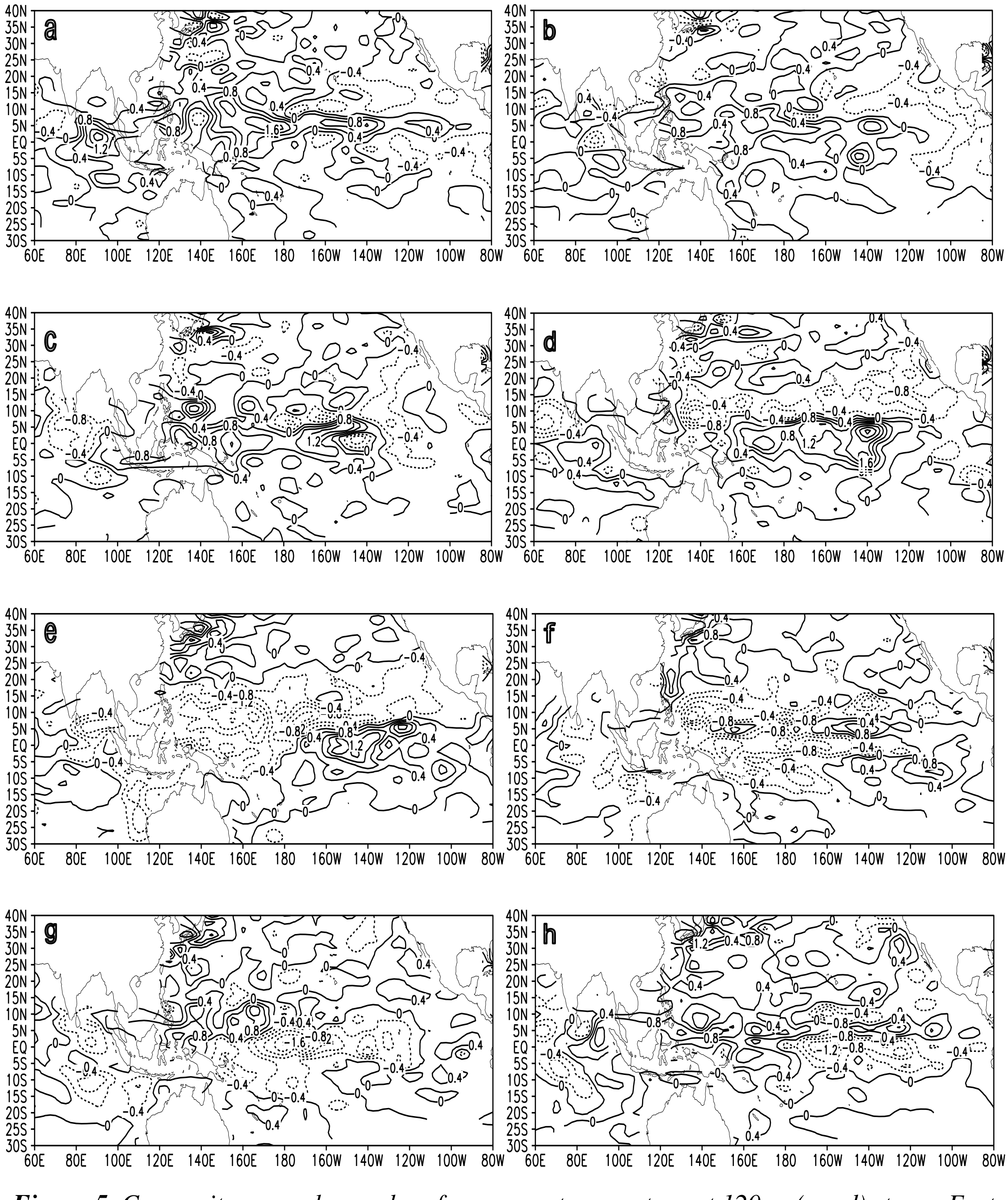

Figure 5. *Composite anomalous subsurface ocean temperature at 120m: (a～d) strong East Asia summer monsoon case: (e～h) weak East Asia summer monsoon case (from front to back:last December, this April, July and November)*

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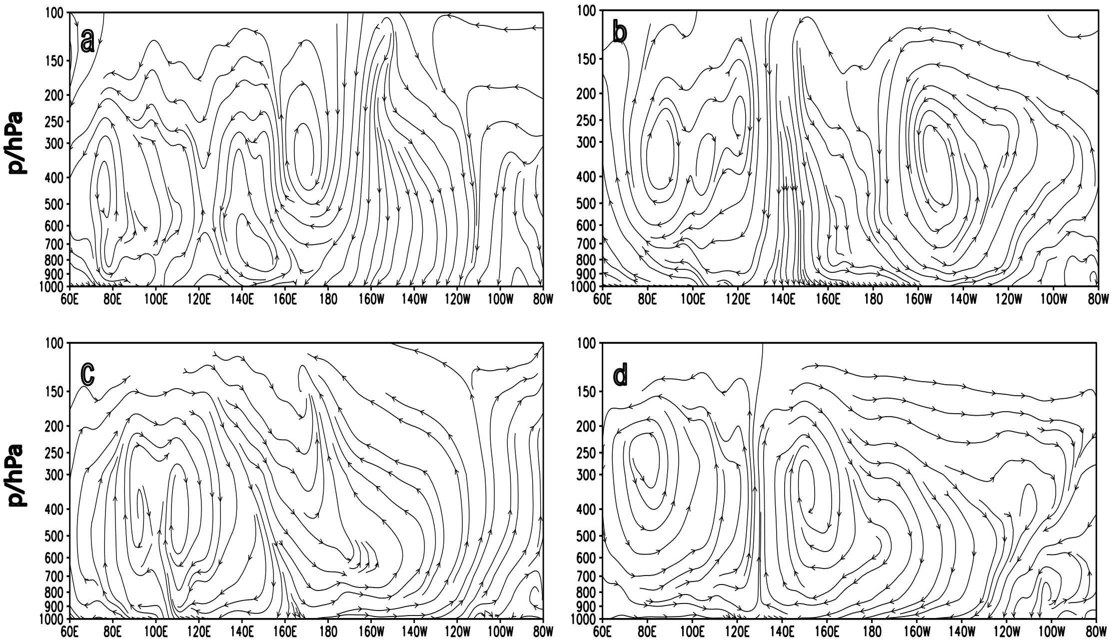

Figure 6.*Height-longitude section for anomalous vertical velocity averaged over (5o S-5o N) a～b last December and this November in strong East Asian summer monsoon case: c～d last December and this November in weak East Asian summer monsoon case*

Discussion

According to the analysis of this paper, we can get some meaningful information on EASM and ENSO prediction: with the strengthening of Walker Circulation on the East Pacific Ocean on the equator in autumn and winter of the previous year, the sea temperature from the East Indian Ocean to West Pacific Ocean Region on the equator at 120m was warming intensively. Meanwhile, the strong westerly wind on the Indian Ocean to the West Pacific motivated the warm Kelvin Wave moved eastward (Huang et al., 1996, 2011), the high temperature center on the West Pacific Ocean began to move to the east, from March to April, there was an easterly wind air current in the East Pacific; Walker Circulation was strengthened and the updraft over the West Pacific Ocean was strengthened, too. Which created the conditions of the strong summer monsoon (Xu et al., 2016). From July to September, there was a weak east wind on the East Pacific Ocean region and Walker Circulation was weakened, and the strong summer monsoon might accelerate the eastward of Kelvin Wave and cause the sea temperature of the West Pacific Ocean reduced and that of the East Pacific Ocean raised and the phenomenon of El Nino-like was formed (Li and Mu, 1999; Mu and Li, 2000). Reversely, in autumn and winter of the previous year, the weak west wind on the Indian Ocean on the equator at 120m, the weak east wind on the East Pacific Ocean and Walker Circulation were weakened, the brace that maintaining the terrain of the sea level of the Pacific Ocean that high in east and low in west was damaged, the warm water of the West Pacific Ocean spread to the east quickly and caused the East Pacific Ocean getting warm and the west

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Pacific Ocean getting cold. In April, the east wind anomaly air current left the East Indian Ocean on the equator, the west wind over the West Pacific Ocean was weakened, the part of the warm pool was warming up. At the same time, the East Pacific Ocean on the equator was still a west wind anomaly air current, Walker Circulation and the updraft over the West Pacific Ocean Region were weakened, which created the conditions of the weak summer monsoon (Xu et al., 2016). From July to September, the east wind on the East Pacific Ocean region was strengthened and Walker Circulation was also strengthened, which cause the sea temperature of the West Pacific Ocean to the East Indian Ocean raised and the phenomenon of La Nina-like was formed.

Through the analysis of the upper sea temperature from tropical Pacific Ocean to Indian Ocean, this paper has found that the sea temperature at 120m is the closest relationship with the intensity of EASM. The study of SOT is more valuable because of its higher stability and memorability. At the same time, the EASM seriously affects the weather and climate in China (Guo et al., 2003; Li et al., 2011), so there is practical significance on forecasting the summer monsoon strength by analyzing the SOT to forecast the weather or climatic conditions. This paper also has found that the strength of EASM can change the distribution of SOT in turn. As for the relationship between the distribution of SOT from tropical Pacific Ocean to Indian Ocean and the ENSO cycle would be the focus of the next work.

In this paper, the studies not only regard SOT as air-sea interaction in the tropical Pacific region, but also begin to pay close attention to the interaction of tropical Indian Ocean and EASM through the atmospheric bridge. However, the relationship between SOT and EASM is not only a tropical problem (Yeh et al., 2009; Chen et al., 2013). There is a lack of further understanding of the influence mechanism of air-sea system in off-tropical area on EASM. Therefore, the interaction between SOT in off-tropical area and EASM may be an important research topic in the future. In addition, the influence mechanism of distribution of SOT and ENSO events on the global weather would receive significant attention.

Conclusion

This paper focused on the correlation of SOT from the Pacific Ocean to the Indian Ocean in the tropics and EASM and discussed the interactions of them. It turned out that:

The analysis of the correlation of EASM index and the SOT from the Pacific Ocean to the Indian Ocean in the tropics showed that the best correlation between the Central Indian Ocean, the Indian Ocean and the West Pacific Ocean to the East Indian Ocean and EASM index is at 120m, the correlation coefficient is weakening above 120m or below 120m (Zhang et al., 2001b, 2002); The positive and negative correlation zone move from west to east over time from the Pacific Ocean to the Indian Ocean in tropics; the correlation distribution of the region on the Indian Ocean, the West Pacific Ocean to the East Pacific Ocean, the East Pacific Ocean and the monsoon index associated with the transition of "- + -" to "+ - +" and in this process, the positive correlation at 120m is exceeded that at 40m.

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The sea temperature on the West Pacific Ocean to the East Indian Ocean at 120m is a little high, while the sea temperature on the Central Indian Ocean and the East Pacific Ocean is a little low, that was to say, the distribution of the sea temperature is demonstrated a phenomenon of La Nina-like, it is easy to cause strong summer monsoon in the next year. Moreover, it is easy to turn into the phenomenon of El Nino-like in autumn and winter. Reversely, the sea temperature from the West Pacific Ocean to the East Indian Ocean on the equator is a little low, while the sea temperature on the Central Indian Ocean and the East Pacific Ocean was a little high, that is to say, the distribution of the sea temperature demonstrates a phenomenon of El Nino-like, it is easy to cause weak summer monsoon in the next year. Moreover, it is easy to turn into the phenomenon of La Nina-like in autumn and winter. Which mainly reflects in the strength of the east wind on the East Pacific Ocean on the equator, the strength difference between the west wind on the Indian Ocean to the West Pacific Ocean and Walker Circulation. This paper can conclude that the distribution of SOT in tropics has influence on EASM; the abnormalities of EASM will change the thermal regime of sea; EASM and the SOT from the Pacific Ocean to the Indian Ocean in the tropics have interactions with each other (Huang et al., 1996; Zong et al., 2010), but the interaction mechanism of them and the relationship between the SOT distribution from the tropical Pacific Ocean to the Indian Ocean and the ENSO cycle need further exploration and study.

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INFLUENCE OF COW MANURE AND ITS VERMICOMPOSTING ON THE IMPROVEMENT OF GRAIN YIELD AND QUALITY OF RICE (*ORYZA SATIVA* L.) IN FIELD CONDITIONS

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Abstract. A field experiment was conducted to assess the impact of cow manure and its vermicompost on the improvement of rice grain yield and quality in Rasht, Iran in 2015 and 2016. The experimental factors were devoted to cow manure (0, 10 and 20 t ha^{-1}) and vermicompost (0, 5 and 10 t ha^{-1}). The results revealed that the application of cow manure and vermicompost increased leaf chlorophyll and grain yield components such as the number of fertile tillers and the number of grain, but, it decreased by 1000–grain weight. The highest grain yield was obtained from the application of 30 t ha^{-1} cow manure + 10 t ha^{-1} vermicompost in the first year (3537 kg ha^{-1}) and in the second year (3958 kg ha^{-1}). In addition, the application of cow manure and vermicompost increased the grain's N, P and K content by 8–20%, 22–23% and 20–33%, but decreased the starch content by 3–7%. Although the combined application of various rates of cow manure and vermicompost improved plant growth and nutrient uptake, the influence of vermicompost on the grain yield and quality of gain was much stronger. In poor areas of the world that experience reduction in rice yield mainly due to lack of soil organic matter, and the farmers cannot buy vermicompost to improve soil fertility, we recommend the combined application of manure and vermicompost, which leads to increased grain qualitative traits and milling percentage, resulting in higher nutritional value of grains and higher grain yield.
Keywords: *biological yield, chlorophyll, nutrient uptake, protein, starch*

Introduction

Rice is a global grain that is grown in about 89 nations and it is a stable food for more than half of the global population (Bodh and Rai, 2015). For many years, rice has been supporting more people than any other cereal. Rice is one of the three most important food crops in Iran (Toorminaee et al., 2017). Fertilizers are the major source of nutrients for rice under intensive cultivation. The use of chemical fertilizers in rice cultivation potentially reduces soil fertility (Biswas et al., 2017). The use of P and K fertilizers in rice cultivation may in the long run lead to nutrient imbalance in the soil, resulting in lower rice productivity (Dong et al., 2012). To improve physical, chemical and biological properties of soil, organic fertilizer can be applied (Mengi et al., 2016). Several studies have shown that the regular application of organic fertilizer, especially manure, could increase the soil org–P fraction (Pant et al., 2009), the availability of C and soil microbial activity and soil organic matter (Dixit and Gupta, 2000), N use efficiency and rice yield (Zhou et al., 2016). Manures of the animals contain valuable

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nutrients and organic compounds that can restore degraded soils and ensure sustainable long−term agricultural activity (Lalandera et al., 2015). Properly treating the organic waste fraction reduces the environmental impact by avoiding greenhouse gas emissions from landfills (Joshi et al., 2015) and decreasing/avoiding the need for chemical fertilizer (Xu et al., 2008). Among the various types of animal waste, the amount of cow manure is 4−9 times than that of pig manure (Guo et al., 2013). Hence, investigating effective measures for disposing of animal waste, especially cow manure, has become an important topic. To this end, earthworms have been widely used to recycle animal manures to vermicompost, thereby rendering the manure harmless. Compared with raw manure materials and its traditional compost, vermicompost possesses a greater capacity for cation exchange and a larger surface area, etc (Lalandera et al., 2015; Meier et al., 2017). Among the various types of animal waste, the amount of cow manure is 4−9 times than that of pig manure (Guo et al., 2013). Hence, investigating effective measures for disposing of animal waste, especially cow manure, has become an important topic. To this end, earthworms have been widely used to recycle animal manures to vermicompost, thereby rendering the manure harmless. Compared with raw manure materials and its traditional compost, vermicompost possesses a greater capacity for cation exchange and a larger surface area, etc. (Meier et al., 2017). Bejbaruah et al. (2013) and Mengi et al. (2016) explored rice response to various rates of compost and animal manure and reported that the application of manure and compost significantly enhanced plant height, tiller number per plant, spike length, and grain and straw yield of rice as compared to control. They related these enhancements to the improvement of soil physical and chemical features and higher availability of nutrients due to the application of organic fertilizers.

In Iran, 20 million tons of manure is produced by animals per annum. The rate of vermicompost production is negligible compared to cow manure. Efforts have been taken in recent years to produce more vermicompost, but its application in farms, especially in rice farms, has not been increased remarkably (Rezaei, 2013). There are some quantitative reports on the application of the mixture of cow manure and vermicompost on grains under field conditions. The present experiment is based on the premise that the application of cow vermicompost will not only improve the grain yield, but also the grain quality of rice from the crop nutritional and marketable viewpoints, as compared to cow manure. Therefore, leaf chlorophyll and grain yield, protein, and NPK uptake were measured to assess this hypothesis.

Materials and methods

Experimental site and plant growth conditions

The field experiment was conducted on clay−loam soil at the Agricultural Research Farm of Islamic Azad University, Rasht, Guilan province, Iran in 2015 and 2016. The area is located at 37°22 N latitude and 49°63 E longitude and 15 m above the sea level. To simplify the comparison of the growing season weather, we considered the monthly total precipitation and temperature from May through August at the Rasht Agricultural Research Farm (*Figure 1*).

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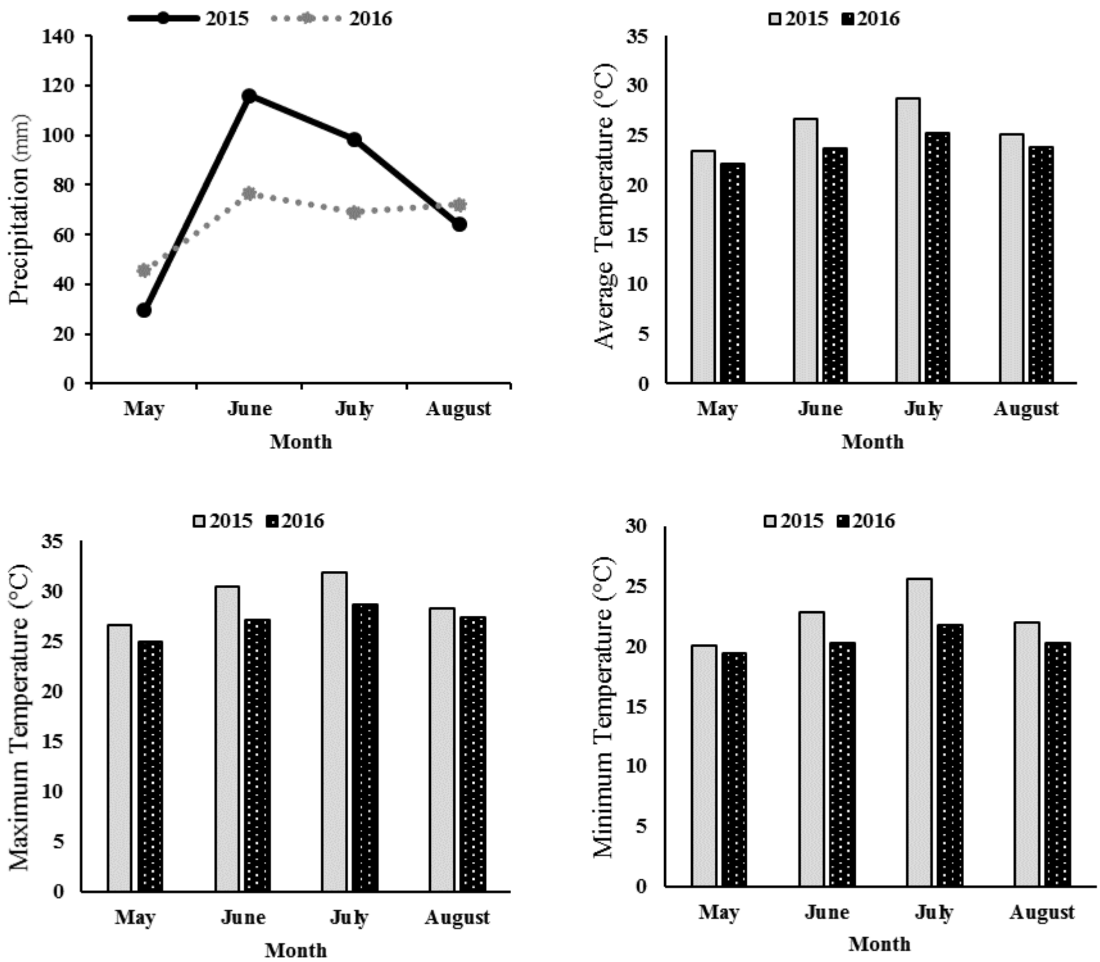

Figure 1. *Monthly precipitation and temperature from May to August for the growing season (2015–2016) at the Rasht Agricultural Research Farm, Iran.*

In order to determine soil characteristics, soil sampling was performed before the experiment. To do this, field soil sampling was done from the depth of 0–30 and 30–60 cm in eight spots. Then the collected samples sent to the laboratory in order to determine soil texture and the chemical composition. Properties of experimental soil samples are given in *Table 1.*

Table 1. *Soil Physical and chemical characteristics of the soil during the two–year study.*

Years	depth (cm)	Sand	Silt (%)	Clay	EC (dS m^{-1})	pH	Organic Carbon (%)	N (%)	P	K	Zn (mg kg^{-1})	Fe	Mn
2015	0–30	29.9	29.4	40.7	1.22	7.6	0.99	0.62	13.2	219	1.32	5.32	8.93
	30–60	28.4	29.1	42.5	1.31	7.9	0.73	0.17	12.5	167	0.76	6.87	8.30
2016	0–30	30.3	34.9	34.8	0.89	7.7	0.58	0.58	14.2	238	0.69	6.5	8.69
	30–60	28.1	31.6	40.3	1.13	7.7	0.70	0.12	12.6	204	0.79	7.1	7.852

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Preparation and applied of Vermicompost

Cow manure, consisting of a mixture of faeces, urine and straw was obtained from the agricultural cow complex Sepidar, in Rasht, Iran. Vermicomposting was carried out in a 3 m^3 vermireactor containing a stable and very active population of the earthworm *Eisenia andrei*. The reactor was fed with different animal manures and mixed agricultural wastes, and supported a population density of 250 g of earthworms kg^{-1} in the top layers. The upper surface of the vermireactor was divided into four independent compartments and 45 kg of cow manure were placed in three successive layers (15 kg each) added to each compartment as the waste was processed by the earthworms. The moisture content of the cow manure in the vermireactor was maintained at 75–80% and the sample was collected from the last layer (40 days of earthworm processing) of the reactor once the manure was processed by the earthworms (Lazcano et al., 2008).

Vermicomposting process significantly changed the physical and chemical properties of cow manure. The vermicompost was much darker in color, had good esthetics and processed into a homogeneous compound after earthworm activity. The total content of waste mixture was reduced 1.8–2.7 times after vermicomposting. The vermicomposting process significantly helps in abatement of organic matter pollution load in the environment and soil. The physicochemical traits and nutrient status of cow manure and its vermicompost are given in *Table 2*.

Cow manure and its vermicompost were applied at 20 days before transplanting. In control plot, the nutritional requirements were supplied by chemical fertilizers in accordance with the results of soil analysis.

Table 2. *Physicochemical properties and elemental composition of the cow manure and its vermicompost as well as their detection method.*

Parameter*	Method	Cow manure	Vermicompost
pH	pH meter	8.10 ± 0.13	7.43 ± 0.06
EC	Conductometer	1.08 ± 0.01	1.26 ± 0.03
Ash content (g kg^{-1})	Burning	197 ± 10.3	441 ± 21.7
Total OC (g kg^{-1})	Walkley black	412 ± 16.9	316 ± 6.5
Total OM (%)	Nitration	78.6 ± 2.3	49 ± 4.3
Total N (g kg^{-1})	Kjeldahl	16.8 ± 0.51	28.3 ± 1.54
Total P (g kg^{-1})	Olsen	6.4 ± 0.91	13.4 ± 0.84
Total K (g kg^{-1})	Film photometry	9.4 ± 1.02	13.7 ± 1.11
C:N	–	24.46 ± 2.3	11.3 ± 0.47
Cu (mg kg^{-1})	Atomic	135.2 ± 7.92	164.8 ± 13.8
Fe (mg kg^{-1})	Atomic	215.4 ± 5.8	496.6 ± 5.7
Mn (mg kg^{-1})	Atomic	109.7 ± 11.4	248.7 ± 4.6
Zn (mg kg^{-1})	Atomic	184 ± 9.83	369 ± 12.06

*Dry matter basis

Sowing condition

Rice seeds (‘Hashemi‘cultivar) were disinfected with 70% thiophanate–methyl WP (Sabz Agrochemical Company (PAC); Tehran, Iran) fungicide at 200 g 100 kg^{-1} seed and were then sown in the nursery. The seedlings were manually transplanted in the experimental field at the 2−3 leaf stage at a spacing of 25 × 25 cm^2. Weeds were

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controlled by hand weeding during the growth season. The permanent flood water level was maintained at 10 cm during the rice growing period.

Plant sampling

At the soft dough stage, eight randomly chosen plants were removed from each plot and in the flag leaves, chlorophyll were determined by Arnon (1949) method. At maturity stage, rice grain yield (based on 14 % humidity) was determined from 2.5 m^2 per plot. Moisture content of grains was measured using a digital grain moisture meter (Model GMK– 303R5–Korea) and grain yield per plot was calculated as ((100 – moisture content of the sample) × fresh grain weight)/86 to convert the sample to 14 % moisture content. Yield components, that is, number of tiller, number of filled grains and 1000–grain weight, were determined from 12 plants (excluding the border ones) sampled randomly from each plot. To determine aboveground biomass, a 1 m^2 sample from each plot was randomly chosen and placed in a separate paper bag, dried at 72 °C for 48 h, weighed, and expressed as the biological yield (dry weight of above–ground plant) per hectare. Harvest index was the proportion (percentage) of filled grain weight to biological yield.

For measuring grain N concentration, rice grains were grounded to pass through a 1-mm sieve. N, P and K concentration was determined using micro–Kjeldahl, vanadomolybdate and flame photometry, respectively (Emami, 1996) and were expressed as the percent of grain dry weight. N, P and K uptake in grain was calculated by multiplying grain dry weight by grain N concentration. Grain protein concentration was calculated as 6.25 × nitrogen content measured by the micro Kjeldahl technique. Similarly grain starch percentage (Amylase) activity is done by using anthrone reagent as suggested by Reyes et al. (1965).

Paddy samples for milling quality evaluation were harvested from the 2.5 m^2 per plot, threshed by a simple motorized thresher and dried up to 8 %, wet basis (w.b.) using the laboratory dryer (Memmart Model 600, Germany) set at 45 ˚C. Paddy moisture content was determined using the digital grain moisture meter (Model GMK–303R5–Korea). After drying process, 200 g of dried paddy from each treatment were dehulled by a laboratory rubber roll huller (SATAKE Co. Ltd, Japan) and then was milled using a laboratory rice whitener (McGill Miller, USA). The milling percentage was calculated by using the following formula and presented in percentage (Zhao and Fitzgerald, 2013):

$$\text{Milling}\,(\%) = \frac{\text{Total weight of milled rice (g)}}{\text{Total weight of rough rice with usk}} \times 100 \qquad \text{(Eq.1)}$$

It should be noted that vermicompost and cow manure in the second year were used as the first year and all the works done in the first year in the second year were repeated.

Experimental design and statistical analysis

The experiments were carried out in a factorial trial based on randomized complete block design (RCBD) with 3 replications. Experimental factors included vermicompost (V0 =0, V1 = 5 and V2 = 10 t ha^{-1}) and cow manure (M0 = 0, M1 = 10, and M2 = 20 t ha^{-1}).

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Basically, various levels of manure and vermicompost as independent variable and measured characters such as seed yield, biologic yield, tiller number, seed number, leaf chlorophyll and the content of nitrogen, potassium and phosphorus as dependent variable were contemplated. In the meantime, plants harvested from 1 m^2 of each plot included experimental samples to measure the mentioned above characters.

All data were subjected to analysis of variance (ANOVA) using SAS 9.3 software. When F test indicated statistical significance at $P<0.01$ or $P<0.05$, the least significant difference (LSD) was used to separate the means.

Results

Leaf chlorophyll

According to the results of ANOVA, the interaction effect of vermicompost × cow manure × year had significant impact on the chlorophyll $a + b$ at the 5% probability level. Means comparison revealed that at vermicompost rates of 0 and 5 t ha^{-1}, the chlorophyll was increased with cow manure, but at vermicompost rate of 10 t ha^{-1}, 20 t ha^{-1} cow manure in the first year was the only treatment that increased the chlorophyll $a + b$ as compared to no cow manure application. It did not result in significant differences in the second year (*Table 3*).

Tiller number, 1000–grain weight, grain number

The interaction effect of vermicompost × cow manure had significant impact on the number of tillers per hill, the 1000–grain weight, and the number of grains per panicle in both years. The application of cow manure and vermicompost, both as a mixture or individually, increased the number of tillers per hill and the number of grains per panicle as compared to the control, but vermicompost had a stronger influence on increasing these two traits so that the application of vermicompost in addition to cow manure further enhanced the number of tillers and the number of grains per panicle than the control. The highest number of tillers and the highest number of grains per panicle were observed in M_2V_2, so that the tiller number was 22.36% higher in the first year and 27.32% higher in the second year than the control, and the number of grains per panicle in the first and second years was 81.22 and 61.52% higher than the control, respectively. However, the application of cow manure and vermicompost reduced the 1000–grain weight as compared to the control (*Table 3*).

Harvest index, grain yield and biological yield

The interaction effect of vermicompost × cow manure × year had significant impact on the harvest index, the grain yield, and the biological yield. According to means comparison, 10 and 20 t ha^{-1} cow manure improved the biological yield by 5.3 and 8.1% in the first year and by 4.3 and 18.1% in the second year as compared to the control, respectively. Also, the application of 5 and 10 t ha^{-1} vermicompost increased it by 9.0 and 17.9% in the first year and 12.3 and 24.8% in the second year, respectively. Likewise, the grain yield showed an 11.26 and 52.66% increase in the first year and a 36.1 and 61.2% increase in the second year when 10 and 20 t ha^{-1} cow manure was incorporated, respectively. The application of vermicompost at the rates of 5 and 10 t ha^{-1} increased grain yield by 41.4 and 69.6% in the first year and by 49.2 and 93.0% in the second year, respectively. The highest grain yield (3537 kg ha^{-1} in the first year and

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3958 kg ha^{-1} in the second year) was obtained from M_2V_2. The highest harvest index was observed in M_1V_1 in the first year and in M_1V_2 in the second year (*Table 4*).

Table 3. *Mean comparison of cow manure and its vermicompost interaction effect on tiller number, 1000 – grain weight, number of filled grain, grain yield, biological yield and harvest index in 2015 and 2016.*

M × V	chlorophyll a+b	Tiller number in hill	1000 – grain Weight (g)	Number of filled grain	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Harvest Index (%)
				2015			
V0M0	2.35 e	56.66 c	26.26 a	64.64 e	1754 d	5283 e	35.0 c
V0M1	3.67 d	59.73 bc	23.56 e	74.96 de	1958 d	5566 e	35.1 c
V0M2	4.46 c	61.63 bc	25.43 c	84.56 cde	2616 bc	5716 de	45.7 a
V1M0	4.79 bc	60.33 bc	24.43 d	94.72 cd	2400 cd	5862 de	41.6 b
V1M1	3.36 d	63.33 abc	23.23 e	110.6 ab	2825 abc	5737 de	48.4 a
V1M2	4.55 bc	63.65 abc	25.63 bc	91.3 cd	2895 abc	6858 bc	42.2 b
V2M0	4.39 c	62.33 abc	25.73 abc	93.3 cd	2666 bc	6229 cd	42.8 b
V2M1	5.23 b	64.33 ab	26.16 ab	98.0 b	3312 ab	7075 ab	46.8 a
V2M2	6.57 a	69.33 a	23.63 e	122.9 a	3537 a	7670 a	46.1 a
LSD (0.05)	0.72	7.19	0.589	20.8	851	637	2.88
				2016			
V0M0	5.61 cd	64.66 e	25.06 a	87.12 e	2208 e	7275 c	30.8 d
V0M1	7.12 bc	70.33 cde	25.46 a	97.6 cd	2879 d	7166 c	38.5 a
V0M2	8.43 ab	72.33 cd	25.50 a	111.4 bcd	3012 cd	8470 b	35.5 bc
V1M0	4.59 d	67.33 de	23.36 bc	107.8 bcd	2937 cd	8250 bc	36.4 b
V1M1	5.65 cd	74.66 bc	23.40 bc	109.7 bcd	3150 bcd	8641 b	36.4 b
V1M2	6.70 c	75.66 bc	22.53 c	105.8 bcd	3516 abc	9016 b	39.0 a
V2M0	8.52 a	73.00 cd	25.63 a	112.9 bc	3429 abcd	8945 b	38.3 a
V2M1	8.87 a	80.66 ab	23.43 b	128.6 ab	3708 ab	10895 a	34.0 c
V2M2	9.66 a	82.33 a	23.53 b	140.7 a	3958 a	10979 a	36.0 b
LSD (0.05)	1.52	6.543	0.872	24.5	585	1121	1.33
				F value			
Y×M×V	2.76*	5.61**	24.12**	7.70**	6.48**	8.92**	6.12**
CV (%)	11.8	5.85	1.75	12.84	14.4	6.99	16.21

Different letters in the same column indicate significant differences by LSD test ($p < 0.05$). (V_0, V_1 and V_2: 0, 5 and 10 t ha^{-1} vermicompost, respectively; M_0, M_1 and M_2: 0, 15 and 30 t ha^{-1} cow manure, respectively).

*, ** Significant at $p < 0.05$ and $p < 0.01$, respectively. Y: Year, V: Vermicompost, M: Cow manure and CV: Coefficient of Variation.

NPK concentration and uptake by grains

The concentration and uptake of the elements were changed significantly under the interaction effect of vermicompost × cow manure × year. When 10 t ha^{-1} vermicompost and 20 t ha^{-1} cow manure were applied individually, N, P and K concentrations of the rice grains showed improvements in both years, but the maximum concentrations were obtained when 20 t ha^{-1} cow manure + 10 t ha^{-1} vermicompost were applied so that this treatment increased N concentrations from 1.34% to 2.02% in the first year and from 1.35% to 2.35% in the second year, K concentration from 0.28% to 0.78% in the first year and from 0.266% to 0.750% in the second year, and P concentration from 0.190% to 0.375% in the first year and from 0.186% to 0.355% in the second year. The uptake rate of N, P, and K by grains was also increased with cow manure and vermicompost rates (although some treatments did not exhibit significant differences with the control) so that

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plants fertilized with 10 t ha^{-1} vermicompost + 20 t ha^{-1} cow manure resulted in higher uptake rate of the nutrients as compared to other organic fertilizer treatments (*Table 4*).

***Table 4.** Mean comparison of cow manure and its vermicompost interaction effect on N, P and K concentration and their uptake by rice grain.*

M × V	K concentration (%)	P concentration (%)	N concentration (%)	K uptake (kg ha^{-1})	P uptake (kg ha^{-1})	N uptake (kg ha^{-1})
2015						
V0M0	0.280 c	0.190 e	1.680 ab	24.58 a	14.01 a	55.65 ab
V0M1	0.420 bc	0.300 bc	1.340 b	7.72 c	8.27 bc	23.56 g
V0M2	0.500 bc	0.300 bc	1.676 ab	19.47 ab	12.66 a	52.08 bc
V1M0	0.490 bc	0.232 de	1.676 ab	28.21 a	13.56 a	43.76 d
V1M1	0.400 bc	0.232 de	2.023 a	10.61 bc	6.17 c	34.99 ef
V1M2	0.500 bc	0.325 ab	1.700 ab	18.33 abc	11.00 ab	45.33 cd
V2M0	0.570 ab	0.262 cd	1.340 b	19.38 ab	8.92 bc	32.16 f
V2M1	0.570 ab	0.325 ab	1.343 b	18.74 ab	8.89 bc	41.86 de
V2M2	0.780 a	0.375 a	1.700 ab	19.65 ab	8.31 bc	60.13 a
LSD (0.05)	0.230	0.057	0.577	10.67	3.39	7.37
2016						
V0M0	0.350 d	0.186 d	2.350 a	9.62 d	5.77 d	51.89 bc
V0M1	0.600 b	0.250 c	1.680 d	23.27 bc	12.60 b	48.37 cd
V0M2	0.600 b	0.187 d	1.690 c	26.57 b	8.30 cd	57.95 b
V1M0	0.266 e	0.262 c	1.400 e	7.08 d	11.22 b	41.12 d
V1M1	0.470 c	0.300 abc	1.350 f	18.85 c	12.03 b	40.66 d
V1M2	0.750 a	0.285 bc	1.350 f	24.01 bc	17.60 a	53.43 bc
V2M0	0.580 bc	0.262 c	1.350 f	26.19 b	11.85 b	47.47 cd
V2M1	0.580 bc	0.325 ab	2.010 b	27.30 b	12.35 b	74.53 a
V2M2	0.575 bc	0.355 a	1.690 c	37.18 a	10.30 bc	53.23 bc
LSD (0.05)	0.0854	0.0577	0.0026	5.9952	2.5893	7.9604
F value						
Y × M × V	6.18**	4.90**	3.29*	8.19**	7.05**	11.38**
CV (%)	20.7	12.14	14.45	24.54	16.19	9.29

Different letters in the same column indicate significant differences by LSD test ($p < 0.05$). (V_0, V_1 and V_2: 0, 5 and 10 t ha^{-1} vermicompost, respectively; M_0, M_1 and M_2: 0, 15 and 30 t ha^{-1} cow manure, respectively).

*,** Significant at $p < 0.05$ and $p < 0.01$, respectively. Y: Year, V: Vermicompost, M: Cow manure and CV: Coefficient of Variation.

Starch and protein content

The results of the analysis of variance showed that the interaction effect of vermicompost × cow manure × year on starch and protein content was significant (*Table 5*). According to the results, the increase in cow manure and vermicompost rate reduced the grain starch content. The lowest starch contents were obtained from M_2V_2 and M_1V_2. The application of vermicompost also resulted in a significant loss of grain starch so that M_2V_2 reduced it by 3.3% in the first year and by 7.4% in the second year versus M_0V_0. Grain protein content was also influenced by cow manure and vermicompost so that they improved grain protein content. When cow manure was applied individually, it did not change protein content significantly, but its incorporation with vermicompost – especially with 10 t ha^{-1} vermicompost – was associated with higher protein content of the grains. This effect of vermicompost was stronger in the second year (*Table 5*).

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Table 5. *Mean comparison of cow manure and its vermicompost interaction effect on on starch content, protein content, milling percentage of rice grain.*

M × V	2015		
	Starch content (%)	Protein content (%)	Milling percent (%)
V0M0	26.97 c	6.834 b	69.6 d
V0M1	27.06 bc	6.851 b	71.3 cd
V0M2	27.67 abc	6.851 b	75.7 abc
V1M0	27.12 abc	6.834 b	71.7 bcd
V1M1	27.74 ab	6.852 b	76.2 abc
V1M2	27.70 ab	6.859 b	77.3 ab
V2M0	27.56 abc	8.201 a	77.4 ab
V2M1	27.81 a	8.985 a	77.4 ab
V2M2	27.83 a	8.670 a	78.1 a
LSD (0.05)	0.717	0.969	5.78
	2016		
V0M0	26.29 e	6.88 b	65.40 e
V0M1	26.79 cde	7.21 b	69.99 cd
V0M2	26.62 de	7.33 b	70.45 bcd
V1M0	27.06 cd	7.14 b	67.22 de
V1M1	27.30 bc	8.25 a	72.14 abc
V1M2	27.77 ab	8.61 a	71.60 abc
V2M0	27.20 c	8.65 a	74.58 ab
V2M1	28.16 a	8.56 a	75.81 a
V2M2	28.26 a	8.61 a	75.92 a
LSD (0.05)	0.533	0.671	4.38
		F value	
Y × M × V	5.84**	3.04*	3.24*
CV (%)	1.33	6.05	4.69

Different letters in the same column indicate significant differences by LSD test ($p < 0.05$). (V_0, V_1 and V_2: 0, 5 and 10 t ha^{-1} vermicompost, respectively; M_0, M_1 and M_2: 0, 15 and 30 t ha^{-1} cow manure, respectively).

*, ** Significant at $p < 0.05$ and $p < 0.01$, respectively. Y: Year, V: Vermicompost, M: Cow manure and CV: Coefficient of Variation.

Milling percent

Milling percent was influenced by cow manure × vermicompost × year (*Table 5*) so that the application of cow manure and vermicompost increased milling percent in both years. In both years, maximum milling percent was obtained from M_2V_2 and it was 12.21% higher than the control in the first year and 16.8% higher in the second year (*Table 5*).

Discussion

The reason for difference of mean of treatments between two years was because there was rain in the first year at the time of plant pollination, also at the time duration of seed filling, temperature in the first year was more than that of the second year. Meanwhile, there was cooler temperature in the second year than the first year at the time of plant tiller formation. Thus, there was a difference between the plant reaction to treatments during the first and second years, so the mean of the most characters in first year was less than second year.

Soil fertility plays a prominent role in the improvement of nutrient uptake by rice, whilst soil organic matter is responsible for soil fertility (Zhou et al., 2016). In addition

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to increasing the organic matter, our aim was to improve soil physical and chemical properties.

EC (Electrical conductivity) of vermicompost was higher than farm soil, which may be due to the presence of more salts in the feed of cow. The micronutrients content was significantly higher in vermicomposts than soil but was within permissible limits as recommended by Iran limits of micronutrients in the compost (Sudkolai and Nourbakhsh, 2017). The C:N ratios of the vermicompost was 11.3 that it was in range of a stabilized product for all types of organic manures. It is reported that if C:N ratio is >20 plants cannot assimilate mineral N (Edwards and Bohlen, 1996) and may affect the growth and yield of rice in field condition.

We observed that applying vermicompost and cow manure can improve the qualitative traits of grains in addition to enhancing NPK uptake and grain yield. Numerous researchers have reported improvement in the growth of the plants in substrates that contain more organic matter. According to Gupta et al. (2014), one consequence of applying organic fertilizer is the reduction of soil C:N. On the other hand, higher soil organic matter increases the activity of soil microorganisms and improves the availability of nutrients to plants (Srivastava et al., 2010).

In both years at various levels of vermicompost (except 10 t ha^{-1} in the second year), higher cow manure rate was related to higher total chlorophyll content of leaves. The impact of cow manure and vermicompost on increasing leaf chlorophyll content has been reported for marigold (Gupta et al., 2014) and pak choi (Pant et al., 2009). Vermicompost stimulates plant growth possibly through supplying nutrients and increasing chlorophyll which, consequently, improves photosynthesis or through having such plant growth hormones as auxin and cytokinin (Ievinsh, 2011). Higher chlorophyll content enhances photosynthesis rate and carbohydrate production, which in turn increases 1000–grain weight, spike number per m^2, grain number per spike, leaf number per plant, and spike length (Xu et al., 2008; Tejada and González, 2009). Basha et al. (2017) demonstrated that the application of organic matter increased the number of fertile tillers and the number of grains per rice plant. They related higher number of tillers and grains to the influence of vermicompost and green manure on the improvement of PNK uptake. On the other hand, Kumar et al. (2016) related the effect of organic matter on increasing tiller number in rice to the improvement of soil physical features. We also observed the highest number of tillers and grains in plants treated with 5 t ha^{-1} vermicompost + 20 t ha^{-1} cow manure (M_2V_2). Cow manure had a negligible impact on the number of tillers and grains per plant when it was applied in the absence of vermicompost, but when it was mixed with vermicompost, the number of tillers was increased by 16–27% as compared to control in both trial years.

The main grain yield components include the number of tillers, the number of grains per panicle, and grain weight (Zhao and Fitzgerald, 2013). Therefore, any parameter that can improve this component can increase grain yield. Although 1000–grain weight was lower in plants fertilized with cow manure and vermicompost, it was offset by the increase in tiller number and grain number per plant. Lower 1000–grain weight in plants treated with cow manure and vermicompost than control may be connected with the inter–grain competition for the uptake of photosynthesis at grain filling phase because when the number of tillers and the number of grains per plant are increased, grains compete more during grain filling period resulting in the loss of their weight (Rusdiansyah and Saleh, 2017). The same fact may be the reason for lower grain starch in plants treated with organic fertilizer as compared to control. Hao et al.

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(2007) reported that grain starch showed a reverse relationship with grain protein and higher N uptake resulted in the loss of starch reserve. We also observed that the treatment with organic fertilizer increased grain protein content. The impact of vermicompost and cow manure on the improvement of grain protein has been reported by Bejbaruah et al. (2013) and Tejada and González (2009), too. Thus, when plants are not faced with N uptake constraint, more N is absorbed and mobilized to grains, resulting in high grain protein content (Bejbaruah et al., 2013). The incorporation of organic matter with soil, especially vermicompost, stimulates the activity of beneficial soil microorganisms and ensures continuous and sustainable supply of mineral nutrients, especially N, to plants. Then, the nutrient availability to roots, soil physical features, and its vital processes are enhanced and the roots are provided with an optimum substrate. Consequently, chlorophyll content is increased and the growth of vegetative parts is induced (Ievinsh, 2011).

Vermicompost contains the nutrients like P, K, Ca, and Mg in a readily available and absorbable form to plants (Gupta et al., 2014; Amanullah, 2016). We observed that P and K content and uptake were higher in grains of plants treated with cow manure and vermicompost, so that the highest P and K content and uptake were obtained from M_2V_2. As well, some researchers have reported that organic matter enhances available P and indirectly hinders the precipitation of phosphate, which is unavailable to plants, in the pH range of 6–9 (Mkhabela and Warman, 2005).

Antil and Singh (2007) stated higher grain P and K content of rice under organic fertilization. In addition, vermicompost contains a considerable amount of micronutrients (Amanullah, 2016), humic acid (Maji et al., 2017) and growth stimulators like auxins, gibberellins, and cytokinins and phosphate, enzyme, and vitamin dissolving bacteria (Tejada and González, 2009; Liu et al., 2017). These compounds improve leaf area, tiller number and finally, biological yield (Joshi et al., 2015). Our results revealed that M_2V_2 which received the highest amount of cow manure and vermicompost exhibited the maximum biological yield and grain yield. These findings are consistent with Hasanuzzaman et al. (2010) and Bejbaruah et al. (2013). It should be noted that the effect of vermicompost on the improvement of rice grain yield and quality was stronger in the presence of cow manure, i.e. when only 10 t ha^{-1} vermicompost was applied (V_2M_0), grain yield was increased at 51% in the first year and at 55% in the second year versus control and when vermicompost + 20 t ha^{-1} cow manure (V_2M_2) was applied, grain yield was increased at 32% in the first year and at 15% in the second year versus V_2M_0. The influence of cow manure on the improvement of nutrient uptake and rice grain yield has been reported by Hasanuzzaman et al. (2010) and Sudarsono et al. (2014).Organic fertilizers increased nutrient uptake and grain protein, resulting in higher milling percentage and grain hardiness so that the highest milling percentage in both years was associated with M_2V_2.

Rice grain milling percentage is one of the major qualitative traits of grain and its loss results in considerable loss of cooking quality and crop price (Ravi et al., 2011; Kumar et al., 2016). Dixit and Gupta (2000) and Yuan et al. (2014) reported milling percentage gain as one effect of organic fertilizers on the quality improvement of rice grains. Davari and Sharma (2010) reported that the integrated application of farmyard manure + vermicompost + plant residue led to the improvement of yield, net profit, and grain quality of basmati rice.

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Conclusion

The results showed that the mixture of vermicompost and cow manure improved the physical and chemical properties as compared to only cow manure. Applying a mixture of cow manure and vermicompost increased the leaf chlorophyll and NPK uptake by rice. Although the organic fertilizers that were used in this study reduced grain starch content, they resulted in grain yield gain and improved the qualitative traits of grains like the milling percent and protein content. In spite of the fact that cow manure, when applied with vermicompost, enhanced nutrient uptake and grain yield, vermicompost was much more efficient than cow manure. In addition, plots that were fertilized with cow manure had more weeds than those fertilized with vermicompost. Since the soil of most paddy farms are suffering from organic matter deficiency, the application of animal residue as vermicompost can improve their organic matter content and can increase the grain quality and yield. On the other hand, the vermicomposting of animal residue curbs environmental problems and cuts down the cost of weed management of the farm. Although vermicompost is a farm input with high cost for farmers, its cost is offset by grain yield gain and it maintains soil organic content at high levels for several years. Though vermicompost has a high potential to sustain rice production, further research is required to find out the long–term effects of the application of vermicomposts derived from plant and animal residue.

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AN ETHNOBOTANICAL SURVEY OF MEDICINAL PLANTS USED FOR THE TREATMENT OF SNAKEBITE AND SCORPION STING AMONG THE PEOPLE OF NAMAL VALLEY, MIANWALI DISTRICT, PUNJAB, PAKISTAN

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Abstract. Snakebite and scorpion sting are the most neglected public problems especially in the poor rural communities of South Asia including Pakistan. The frequency of snakebites and scorpion stings in Pakistan has increased tremendously due to destruction of habitats of snakes and scorpions as a result of deforestation and the consequent migration of these poisonous animals towards human settlements. The management of the frequency of snake bites and scorpion stings has been unsatisfactory in this region. In order to investigate and collect information from the inhabitants on the curative values of plants for treatment of snakebite and scorpion sting, an ethnobotanical survey of medicinal plants was carried out from September 2015 to July 2016 to document information from 14 villages of Namal Valley of District Mianwali, Punjab, Pakistan. The Martin method was followed for collecting information from informants about the medicinal plants used for these poisonous bites. A total of 68 plant species belonging to 37 families and 61 genera was recorded. Information obtained included the common names and their families while photographs of all plants recorded were obtained using the Olympus digital camera. The plants documented were herbs (54%), shrubs (26%) and trees (20%). The family Solanaceae was found to be the most frequently used. The dominant plant part in the recipes obtained is the leaf (22%). The most frequently used mode of preparation in the recipes was the paste. *Calotropis procera, Arisaema jacquemontii, Albizia lebbeck* and *Portulaca oleracea* were the major medicinal plants recorded. The present study is the first ethnobotanical survey of the area and the first report of the medicinal plants used in the treatment of poisonous snake bites and scorpion stings among the indigenous communities of Namal Valley of District Mianwali, Punjab, Pakistan.
Keywords: *ethnobotany, poisonous bites, Solanaceae, Arisaema jacquemontii, antidotes*

Introduction

Medicinal plants play a pivotal role in the treatment of various health problems due to their therapeutic value. Since ancient times the use medicinal plants has been preferred due to their safety, effectiveness, cultural preferences, inexpensiveness, abundance and availability. Even at present, many indigenous communities depend on herbal medicines for the treatment of various diseases including snake and scorpion bites (Singh et al., 2012; Naidu et al., 2013). Thus, studies on herbal antidotes are important in the management of poisonous bites from snakes and scorpions (Mukherjee and Wahile, 2006).

An accurate measure of the global burden of snakebite envenoming remains elusive despite several attempts to estimate it and, apart from a few countries, reliable figures on the incidence, morbidity, and mortality are limited (Chippaux, 1998; Kasturiratne et al., 2008). According to World Health Organization (WHO) the estimated number of cases of snakebites around the world is about 5 million per year and the cases are often fatal resulting to death of 2 million people while 0.4 million victims are disabled for whole life (WHO, 2010; Gutiérrez et al., 2013). On the other hand, approximately two billion people are at risk from scorpion stings, with over one million accidents occurring annually worldwide (Chippaux and Goyffon, 2008).

South Asia is by far the most affected region in this context (Chippaux 1998; Kasturiratne et al., 2008). According to World Health Organization's direct estimates, India has the highest number of deaths from snakebites in the world with 35,000–50,000 people dying per year (Chippaux, 1998; Kasturiratne et al., 2008). Moreover, envenoming by snakebites and scorpion stings impose a high burden on humans worldwide and result in considerable social and economic impact. The countries most affected by snakebites are located in the inter-tropical zone; areas with high rates of field use for agriculture involving adult males who are the most affected (WHO, 2007). In Pakistan, expiries from snake bites and scorpion stings have been reported to increase every year with about 40,000 reported cases annually resulting in up to 8,200 fatalities (Kasturiratne et al., 2008; Ali, 1990). The frequency of scorpion stings and snakebites in Pakistan has been on the increase due to destruction of the habitats of these animals and their consequent migration towards human populations (Nasim et al., 2013). Snakebite cases are reported from most populated parts of the Indus Valley [Punjab] and the Indus Delta [Sindh] where about 95% of country's agricultural activity takes place. It is estimated that snakebite reports from Punjab150, Sindh 500, Khyber Pakhtunkhwa and Balochistan are less than 50 per year (Khan, 1990).

At present, the use of anti-venoms from plants has been reported and documented among many indigenous communities in various regions of the world. In spite of the success of this therapy, there is need to search for more plant based venom inhibitors. Poisonous bites from snakes and scorpions constitute problems for medical researchers because they are neither infectious nor preventable by vaccination thus they are neglected conditions with no associated WHO programmes for control and prevention. In Pakistan, lack of proper facilities in rural areas, treatment by quacks, travelling time to hospital for definitive treatment add considerably to morbidity and mortality of cases involving snakebites in particular. A rich and diverse flora of Pakistan provides valuable storehouse of plants used by the tribal communities in remote areas for medicinal purposes such as cure for snakebite and scorpion sting. Previous reports recorded sixty two (62) medicinal plants for the management of snakebite and scorpion sting in northern Pakistan (Butt et al., 2015).

The main objective of our study was to document how the inhabitants - Awan tribes of Namal Valley, Pakistan, who live as pastoralists, peasants and agriculturists, have employed the plant resources of their area as antidotes for scorpion and snake toxin as with many other health challenges.

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Materials and methods

Study area

Namal Valley is one of the most important valleys in Pakistan and a priceless place from an ethnomedicinal point of view. Namal Valley is located at 71°48°45° E longitude and 32°40°10° N latitude, on the eastern border of Mianwali District, Punjab, Pakistan and is nestled amidst the mountains of Salt Range along the border area of Districts Mianwali, Chakwal and Khushab. It has a distinct topography with many lakes, of which Namal Lake is the most visible and largest with a surface area of approximately 5.5 km². The valley is speckled with a number of smaller and larger patches of forests and lakes that are stretched up to the Soon Valley (Sakesar) which is the highest point of the Salt Range. Main villages of the valley include Namal, Rikhi, Kalri, Nawan, Ban Hafiz Jee, Chakda, Dhok Ali Khan, Dhurnaka, DhibbaKarsial, Chaki Sheikh Jee, DhokeAyub, DhokStala, DhokMiani and DhokPeerha. The dominant tribal community is the Awan; others include the Malik (with many sub castes), Pathan and Mian (*Figure 1*).

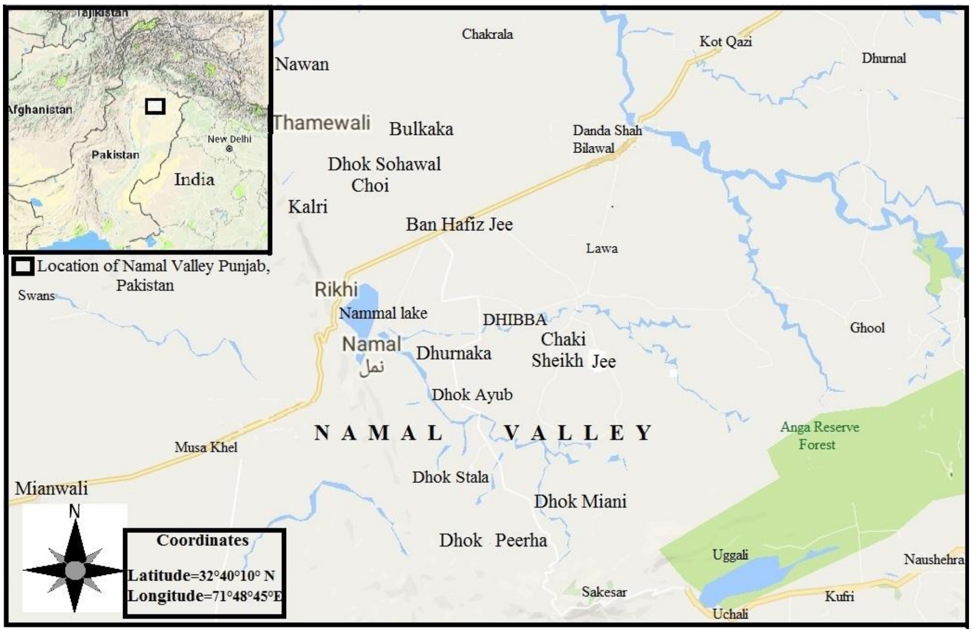

Figure 1. *Map of the study area*

Namal Valley represents one of the ancient civilizations of Pakistan with centuries-old vaults on hills. The dominating economic activity is farming and livestock; people mainly depend on farming for livelihood. Sulphur water spring, shrine of Baba Hafiz Jee, Baba Khaki Shah, Namal College, Namal Lake and Namal Dam are the most fascinating places of the valley. The climate of Namal Valley is extreme; with a minimum temperature of 1°C in winter and a maximum of 45°C in summer and an average annual rainfall of about 250 mm. A unique topography and climatic conditions impart the valley with distinctive characteristics that support biodiversity harboring a wide variety of medicinal plants. The valley is blessed with *Prosopis glandulosa*, *Dodonaea viscosa* and *Tamarix dioica* as the dominant plants in low altitudes while *Olea ferruginea* mixed with *Acacia modesta* represent the evergreen tree population of

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the forests at high altitudes. Other valuable species found in the valley are *Pupalia lappacea*, *Viola cinerea*, *Capparis cartilaginea*, *Capparis spinosa*, *Pluchea arabica*, *Grewia tenax*, *G. villosa*, *Typha angustifolia*, *Phoenix sylvestris*, *Tamarix aphylla, Prosopis juliflora, Capparis decidua, Tephrosia purpurea, Peganum harmala* and *Salvadora oleoides*. These plant species are commonly used as a source of food, fodder, forage, fuel and medicinal purposes by the local communities since ages. This study is the first report of the documentation of plants for the management of snakebite and scorpion sting in the Awan tribes of Namal Valley of Mianwali District, Pakistan.

Methods

Ethnobotanical investigation was carried out from September 2015 to July 2016 in 14 different villages of Namal Valley to document plants utilized for the treatment of snakebite and scorpion sting by the aboriginal people. Ethnomedicinal data was collected following Martin methodology. Informal meetings were held in the 14 different villages of the valley. A semi structured questionnaire was prepared to document the collection, dispensation and usage of the local plants. Information of the ethnic use, common name, part utilized, growing season of the plants for the collection of specimen, mode of administration and locally used recipes were obtained and documented (Martin, 1995). Photographs of all plants recorded were obtained using the Olympus digital camera. All plant specimens collected were pressed, dried and mounted on herbarium sheets. The dried specimens were labeled and allotted voucher numbers. Scientific names of collected plant specimens were confirmed with help of “Flora of Pakistan” (Nasir and Ali, 1970-2003). Preserved voucher specimens were deposited in the Herbarium of the University of Sargodha for future reference.

A total of 130 traditional healers (40 women, 80 men and 10 men) of the area were interviewed. The informants were divided into three different age groups, i.e. 20–40, 41–60, 61–80 years old (*Figure 2*).

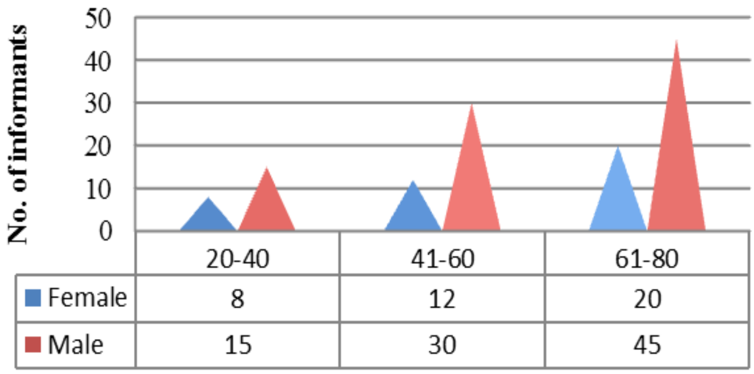

Figure 2. *Gender data of participating informants devided into three age (years) groups*

Results

68 plant species belonging to 61 genera and 37 families were recorded as antidotes used in the Namal Valley. The families, common names, and uses with pictures and reported phytochemical compounds are presented in *Table 1*.

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Table 1. *Plants used for the treatment of snakebite and scorpion sting in Namal Valley, Mianwali, Punjab, Pakistan*

Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
1	*Achyranthes aspera* L./SAN-V-18	Puth Kanda	Amaranthaceae	Seeds and leaves	Alkaloids, steroids, triterpens and saponins possessing oleanolic acid (Barua et al., 2010; Sumeet et al., 2008)	Paste of seeds and leaves is dried in the form of capsule and given to the victim for snakebite. Leaf juice is also effective	
2	*Albizia lebbeck* (L.) Benth./SAN-V-46	Kala Shreen	Fabaceae	Whole plant	Steroids, terpenoids, coumarins,tannins, flavanoids, anthraquinones, and saponins (Babu et al., 2009)	Roots, bark, leaves and fruit all parts are very effective in snakebite	
3	*Albizia procera* L./SAN-V-41	Chhita Sirin	Fabaceae	Whole plant	Acylated Triterpenoid Saponins, proceraosides A–D, (Yoshikawa et al., 1998)	Paste of any part of plant is very effective in snakebite	
4	*Allium cepa* L./SAN-V-16	Pyaaz	Amaryllidaceae	Stem	Albuminoids, Volatile Oil, Quercetin , Sulfur, Ether Organic Sulfur, Moisture,Carbohydrates, Essential Oil, Ash and Sugar (Odhav et al., 2007)	The juice of onion is applied on the effected part of skin for the neutralization of the snake venom as well as scorpion venom	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
5	*Allium sativum* L./SAN-V-10	Lehsan	Amaryllidaceae	Roots	Saponins, steroids, tannins, carbohydrates, cardiac glycosides (Mikail, 2010)	Cut the garlic into two pieces and then tie them on the wounded site. Mix the paste in vinegar and apply it on snake or scorpion bitten site	
6	*Aloe vera* (L.) Burm.f./SAN-V-48	KnwarPharra	Xanthorrhoeaceae	Latex	Flavonoids, terpenoids, tannins, Saponin (Arunkumar and Muthuselvam, 2009)	The latex is applied on the wounded site and also prescribed to the patient orally	
7	*Amaranthus viridis* L./SAN-V-02	Chulai	Amaranthaceae	Whole plant	Flavonoids, saponins, glycosides, terpenoids, amino acids, alkaloids, carbohydrates, phenolic compounds and proteins (Kumar et al., 2012)	Decoction of whole plant is used for scorpion bite. Paste is applied externally to the part of skin bitten by snake	
8	*Anagallis arvensis* L./SAN-V-54	NeeliBooti	Asteraceae	Whole plant	Saponins, flavonoids (Napoli et al., 1992; Kawashty et al., 1998)	Decoction of whole plant is used for snakebite	
9	*Arisaema jacquemontii* Blume/SAN-V-14	Zahr Mora	Araceae	Stem tuber	Terpenoids, Coumarins, Quinones, Glycosides, Alkaloids and Anthraquinones (Sudan et al., 2014)	Paste is used for scorpion sting and snakebite	

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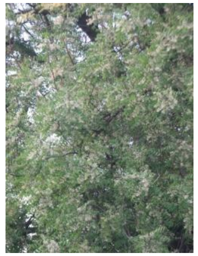
Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
10	*Azadirachta indica* A. Juss/SAN-V-18	Neem	Meliaceae	Leaves	Alkaloids, steroids, flavonoids, glycosides, terpenoids, carbohydrates, antiquonons (Raphael, 2012)	The paste of the leaves is tied on the snake bitten site or scorpion sting site with the help of a bandage or leaf extract is given orally to the victim	
11	*Bauhinia variegata* L./SAN-V-13	Kachnaar	Fabaceae	Root	Tannins, alkaloids and saponins (Parekh et al., 2006)	The decoction of the roots is given orally to the victim of snakebite	
12	*Bombax ceiba* L./SAN -V-67	Simbal	Malvaceae	Leaves	Carbohydrates, glycosides, flavones, flavanones, tanins, phenolic compounds, proteins, saponins, sterols, triterpenoids (Anandarajagopal et al., 2013)	Paste of leaves of this plant is tied on the snakebite and scorpion sting part of the body	
13	*Calotropis procera* R.Br./SAN-V-04	Akra	Apocynaceae	Whole plant	Reducing sugars, tannins, steroid glycosides, resins, saponins, flavonoids (Kawo et al., 2009)	The victim is asked to eat the leaves continuously in raw form until it taste turn bitter. This is helpful to neutralize the poison	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
14	*Cannabis sativa* L./SAN-V-36	Bhang	Cannabinaceae	Leaves	Cannabinoids ((Ahmed et al., 2008; ElSohly and Slade, 2005; Radwan et al., 2008)	Paste of the plant is applied to reduce the effect of poisonous bites	
15	*Capsicum annuum* L./SAN-V-24	Laal Mirch	Solanaceae	Fruit	Vitamin C, polyphenols, particularly flavonoids, quercetin and luteolin (Guil-Guerrero et al., 2006; Topuz and Ozdemir, 2007; Chuah et al., 2008; Materska and Perucka, 2005)	Paste is used for snake and scorpion bite	
16	*Carica papaya* L./SAN-V-26	Papeeta	Caricaceae	Fruit	Cardenolides, saponins (Oloyede, 2005)	Sliced piece of fruit is rubbed on the skin for wasp and scorpion sting	
17	*Cassia fistula* L./SAN-V-51	Gardanali/Amaltas	Caesalpinaceae	Fruit	Rhein (Chewchinda et al., 2014)	The pulp of the fruit is applied on snake bitten place on skin. Paste of the fruit is applied on the bitten site and also taken orally	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
18	*Catharanthus roseus* (L.) G. Don/SAN-V-64	SadaBahaar	Apocynaceae	Flower	Triterpenoids, tannins and alkaloids (Nayak and Pereira, 2006)	The juice of the flower is applied on the sting area to neutralize the wasp sting poison	
19	*Chenopodium album* L./SAN-V-03	Bathu	Chenopodiaceae	Leaves	Quercetin, Isorhmnetin (Jain et al., 1990)	The paste of leaves is applied on the wasp sting part to reduce swelling and neutralize the poison	
20	*Citrullus colocynthis* (L.) Schrad./SAN-V-35	Tuma	Cucurbitaceae	Fruit	Tannins, saponins, proteins, reducing sugars, alkaloids, flavonoids, glycosides (Najafi et al., 2010)	Pulp of the fruit is applied directly on the snake bitten part and also used to treat scorpion stings	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
21	*Citrus limon* (L.)Burm. f./SAN-V-59	Nibu	Rutaceae	Fruit	Taninns, glycosides, reducing sugars, flavonoids (Pandey et al., 2011)	*Citrus* neutralize the venom. Just cut a lemon into two halves and apply it on the wasp sting site firmly	
22	*Convolvulus arvensis* L./SAN-V-05	Vehri	Convolvulaceae	Whole plant	Steroids, tannins, flavonoids, coumarins, cardiac glycosides, saponins, phlobatannins (Khan et al., 2015)	Juice extracted from this plant used for the snakebite	
23	*Coriandrum sativum* L./SAN-V-32	Dhania	Apiaceae	Whole plant	Monoterpenes, aldehydes, alcohols, alkanes (Matasyoh et al., 2009)	Extracted juice is applied externally to reduce the effect of scorpion sting	
24	*Cucumis sativus* L./SA N-V-60	Kheera	Cucurbitaceae	Fruit	Glycosides, steroids, flvonoids, carbohydrates, terpenoids, and tannins (Kumar et al., 2010)	Sliced the fruit and place it on the wasp sting site	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
25	*Cucurbita pepo* L./SAN-V-56	Kadu	Cucurbitaceae	Whole plant	Saponins flavonoids alkaloids, steroids Saponins and tannins (Chonoko and Rufai, 2011)	Paste is applied on the scorpion sting site. Juice of this plant is also given orally	
26	*Cupressus sempervirens* L./SAN-V-31	Saru	Cupressaceae	Fruit	Flavonoids, saponons, tannins (Hassanzadeh Khayyat et al., 2005)	Paste of fruit is eaten for curing poisonous bites	
27	*Cynodon dactylon* (L.) Pers./SAN-V-52	Tala	Poaceae	Whole plant	Tannin, quinones and phenols (Kaleeswaran et al., 2010)	Plant is crushed with the black pepper and applied on the sting site to minimize the pain	
28	*Datura alba* Nees./SAN-V-08	Dhatura	Solanaceae	Roots	Flavonoids, phenols, tannins, saponins and sterols (Donatus and Ephraim, 2009)	The roots of this plant are crushed and mixed with garlic juice and applied externally on the snake bitten site	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
29	*Eclipta alba* L./SAN-V-57	Bhangra	Asteraceae	Whole plant	Alkaloids, coumestans, phenolics, saponins, steroids, proteins, amino acids, reducing sugars, flavonoids (Dalal et al., 2010)	Drinking extract of whole plant is used as antidote	
30	*Eruca sativa* Mill./SAN-V-65	Jhamayon	Brassicaceae	Flower	Allyl isothiocyanate, 3-butenyl isothiocyanate, 4-methylsulfinybutyl isothiocyanate, sulforaphane), 2-phenylethyl isothiocyanate and bis(isothiocyanatobutyl)disulphide, fatty acids (Khoobchandani et al., 2010)	Paste of flowers applied on the scorpion sting site	
31	*Euphorbia hirta* L./SAN-V-55	Dodhak	Euphorbiaceae	Root	Ducing sugars, terpenoids, alkaloids, steroids, tannins, flavanoids and phenolic compounds (Basma et al., 2011)	Paste of root is applied on the snake bitten area of skin	
32	*Ficus benghalensis* L./SAN-V-25	Bargad	Moraceae	Leaves	Carbohydrates, flavonoids, proteins, steroids, saponins, tannins, glycosides (Gabhe et al., 2006)	Paste of leaves is effective for scorpion and wasp sting	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
33	*Foeniculum vulgare* Mill./SAN-V-28	Sonf	Apiaceae	Seeds	Alkaloids, flavonoids, tannins, saponins (Kaur and Arora, 2009)	Decoction of seeds is given to the patient of snakebite and scorpion stings	
34	*Fumaria indica* (Hausskn.) Pugsley/SAN-V-37	Paprra	Fumariaceae	Whole plant	Alkaloids, flavonoids, glycosides, tannins, saponins, steroids and triterpenoids (Rao et al., 2007)	Juice of plant is used for snakebite	
35	*Gossypium hirsutum* L./SAN-V-19	Kpa	Malvaceae	Leaves	Alkaloids, saponins, flavonoids, tannins and cardiac glycosides (Ayeni et al., 2015)	Paste of leaves aling with milky juice of *Calotropisprocera*is applied on the bitten part of skin	
36	*Helianthus annuus* L./SAN-V-50	Suraj Mukhi	Asteraceae	Seeds	Tannins, saponins, flavonoids, carbohydrates, steroids, fixed oils and fats (Subashini and Rakshitha, 2012)	Oil extracted from seeds is applied on skin for snakebite and scorpion sting	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
37	*Jatropha curcas* L./SAN-V-63	Jamal Ghota	Euphorbiaceae	Seed	Alkaloids, saponin tannin, Terpenoid, Steroid, Glycosides, Phenolic Compound, Flavonoid (Sharma et al., 2012a)	The seed powder with water is given orally to the victim to drain the poison out of the body through urine	
38	*Justicia adhatoda* L./SAN-V-22	Bhaikar	Acanthaceae	Leaves	Quinazoline alkaloids (vasicoline, vasicolinone, vasicinone, vasicine, adhatodine and anisotine)- (Jha et al., 2012)	Paste of fresh leaves is applied on the skin for snakebite	
39	*Luffa acutangula* (L.) Roxb./SAN-V-49	Tori	Cucurbitaceae	Leaves	Steroids, tannins, flavonoids, anthroquinone (Anitha and Miruthula, 2014)	The juice of leaves is applied on the scorpion sting site	
40	*Mangifera indica* L./SAN-V-33	Amb/Aam	Anacardiaceae	Flowers	Tannins, Phenols, Pentagalloyl, Glucopyranose (Sahreen et al., 2011; Bhatt et al., 2012; Pithayanukul et al., 2009)	Crushed flowers are externally applied to reduce the effect of scorpion and wasp sting	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
41	*Mentha longifolia* (L.) Huds./SAN-V-42	Podina	Lamiaceae	Whole plant	Essential oils, flavonoids (Ghoulami et al., 2001)	Paste is applied on the body for wasp and scorpion sting. Used in snakebite treatment to avoid the sleepiness	
42	*Momordica charantia* L./SAN-V-53	Karaila	Cucurbitaceae	Fruit	Alkaloids, tannins, saponins, cardiac glycosides and steroids (Bakare et al., 2010)	The juice of fruit causes vomiting in this way removes poison or venom	
43	*Moringa oleifera* Lam./SAN-V-09	Suhanjran	Moringaceae	Roots	Flavonols, carotenoids, quercetin, kaempferol, b-carotene (Lako et al., 2007) tannins, anthraquinones (Kasolo et al., 2010)	Paste of the roots is used for snakebite or scorpion bite	
44	*Morus alba* L./SAN-V-34	Jangli Toot	Moraceae	Leaves	Saponins, phenolics, alkaloids, flavonoids, (Toyinbo et al., 2012)	Leaves are applied as a poultice to snakebite	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
45	*Musa paradisiaca* L./SAN-V-44	Kaila	Musaceae	Fruit	Alkaloids, flavonoids, tannins, phenols, saponins, phytates, oxalates (Adeolu and Enesi, 2013)	The peel of fruit is rubbed on the wasp sting site. Fresh juice is given to the victim of snakebite	
46	*Nerium oleander* L./SAN-V-11	Knair	Apocynaceae	Roots	Terpenoids, cardiac glycosides, alkaloids, saponins, tannins, carbohydrates (Bhuvaneshwari et al., 2007)	Paste of roots is used for snakebite and scorpion sting	
47	*Nicotiana tabacum* L./SAN-V-20	Tambaku	Solanaceae	Leaves	Alkaloids, phenols, flavonoids, phytosterols, triterpinoids, tannins and carbohydrates (Kaushik et al., 2010)	Dried leaves are tied on the wounded skin	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
48	*Ocimum basilicum* L./SAN-V-62	Niazbo	Lamiaceae	Whole plant	Saponins, tannins and glycosides (Daniel et al., 2011)	Decoction taken orally to minimize wasp sting poisonous. Juice of the leaves is also applied on the sting area	
49	*Olea ferruginea* Royle./SAN-V-61	Zaitoon	Oleaceae	Fruit	Quercetin,β-amyrin, oleuropein, and ligstroside (Hashmi et al., 2015)	Oil is extracted from fruit is rubbed on the wasp or scorpion sting part	
50	*Opuntia dillenii* (Ker Gawl.) Haw./SAN-V-43	Thor	Cactaceae	Roots	Phenols, Alkaloids, Flavonoids, Saponins, glycosides, Terpeonids Steroids, Tannins (Pooja and Vidyasagar, 2016)	Roots are grinded and given orally victim to vomit out the venom of snake	
51	*Portulaca oleracea* L./SAN-V-66	Loonak	Portulacaceae	Leaves	Fatty acids, organic acids, and phenolic compounds (Oliveira et al., 2009)	Juice of the leaves is applied on the stings of wasp and scorpion and paste of the plant is applied on the snake bitten area	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
52	*Punica granatum* L./SAN-V-39	Anaar	Punicaceae	Leaves	Triterpenoids, steroids, glycosides, saponins, alkaloids, flavonoids, tannins (Bhandary et al., 2012)	Paste of leaves is applied on wasp sting site	
53	*Raphanus sativus* L./SAN-V-21	Mooli	Brassicaceae	Root	Alkaloids, flavonoids, and saponins (Jahan and Rahmatullah, 2014)	Slices of root rubbed on wasp and scorpion sting site. Paste is applied on the wounded skin	
54	*Rhazya stricta* Dcne./SAN-V-06	Sava Winraan	Apocynaceae	Leaves	Phenolics (Iqbal et al., 2006)	Infusion of leaves is used to cure snakebite	
55	*Ricinus communis* L./SAN-V-29	Arind	Euphorbiaceae	Seeds root	Toxalbumin, Ricin, Alkaloid Ricinine, Beans yield Fixed Oil Flavonoids, Tannins (Khafagy et al., 1979; Kang et al., 1985; Nadkarni, 1976)	Seeds are used to cure scorpion bite also the root is taken orally for this purpose in raw form	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
56	*Rosa indica* L./SAN-V-40	Gulaab	Rosaceae	Flowers	Triterpenoids, steroids, saponins, alkaloids carbohydrates phenolic compounds, tannins (Bakshi et al., 2015)	Infusion is taken orally to avoid the sleepiness during snakebite	
57	*Saussurea heteromalla* L./SAN-V-47	KaaliZeeri	Asteraceae	Leaves	Arctiin, arctigenin and chlorojanerin (Saklani et al., 2011)	The leaves are boiled in water and then the water is used to wash the wasp, scorpion and snake bitten area	
58	*Silybum marianum* (L.) Gaertn./SAN-V-30	Dhmaan/ Kandiara	Asteraceae	Whole plant	Flavonoids, phenols and tannins (Shah et al., 2011)	Juice is extracted from the green plant is given orally to the snakebite victim	
59	*Solanum nigrum* L./SAN-V-38	Mako	Solanaceae	Whole plant	Saponin, phytosterols, tannins oils, fats, carbohydrates, coumarins, phytosterols, flavonoids (Ravi et al., 2009)	Infusion of plant is used to wash the snake bitten part of body	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
60	*Solanum surattense* Burm. f./SAN-V-23	Mahorri	Solanaceae	Fruit	Alkaloids, flavonoids, tannins, glycosides, triterpenoids and sterols (Muruhan et al., 2013)	The ripened fruit is used for the snakebite	
61	*Solanum tuberosum* L./SAN-V-17	Aalu	Solanaceae	Stem	Flavonoids, ferulic acid, pcoumaric acid, rutin, quercetin, myricetin, kaempferol, naringenin other (Nara et al., 2006; Reyes, 2005).	The sliced tuber of potato with salt is rubbed at the wasp sting site to reduce pain and swelling	
62	*Solanum americanum* Mill./SAN-V-68	Jangli niazbo	Solanaceae	Fruit	Solasonine and solamargine (Fukuhara and Kubo, 1991)	The ripened fruit is used for the snakebite	
63	*Trianthema portulacastrum* L./SAN-V-15	It Sit	Aizoaceae	Leaves	Carbohydrates, protein, volatile oils, glycosides, saponins, flavonoids, alkaloids (Verma, 2011)	Used as a paste on the bitten site to avoid the severely swollen body part. Fresh leaves of this plant are eaten in snakebite and scorpion sting cases	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
64	*Withania coagulans* (Stocks) Dunal/SAN-V-12	Khamjeera	Solanaceae	Leaves, fruits	Alkaloids, steroids, phenolic compounds, tannins, saponin, carbohydrates, proteins, amino acids and organic acids (Mathuret al., 2011)	Leaves and fruit are used in scorpion sting cases	
65	*Withania somnifera* (L.) Dunal/SAN-V-01	Asgandh	Solanaceae	Root	Glycoprotein (WSG) (Machiah et al., 2006)	Root of plant is crushed and extract is used for snakebite. Decoction of root is also used for snakebite	
66	*Zea mays* L./SAN-V-58	Makai	Poaceae	Seeds	Sesquiterpene hydrocarbons (Köllner et al., 2004)	Corn flour is mixed with water and applied as a paste on the wasp sting site	
67	*Zingiber officinale* Roscoe/SAN-V-27	Adrak	Zingiberaceae	Rhizome	Diarylheptanoids (Ma et al., 2004)	Sliced rhizome is applied on skin for wasp sting	

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Sr. No.	Scientific name/voucher number	Common name	Family	Part(s) used	Phytochemical (s)	Recipes (mode of utilization)	Picture of plant
68	*Ziziphus nummularia* (Burm. f.) Whight&Arn./SAN-V-45	Bair	Rhamnaceae	Roots	Flavonoids, tannins, sterols, saponins, pectin, glycosides and triterpenoic acid (Morel et al., 2009; Goyal et al., 2012)	The roots are taken orally to cure scorpion sting	

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The family Solanaceae is the most dominant (9 species) followed by the Cucurbitaceae (5 species), the Asteraceae, Fabaceae and Apocynaceae (4 species) Euphorbiaceae (3 species) and Amaranthaceae, Amaryllidaceae, Apiaceae, Brassicaceae, Malvaceae, Lamiaceae, Moraceae and Poaceae (2 species each) (*Table 2*). The plant species are mainly herbs (54%), shrubs (26%) and trees (20%) (*Figure 3*). The mostly used plant parts are leaves (22%), followed by whole plant (22%), Fruit and roots (17%), stem and seeds (8%) and flowers (7%) (*Figure 4*). The frequent mode of utilization is paste (51%) followed by juice (26%), powder (8%), raw form (7%), decoction (5%) and oil (3%) (*Figure 5*).

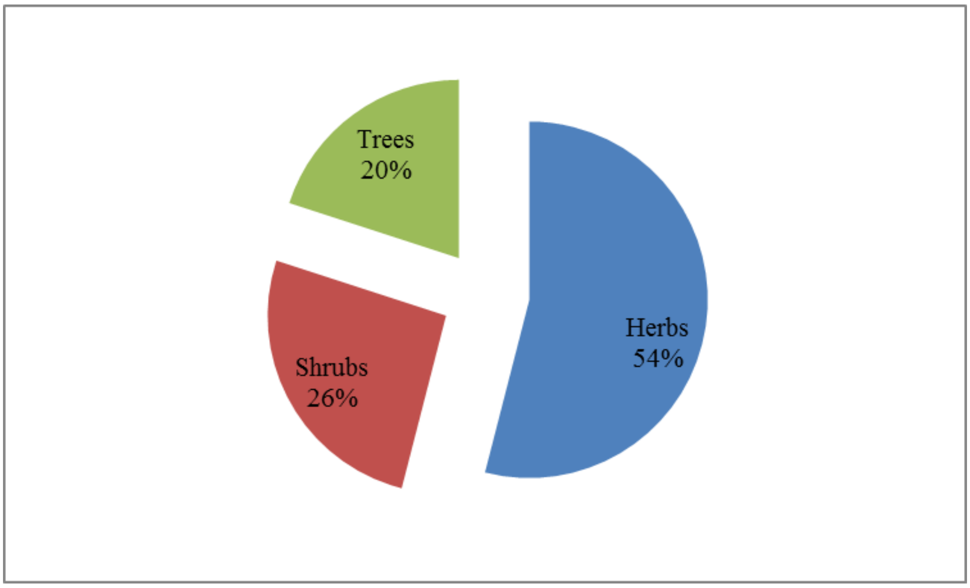

Figure 3. *Habit of medicinal plants used*

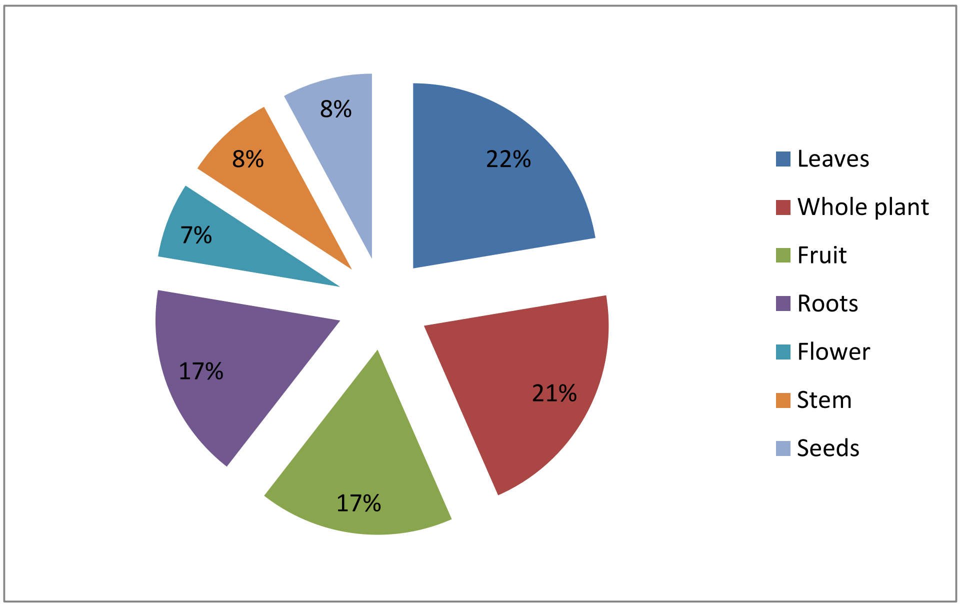

Figure 4. *Part used of medicinal plants*

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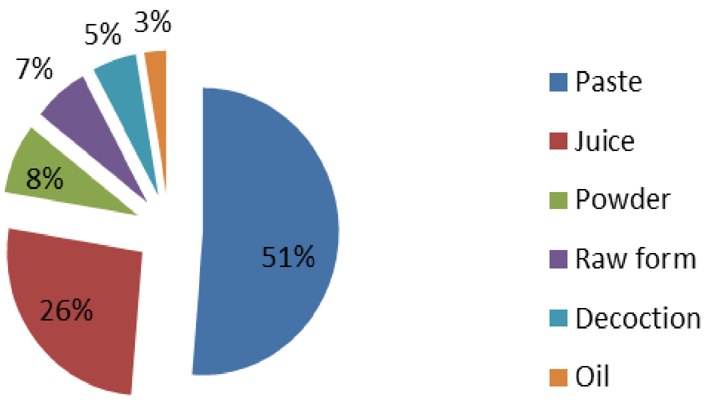

Figure 5. *Mode of utilization of reported medicinal plants*

Table 2. *List of plant families with species name and number of species in each family*

Sr. No.	Family	Scientific name of species	No. of species
1	Amaranthaceae	*Achyranthes aspera* L.	2
		Amaranthus viridis L	
2	Anacardiaceae	*Mangifera indica* L.	1
3	Acanthaceae	*Justicia adhatoda* L.	1
4	Aizoaceae	*Trianthema portulacastrum* L.	1
5	Amaryllidaceae	*Allium cepa* L.	2
		Allium sativum L.	
6	Apiaceae	*Coriandrum sativum* L.	2
		Foeniculum vulgare Mill.	
7	Apocynaceae	*Catharanthus roseus* (L.) G. Don	4
		Calotropis procera (Aiton) W. T. Aiton	
		Rhazya stricta Dcne.	
		Nerium oleander L.	
8	Araceae	*Arisaema jacquemontii* Blume	1
9	Asteraceae	*Eclipta alba* (L.) Hassk.	4
		Helianthus annuus L.	
		Saussurea heteromalla (D. Don) Hand.-Mazz.	
		Silybum marianum (L.) Gaertn.	
10	Brassicaceae	*Eruca sativa* Mill.	2
		Raphanus sativus L.	
11	Cactaceae	*Opuntia dillenii* (Ker Gawl.) Haw.	1
12	Cannabaceae	*Cannabis sativa* L.	1
13	Caricaceae	*Carica papaya* L.	1
14	Chenopodiaceae	*Chenopodium album* L.	1

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Sr. No.	Family	Scientific name of species	No. of species
15	Convolvulaceae	*Convolvulus arvensis* L.	1
16	Cucurbitaceae	*Luffa acutangula* (L.) Roxb.	5
		Momordica charantia L.	
		Citrullus colocynthis (L.) Schrad.	
		Cucumis sativus L.	
		Cucurbita pepo L.	
17	Cupressaceae	*Cupressus sempervirens* L.	1
18	Euphorbiaceae	*Ricinus communis* L.	3
		Euphorbia hirta L.	
		Jatropha curcas L.	
19	Fabaceae	*Bauhinia variegata* L.	4
		Cassia fistula L.	
		Albizia lebbeck (L.) Willd.	
		Albizia procera L.	
20	Fumariaceae	*Fumaria indica* (Hausskn.) Pugsley	1
21	Lamiaceae	*Mentha longifolia* (L.) Huds.	2
		Ocimum basilicum L.	
22	Liliaceae	*Aloe vera* (L.) Burm. f.	1
23	Malvaceae	*Gossypium hirsutum* L.	2
		Bombax ceiba L.	
24	Meliaceae	*Azadirachta indica* A. Juss.	1
25	Moraceae	*Ficus benghalensis* L.	2
		Morus alba L.	
26	Moringaceae	*Moringa oleifera* Lam.	1
27	Musaceae	*Musa paradisiaca* L.	1
28	Oleaceae	*Olea ferruginea* Royle.	1
29	Poaceae	*Zea mays* L.	2
		Cynodon dactylon (L.) Pers	
30	Portulacaceae	*Portulaca oleracea* L.	1
31	Lythraceae	*Punica granatum* L.	1
32	Primulaceae	*Anagallis arvensis* L	1
33	Rhamnaceae	*Ziziphus nummularia* (Burm. f.)Whight&Arn.	1
34	Rosaceae	*Rosa indica* L.	1
35	Rutaceae	*Citrus limon* (L.) Burm. f.	1
36	Solanaceae	*Datura alba* Nees.	9
		Nicotiana tabacum L.	
		Solanum nigrum L.	
		Withania coagulans (Stocks) Dunal	

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Sr. No.	Family	Scientific name of species	No. of species
		Withania somnifera (L.) Dunal	
		Capsicum annuum L.	
		Solanum surattense Burm. f.	
		Solanum tuberosum L.	
		Solanum americanum Mill.	
37	Zingiberaceae	*Zingiber officinale* Roscoe	1
Total			**68**

Discussion

Ethnomedicinal survey is a veritable strategy which provides insight into the potentials of plants. Such knowledge can then be explored for pharmaceutical and medical uses. Ethnopharmacological information is imperative for knowledge about human- plant relationship. This can be helpful when applied in the selection of plants for phytochemical as well as pharmacological studies. The present study brought to the fore the immense hidden knowledge of the Awan tribes of Namal Valley in Pakistan on plant remedies for poisonous bites and stings from Snakes and Scorpions. Members of the Solanaceae which are ubiquitous in the valley are the frequently used plants for these bites. This might not be unconnected with their chemical constituents such as alkaloids, quassininoids, sesquiterpene, lactones, coumarins, triterpenoids, limonoids, and quinolone alkaloids (Saxena, 2003). Herbs dominated the major plant forms in the recipes and are the most abundance in the area (Shah and Rahim, 2017). The easy accessibility of leaves by plant harvesters in the region may be responsible for their high frequency of use in the recipes. Leaves are a major source of bioactive compounds (Bhattarai et al., 2006) and less dangerous to plant survival (Giday et al., 2003). Some of the plants have been screened and found to have anti-venom properties (Murti et al., 2010). *Calotropis procera* ranked highest among the documented plants in the treatment of poisonous animal bites and stings as documented in other parts of the world. Its leaf extract are known to have good antidote properties (Sharma et al., 2012b). In vitro studies showed that *Calotropis procera* contains sugars, phenols, flavonoids, saponins, steroids, terpenoids, tannins and glycosides (Sharma et al., 2012a). Several bio-chemicals have been identified for anti-snake scorpion and wasp venom from *Withania somnifera* (Lizano et al., 2003; Machiah et al., 2006), Azadirachta *indica* (Mukherjee et al., 2008) and *Mangifera indica* (Pithayanukul et al., 2009). *Achyranthes aspera* is a widely used herb in the sub-continent for poisonous animal bites and it is one of the major plants utilized in a variety of ways to treat snake and scorpion bites. Some of the chemicals derived from *Achyranthes aspera* include saponin, achyranthine, alkaloids, alkaline ash containing potash, tannins, flavonoids, oil and fats, steroids, carbohydrates, and terpenoids (Kadel and Jain, 2008; Rahmatullah et al., 2010).

Furthermore, *Arisaema jacquemontii* locally known as “zahr mora” that is widely used as treatment here is considered to be one of the unique plant species being used to cure poisonous bites and stings in the subcontinent and in North America. It is collected by only by professionals and old farmers (Turner and Szczawinski, 1991). *Albizia lebbeck* and *Albizia procera* are also abundantly cited species for the same purpose in Pakistan and other countries of the world. The chemical constituents known from these

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trees are leanolic acid, lupeol, acyclic ester, alkaloid, heneicos, tannins, carbohydrate, proteins, flavanoids (Baquar, 1989; Saha and Ahmed, 2009). Similarly *Allium cepa* and *A. sativum* are two other worth mentioning plant species in the treatment of poisonous bites and stings. Their chemical constituents includes volatile oil, albuminoids, quercetin, sulfur, carbohydrates, ether organic sulfur, moisture, essential oil (Odhav et al., 2007).

Conclusion

The present study has highlighted the potential of the plants document in the search for essential drugs in the treatment of poisonous bites by animals and by the snake and scorpion in particular. The Namal Valley in the Mianwali District, Pakistan is under-explored for its exceptionally rich medicinal plants that can serve as important source of crude drugs of plant origins for antidote medicine. This study has therefore, provided a baseline report for new plant based anti-venomous compounds. The leaves extract of documented plant species could be tested for effective anti-venomous compounds.

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SULPHUR CONTENT IN TEST PLANTS AND ARYLSULFATASE ACTIVITY IN SOIL AFTER APPLICATION OF WASTE MATERIALS

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Abstract. Sulphur available for plants may come from organic compounds introduced to the soil with waste materials which undergoes arylsulfatase-assisted mineralisation. Studies were undertaken to assess the effect of fertilisation with sewage sludge and hard coal ash on sulphur content and uptake by test plants, and arylsulfatase activity in the soil. The experimental design was a completely randomised arrangement with three replicates. The following factors were examined: I fertilisation with organic and mineral materials: fresh sewage sludge, hard coal ash, calcium carbonate; II mineral fertilisation: no fertilisation; NPK fertilisation. The sulphur content in orchard grass was significantly higher following an application of composted sewage sludge and hard coal ash, and in maize after an application of sludge/ash mixtures. The waste materials and NPK fertilisation significantly increased sulphur uptake by both the plants tested. Arylsulfatase activity was significantly higher in fresh sludge-amended soil compared with the remaining amended units whereas an application of ash reduced the activity of the enzyme. NPK fertilisation had no significant influence on the enzyme studied.
Keywords: *sewage sludge, hard coal ash, manure, orchard grass, maize, enzyme*

Introduction

Sulphur is widely found in nature as it takes part in life processes occurring in all living organisms and determines their proper functioning. Sulphur deficiency leads to a decline in plant protein, which contributes to a decline in yield quantity and quality (Millard et al., 2006). Soil sulphur has been on the decline in recent years due to an introduction of restrictions on sulphur dioxide amounts released into the atmosphere (Scherer, 2009), an application in cultivation of high-yielding cultivars and highly-concentrated mineral fertilisers, and a drop in farmyard manure production. Organic compounds in manures obtained from natural waste materials may be a source of plant-available elements, including sulphur (Ciepiela et al., 2016). The fertilisers include sewage sludge which, when introduced into the soil, undergoes arylsulfatase-assisted mineralisation.The enzyme is abundant in the soil as it takes part in the processes of oxidation of sulphur which it available for plants (Siwik-Ziomek et al., 2013). Waste materials are a valuable source of nutrients (Farasat and Namli, 2016), including sulphur which, in addition to nitrogen is the main factor determining soil fertility and hence its productive potential. It is worth considering the possibility of using sewage sludge and ashes emitted by power plants in crop fertilisation as they contain considerable amounts of sulphur (Kalembasa et al., 2008) which, however, occurs in various forms, some of them being unavailable for crop plants. When introduced into the soil, these waste materials may improve the nutrient balance in the environment (Palumbo et al., 2007; Antonkiewicz, 2010) and, at the same time, limit the effect of their excess concentration in the place where they are stored, thus solving the problem of their utilisation (Bernoud et al., 2016).

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Studies were undertaken to assess the effect of fertilisation with sewage sludge and hard coal ash on sulphur content and uptake by test plants, and arylsulfatase activity in the soil.

Materials and methods

A pot experiment was established in a glasshouse located at the experimental unit of Siedlce University of Natural Sciences and Humanities in Poland in 2007-2008. The experimental design was a completely randomised arrangement with three replicates. The following factors were examined:

I. fertilisation with organic and mineral materials:

a) fresh sewage sludge;

b) sludge obtained from the sewage treatment works in Siedlce and composted for three months (industrial and domestic waste water);

c) hard coal ash obtained from the electricity distribution company in Siedlce;

d) calcium carbonate.

Sewage sludge was applied once, adding 5% relative to soil weight. Sewage sludge and coal ash were mixed at the ratio of 2:1 when converted to dry matter.

II. mineral fertilisation:

a) no fertilisation;

b) NPK fertilisation.

Mineral fertilisers: urea, triple superphosphate and potassium sulphate, were applied pre-plant. The soil used in the experiment was very loamy sand obtained from the 0-20 cm layer of grey brown podzolic soil. Before the experiment was set up, soil contents of nitrogen, carbon, available phosphorus and potassium were determined (respectively: 1.10, 8.20, 0.052 and 0.071 g kg^{-1}). Pots were filled with 10 kg of soil and kept during the growing season at the moisture level of 60% maximum water holding capacity of the soil.

Orchard grass (*Dactylis glomerata)* was tested in the first study year. It was sown at 1.0 g pot^{-1} and harvested three times during the growing season. In the second year, maize was grown at a density of 3 plants per pot. After harvest, plants were dried and ground to determine the total sulphur content by ICP – AES after dry mineralisation. The plant material was incinerated in a muffle furnace at 450ºC, temperature being increased gradually, and HCl solution (1:1) was added to mineralised samples which were then evaporated to dryness in order to decompose carbonates and precipitate silica. 10% HCl was added to the resultant crude ash which was moved to volumetric flasks by pouring it through a hard filter paper. The total sulphur content in sewage sludge and hard coal ash were determined by ICP-AES following mineralisation of material to dryness. Arylsulfatase activity was determined by colorimetry of *p*-nitrophenol released when soil samples were incubated one hour at 37^0 C with *p*-nitrophenyl sulfate (Tabatabai and Bremner, 1970). All colorimetric data was determined with a spectrophotometr UV-VIS Lambda 25 (Perkin Elmer, Whaltham, USA).

The program STATISTICA (data analysis software system), version 12 (www.statsoft.com) was used to statistically analyse the results. Significance of differences between means for the experimental factors were checked using Tukey's test at the significance level of $\alpha \leq 0.01$.

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Results and discussion

In addition to nitrogen and phosphorus, sulphur is an element which markedly affects crop yields. Sulphur content in sewage sludge and hard coal ash used in this experiment was, respectively, 8.47 and 5.45 g kg^{-1} in d.m. This high sulphur content in sewage sludge and coal ash is several times higher compared with farmyard manure (Kalembasa and Godlewska, 2010).

Sulphur content in the biomass of orchard grass in the first study year (*Table 1*) was affected by all the experimental factors and averaged 3.46 g kg^{-1} d.m. Such an amount of sulphur is believed to be high from the standpoint of livestock nutritional needs (Falkowski, 2000). An application of either fresh or composted sewage sludge reduced sulphur content in orchard grass compared with the control grass. The fact can be indicative of an occurrence in sewage sludge of barely soluble sulphur forms which are unavailable for plants and undergo mineralisation in the soil. An application of hard coal ash increased sulphur content in control plants and sludge-manured plants. However, significant differences in sulphur content were found between plants harvested from hard coal ash-manured plots and plants manured with composted sewage sludge. An addition of ash to composted sludge contributed to an increase in sulphur content in orchard grass biomass. NPK fertilisation only slightly increased sulphur content in the plants tested but the differences were not statistically significant.

Table 1. *The content of S (in g kg^{-1} DM) in orchard grass*

NPK fertilization	0				NPK				Mean
Organic and mineral materials / Cuts	I	II	III	Mean	I	II	III	Mean	
control object	3.90	3.21	3.83	3.65	3.98	3.21	3.86	3.68	3.67bcd
sewage sludge	2.86	3.18	4.00	3.35	3.57	3.01	4.38	3.65	3.50abcd
composted sewage sludge	3.26	2.91	3.84	3.34	3.32	2.68	2.72	2.91	3.12ab
hard coal ash	3.90	3.35	3.35	3,60	4.70	3.84	3.98	4.17	3.85cd
sewage sludge/ash	2.09	2.66	3.59	3.04	4.16	2.79	3.77	3.57	3.18abc
composted sewage sludge/ash	3.38	3.51	4.38	4.76	3.09	2.80	4.64	3.51	3.63bcd
liming	4.74	3.78	3.96	4.16	5.42	2.85	3.26	3.84	4.00d
liming/sewage sludge/ash	2.89	2.13	3,18	2.73	4.06	2.07	2.95	3.03	2.88a
liming /composted sewage sludge/ash	2.70	2.76	3.70	3.05	3.78	2.07	4.68	3.51	3.28abc
Mean	3.30	3.05	3.76	3.37	4.01	2.81	3.80	3.54	3.46

a,b,c,d – groups of means which do not differ significantly at $\alpha < 0.01$

Maize needs average amounts of sulphur but it produces high dry matter content so it requires intensive fertilisation. Maize cultivated in the second study year (*Table 2*) contained on average 1.02 sulphur per kg^{-1} d.m. The organic materials and coal ash

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markedly influenced the plant content of sulphur. The effect of fertilisation with sewage sludge was visible in the second study year, which can indicate that the sludge organic compounds underwent mineralisation and sulphur was transformed into forms available for plants. Nutrients in sewage sludge occur mainly in organically-bound forms, which means they are available only when these materials have undergone mineralisation (Fijałkowski and Kacprzak, 2009). The biomass of maize harvested from sewage sludge-amended units and sewage/coal ash-amended units contained significantly more sulphur compared with control plants. Research conducted by Gondek (2010a) demonstrated that plant biomass content of sulphur increased following an application of sewage sludge. Also Gondek and Filipek-Mazur (2008) reported a significant increase in sulphur content in the biomass of sewage sludge/peat-fertilised maize. An addition of hard coal ash to composted sewage sludge contributed to an increase in the plant biomass content of sulphur but the differences were not substantial, which seems obvious as the mineral fertilisers that had been applied contained sulphur (triple superphosphate and potassium sulphate).

Table 2. *The content of S (in g kg^{-1} DM) in maize*

NPK fertilization / Organic and mineral materials	0	NPK	Mean
control object	0.620	0.858	0.739a
sewage sludge	0.820	1.52	1.17cd
composted sewage sludge	0.705	1.25	0.978bc
hard coal ash	0.615	1.28	0.948ab
sewage sludge/ash	0.760	1.30	1.03bcd
composted sewage sludge/ash	0.890	1.35	1.12bcd
liming	0.828	1.09	0.959abc
liming/sewage sludge/ash	1.01	1.43	1.22d
liming/composted sewage sludge/ash	0.98	1.07	1.03bcd
Mean	0.803a	1.24b	1.02

See explanation in *Table 1*

Sulphur uptake by plants in the first study year differed and depended on the waste materials applied (*Table 3*). Statistical analysis demonstrated that sulphur uptake by plants was significantly higher following an application sewage sludge (fresh and composted) and its mixture with hard coal ash. Sulphur uptake was the highest after an application of composted sewage sludge and hard coal ash, the increase being over 140% compared with control plants. Also grasses grown in pots where only liming or hard coal ash had been applied took up more sulphur compared with control but the differences were insignificant. Hard coal ash is a waste material which contains substantial amounts of calcium and magnesium (Kalembasa et al., 2008), which might contribute to an increase in sulphur uptake by plants. Moreover, a tendency was

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observed for orchard grass to take up less sulphur in successive cuts. However, in pots without NPK fertilisation where soil had been amended with sewage sludge and its mixture with coal ash, sulphate uptake was the highest in the second cut. The fact can indicate how fast the rate of mineralisation was.

Table 3. *The total sulphur uptake (mg pot^{-1}) by orchard grass*

NPK fertilization	0				NPK				Mean
Organic and mineral materials / Cuts	I	II	III	Mean	I	II	III	Mean	
control object	31.2	13.9	12.2	19.1	19.5	10.9	23.5	18.0	18.5a
sewage sludge	48.3	54.4	45.6	49.4	32.6	49.7	29.5	37.3	43.4b
composted sewage sludge	47.3	49.8	32.3	43.1	24.3	43.4	30.2	32.6	37.9b
hard coal ash	34.6	14.6	8.81	19.3	63.5	50.3	14.4	42.7	31.0ab
sewage sludge/ash	26.2	43.6	39.1	36.3	49.9	46.2	32.4	42.8	39.6b
composted sewage sludge +ash	49.1	59.6	36.8	48.5	62.0	33.2	27.2	40.8	44.7b
liming	53.7	19.8	26.3	33.3	77.0	17.8	5.97	33.6	33.4ab
liming/sewage sludge/ash	31.3	18.4	18.8	22.8	74.7	40.2	24.9	46.6	34.7b
liming/composted sewage sludge/ash	35.6	35.2	30.3	33.7	64.6	35.1	28.1	42.6	38.2b
Mean	39.7	34.3	27.8	33.9a	52.0	36.3	24.0	37.4a	35.7

See explanation in *Table 1*

Analysis of data revealed that mineral (NPK) fertilisation had an insignificant effect on sulphur uptake by orchard grass.

In the second study year, differences in sulphur uptake by maize remained significant (*Table 4*). An application of hard coal ash increased sulphur uptake by maize compared with control, the differences being insignificant. A significantly lower amount of sulphur was taken up by control maize compared with plants grown in all the remaining experimental units. However, the greatest increase in sulphur uptake by maize (by almost 130% compared with control) was observed in fresh sewage sludge-amended units. Liming increased sulphur uptake by plants, which agrees with findings reported by Gondek (2010b). NPK fertilisation increased sulphur uptake by maize by 239%, the differences being significant.

Arylsulfatase activity in the soil studied (*Table 5*) averaged 18.5 µg PNP g^{-1} h^{-1}. Koper and Siwik-Ziomek (2004) obtained similar results ranging from 16.9 to 30.8 µg PNP g^{-1} h^{-1}, for soils amended with farmyard manure and NPK. According Balota and Chaves (2010), arylsulfatase activity cited in literature varies widely from 4 to 770 µg PNP g^{-1} h^{-1} and depending on different factors. Statistical analysis demonstrated a varied significant effect of waste materials on arylsulfatase activity in the soil. The greatest increase in the activity of the enzyme (by 33.9%) was found in soil amended with fresh sewage sludge compared with the control. The findings confirm that organic matter significanly affects enzyme activity (Bielińska and Mocek-Płóciniak, 2009;

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Siwik-Ziomek and Lemanowicz, 2014). When carbon compounds are present, they stimulate the biosynthesis ofenzymes by soil microrgansims so fresh organic matter incorporated into the soil in the form of sewage sludge significantly increased arylsulfatase in the soil studied.

Table 4. *The total sulphur uptake (mg pot^{-1}) by maize*

NPK fertilization / Organic and mineral materials	0	NPK	Mean
control object	14.8	75.7	45.3a
sewage sludge	47.6	158	102.8c
composted sewage sludge	33.0	101	67.0b
hard coal ash	20.1	104	62.1ab
sewage sludge/ash	50.2	102	76.1b
composted sewage sludge/ash	38.9	113	76.0b
liming	21.2	121	71.1b
liming/sewage sludge/ash	37.6	124	80.8b
liming/composted sewage sludge/ash	33.6	111	72.3b
Mean	33.0a	112b	72.6

See explanation in *Table 1*

Table 5. *Aryslulfatase activity in soil (µg PNP g^{-1} h^{-1})*

NPK fertilization / Organic and mineral materials	0	NPK	Mean
control object	13.8	24.5	19.2cd
sewage sludge	27.1	24.3	25.7e
composted sewage sludge	16.6	15.8	16.2bc
hard coal ash	17.3	7.00	12.2ab
sewage sludge/ash	10.1	10.0	10.1a
composted sewage sludge/ash	17.6	13.0	15.3abc
liming	19.5	21.0	20.3cde
liming/sewage sludge/ash	21.4	23.2	22.3de
liming/composted sewage sludge/ash	25.8	24.2	25.0e
Mean	18.8a	18.1a	18.5

See explanation in *Table 1*

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An application of hard coil ash and its addition to fresh and composted sewage sludge reduced soil enzymatic activity compared with control. Soil liming increased arylsulfatase activity in the soil but the differences were insignificant. According Bielinska and Baran (2009) that an addition of fluidal ashes from hard coal increased the enzymatic activity. Mineral fertilisation only slightly reduced arylsulfatase activity in the soil, which may be indicative of an inhibitory effect of mineral fertilisers on the activity of the enzyme, which has been confirmed by other authors, too (Siwik-Ziomek and Koper, 2008). Vong et al. (2004) has pointed out that high rates of nitrogen fertilisation contribute to a decline in soil enzymatic activity. Also Sun et al. (2016) observed sufficient N increased the enzyme activity, but excess N did not stimulate activity.

In the present work, the linear correlation coefficient was calculated for the comparison between sulphur content and uptake by the biomass of maize and orchard grass harvested in individual cuts. The correlation (mean values) between these parameters determined in orchard grass biomass was significant and positive for the first cut only and amounted to $r = 0.54^{**}$. The value of the coefficient of linear correlation between sulphur content and uptake in maize biomass was $r = 0.89^{**}$.

Conclusions

The sulphur content in orchard grass was significantly higher following an application of composted sewage sludge and hard coal ash, and in maize after an application of sludge/ash mixtures. The waste materials and NPK fertilisation significantly increased sulphur uptake by both the plants tested. Arylsulfatase activity was significantly higher in fresh sludge-amended soil compared with the remaining amended units whereas an application of ash reduced the activity of the enzyme. NPK fertilisation had no significant influence on the enzyme studied.

In summary, it should be noted that the degree of organic matter decomposition and origin has a considerable effect on the characteristics discussed in the work. Further studies are needed to develop more comprehensive results and evaluate the impact of different rates of organic and mineral materials.

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IMPACT OF LEAF EATING CATERPILLAR CONTROLS ON THE DIVERSITY OF INSECTS IN ASIATIC PENNYWORT FARMS

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Abstract. Annual loss of Asiatic pennywort production is primarily due to lepidopterous larvae. The pennywort cutworm (*Zonoplusia ochreata*) is one of the most destructive leaf feeders of pennywort. Most Thai growers prefer chemical controls to prevent production loss. Bioinsecticide is an alternative to synthetic insecticides. Some organic growers used *Bacillus thuringiensis* and neem extract for lepidopterous control in pennywort farms. We should understand how diversity of target and non-targeted species in the areas is impacted by different control management. Therefore, four preventative treatments were trialed: natural control in organic farming, *Bacillus thuringiensis,* neem extract and abamectin, to evaluate their effect on the biodiversity of insects in Chainat, Nonthaburi and Bangkok provinces, all located in the central region of Thailand. After 8 weekly spray applications, the insects were collected using the sweep sampling method. A total of 561 insects were identified belonging to 38 different species, including 34 predator species, and 29 parasitoid species. The effect of different treatments on the species diversity index and evenness index was calculated. Shannon's species diversity index (H') was higher in the natural control area (2.74) compared to areas treated with Bt (2.09), neem extract (1.90) and abamectin pesticide (1.88). The evenness index showed a similar trend; natural control (0.82), Bt (0.57), neem extract (0.62) and abamectin (0.55). The results support the hypotheses that organic farming promote insect diversity. Environmentally insect control approaches enhance biodiversity and ecological service in farmland.
Keywords: *biological control agents, bioinsecticides, natural control, Shanon diversity index, phytophagous lepidopterans, ecological balance*

Introduction

The Asiatic pennywort (*Centella asiatica*) is a ground creeping plant belonging to the genus *Centella* of the *Apiaceae* family (De Padua and Bunyapraphatsara, 1999), and is a common medicinal herb (Yadav et al., 2012; Yasmeen et al., 2011). In the 1990s, 60% of Thailand's exports were agricultural products. Twelve kinds of vegetables were exported to the European market including Asiatic pennywort which was found to contain excessive chemical residues, due to inappropriate and excessive use of pesticides for controlling insects (Devarrewaerre, 1995; FAO, 1998). The main issue of pennywort production for local consumption and for export is insect infestation resulting in decreased productivity (Pathak and Khan, 1994). There are many studies which suggest chemical usage for pest control can risk pest resurgence and decreased diversity (Montanez and Amarillo, 2014). Organic farming systems rely on ecological services and natural balance to provide agricultural sustainability. Organic agriculture is mainly defined as the ban of chemical pesticides, chemical fertilizers, growth hormones, antibiotics, and genetically modified organisms. Furthermore, habitat management is recommended to increase biodiversity and especially the abundance and diversity of

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natural enemies (Zehnder et al., 2007). Modern farming practices (mechanization, mono-cropping, hybrid varieties and genetically modified (GM) crops) combined with the heavy use of agri-chemicals (fertilizers, pesticides and herbicides) have resulted in a loss of biodiversity in agricultural landscapes and surrounding areas. Agriculture that is rich in biodiversity, possess greater resilience and are, therefore, able to recover more readily from biotic and abiotic stresses such as drought, environmental degradation, pests, diseases, and epidemics. Furthermore, biodiversity conservation in agricultural landscapes promotes higher species richness and facilitates metapopulation processes between habitats (Wittebolle et al., 2009; Crowder et al., 2010).

Many different types of caterpillars, especially *Zonoplusia ochreata*, devour the leaves of pennywort and can strip the leaves in days during peak seasons. Organic insecticides are available for caterpillar control including *Bacillus thuringiensis* (Bt) and neem oil. Jun-Ce et al. (2015) compared arthropod populations in spray Bt and organic cotton crops and found no significant differences in the population of arthropod taxa collected namely, stink bugs, plant bugs, *Geocoris* spp., *Orius* spp., *Solenopsis invicta*, ladybeetles, and spiders. However, some previous studies suggest that transgenic Bt cotton has detrimental impact on biodiversity (Cattaneo et al., 2006; Sisterson et al., 2004), especially when compared with conventional chemical sprays for insect control (Marvier et al., 2007). Lawo et al. (2009) reported that transgenic Bt cotton will alter the arthropod community directly by reducing the abundance of *Helicoverpa* spp. and some other lepidopteran species. Bt cotton may also have indirect, effects on the abundance of predators and parasitoids that specialize on larvae of *Helicoverpa* spp. or other lepidopteran species controlled by Bt. Neem products can use for lepidopterous larva control (Ukeh et al., 2007). Its bioactivity includes antifeedant, insecticidal activity and disruption to growth and development of insects (Senthil-Nathan, 2013). Both Bt and neem extract has long been recognized as environmental friendly biopesticides. Many synthetic insecticides are available on the market but growers generally prefer abamectin mostly for insect control in Asiatic pennywort growing areas (Ngernyu and Bumroongsook, 2012). However, organic Asiatic pennywort farming rely on biological control agents which are currently implemented to resolve the chemical contamination problems of export vegetables.

Therefore, we need to assess the impact of various methods for controlling leaf eating caterpillars of Asiatic pennywort using different insecticides as compared to natural control in organic farming on insect diversity. The diversity and evenness index approaches is used to evaluate whether these treatments are suitable as eco-friendly products for organic farming.

Materials and methods

Study area

The studies were conducted from April to July 2016 in Chainat, Nonthaburi and Bangkok provinces, all located in the central region of Thailand (*Fig. 1*). These three provinces received 82.5-456.5 mm of rainfall in 2015-2016. During the sampling period, mean temperatures ranged from 30.2°C to 34.9°C while, the average relative humidity varied from 54.6-75.5%.

Chainat: located at Sankhaburi, a district in the south of Chainat province (Lat. 15° 11’N: Long. 100° 7’ E), surrounded by rice fields. The study area was three hectares.

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Nonthaburi: located at Sai Noi, in the north west of Nonthaburi province (Lat. 13° 47’ N: 100° 15’ E) with small irrigation canals situated in an Asiatic pennywort plantation; one side bordered by the local road and the other side by a rice field.

Bangkok: located at Ladkrabang, to the east of Bangkok (Lat. 13° 43’ N: Long. 100° 47’ E), both sides connected to small irrigation canals and surrounded by a vegetable crop plantation.

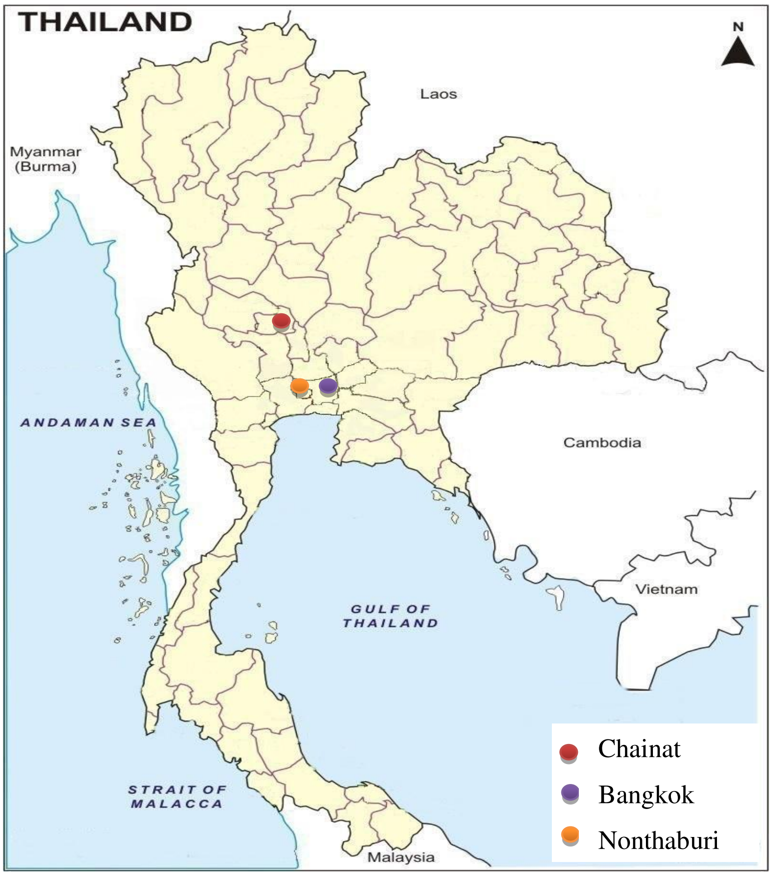

***Figure 1.** The map showing the three study location of Asiatic pennywort farms*

Treatment application

One-month old Asiatic pennywort with almost uniform growth were selected for treatment. The experiment comprised Asiatic pennywort farmed using natural control in organic farming methods (T1); neem extract at 300 ml per 20 L water (T2); *Bacillus thuringiensis* var. aizawai at 80 ml per 20 L water (T3); and abamectin 1.8% w/v 80 ml per 20 L water (T4). The insecticides were sprayed weekly onto the leaves of Asiatic pennywort for 8 weeks.

Insect Sampling of Asiatic pennywort farms

At each site, insect sampling was conducted between 9-11 am in April to July 2016. The insects were collected using the sweep sampling method described by Gadagakar et al. (1990). Nets made of thick cotton cloth were used with a diameter of 30 cm at the mouth and a bag length of 60 cm. The sampling method was to sweep on and around

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the plants 100 times/site. The collected insects were transferred into vials in 70% alcohol for identification. Insects were identified based on morphological characteristics and classified according to family, order and species (Heisswolf et al., 2010).

Data analysis

Study area diversity was calculated based on species diversity using the Shannon diversity index (Shannon, 1948; *Eq. 1*):

$$H = -\sum_{i=1}^{s}\left(P_i \ln P_i\right) \qquad \text{(Eq. 1)}$$

where H is the Shannon diversity index, P_i is the proportion of individuals found in species i, s is the total number of species.

We also adopt a Simpson index of diversity which is widely used for measurement of biodiversity (Simpson, 1949; *Eq. 2*):

$$1 - D = 1 - \frac{\sum n(n-1)}{N(N-1)} \qquad \text{(Eq. 2)}$$

where 1-D is the Simpson index of diversity, n = the total number of organisms of a particular species, N = the total number of organisms of all species.

The evenness index was calculated using Pielou's formula (Pielou, 1966; *Eq. 3*):

$$E = H' / \ln S \qquad \text{(Eq. 3)}$$

where E is the evenness index, H is the Shannon diversity index, S is the total number of species.

Results

Differences among predators, parasitoids and insect pest communities in the Asiatic pennywort farms

A total of 561 insects belonging to 38 different species, including 34 predator species, 29 parasitoid species and 498 insect pest species were collected using the sweep sampling method. The effect of different treatments on the species diversity index and evenness index was higher in the natural control compared to Bt (2.09, 0.62), neem extract (1.90, 0.57) and abamectin pesticide (1.88, 0.55) (*Table 1, Fig. 2*). The Shannon's species diversity index (H') showed a negative effect of abamectin treatment compared with the natural control of organic farming in three Asiatic pennywort plantations. There was no discernible effect of Bt and neem treatment on Shannon's species diversity index (H'). Shannon's species evenness index (E) was higher in organic farming compared to the abamectin treatment. The Bt and neem extract had little impact on the diversity of predator, parasitoid and insect pests. The results for predator, parasitoid and insect pest species diversity and evenness were similar for all insect species, which confirms that insecticide treatment has a considerable negative effect on Shannon's species diversity and evenness.

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The results showed a significant effect on mean abundance and species richness, with higher values in organic farming, but no effect on diversity (H') or evenness (E'). The mean abundance of the predator, parasitoid and insect pests per sweeping net was significantly lower in the abamectin treatment compared to the organic farming, Bt and neem treatments (*Table 1*).

Table 1. *Diversity of predator, parasitoid and insect pest for the natural control, Bt, neem and abamectin treatments of Asiatic pennywort farms*

Treatment	Species	Simpson index of diversity (1-D)	Shannon diversity index (H)	Evenness (E)	Total of insect
Natural control	38	0.10	2.74	0.82	250
Neem	38	0.07	1.90	0.57	124
Bt	38	0.08	2.09	0.62	135
Abamectin	38	0.05	1.88	0.55	52

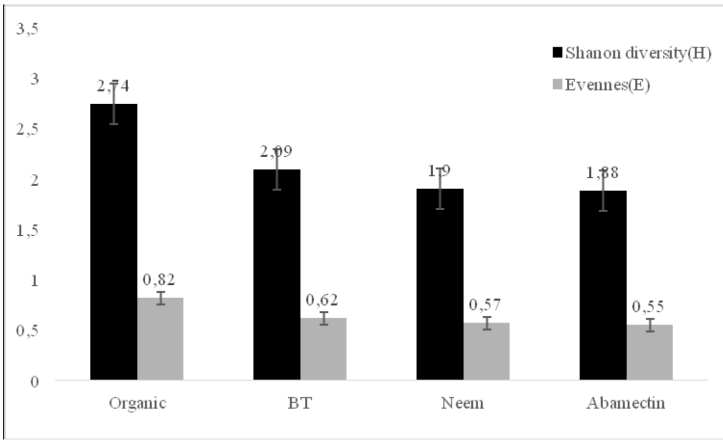

Figure 2. *Shanon diversity index (H) and evenness (E) for the natural control in organic farming, Bt, neem extract and abamectin of Asiatic pennywort farms*

Composition of pest insect communities

The main predators found in organic farming, Bt, neem extract and abamectin on Asiatic pennywort are shown in *Table 2*. A total of 498 pest insects were counted in Asiatic pennywort (218 in organic farming, 118 in Bt, 111 in neem extract and 51 in abamectin). Different insect pests were recorded on the organic, neem, Bt and abamectin treatment. The dominant groups of insect found in the Asiatic pennywort were *Cicadellidae*, *Agromyzidae*, *Thripidae* and *Noctuidae*. In the organic farms, a significantly higher abundance of insect was observed in comparison to the Bt, neem, and abamectin treatment namely, *Agromyzidae* ($p < 0.001$), *Chrysomelidae* ($p < 0.001$), *Thripidae* ($p < 0.001$), *Cicadellidae* ($p < 0.001$), *Noctuidae* ($p < 0.001$), *Arctiidae* ($p < 0.001$) and *Agromyzidae* ($p < 0.001$). The following were significantly more abundant in Bt and neem than in organic namely, *Delphacidae* ($p \leq 0.001$), *Acrididae* ($p \leq 0.001$), *Aphididae* ($p \leq 0.001$), *Chrysomelidae* ($p \leq 0.001$), *Tetrigidae* ($p \leq 0.001$), *Cecidomyiidae* ($p \leq 0.001$) and *Tridactylidae* ($p \leq 0.001$). Generally, the phytophagous *Lepidopteran* of pennywort in organic farms were *Zonoplusia ochreata*, *Diasemia accalis*, and *Spodoptera exigua* (*Fig. 3*).

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Table 2. *Mean presence per sampling point of insect pest on Asiatic pennywort in April to July 2016. Each value was the mean of three fields; SE was calculated using each sampling data in each farm as an independent data. Statistical significance based on ANOVA*

Pest taxa	Organic [Mean/3 sites (S.E.)]	Bt [Mean/3 sites (S.E.)]	Neem [Mean/3 sites (S.E.)]	Abamectin [Mean/3 sites (S.E.)]	*P*
Delphacidae	2.00(0.01)	3.00(0.61)	1.00(0.01)	1.67(0.08)	0.001
Agromyzidae	24.33(0.57)	6.00(0.81)	14.33(0.77)	1.67(0.08)	0.001
Chrysomelidae	2.33(0.53)	0.33(0.08)	4.00(0.58)	0.33(0.08)	0.001
Tetrigidae	2.67(0.15)	3.33(0.21)	6.33(0.51)	2.67(0.31)	0.001
Acrididae	1.33(0.53)	2.00(0.05)	0.33(0.08)	1.33(0.31)	0.001
Thripidae	5.67(0.04)	2.00(0.01)	4.00(0.20)	2.00(0.46)	0.001
Cicadellidae	27.00(0.29)	14.00(0.94)	4.67(0.51)	3.33(0.31)	0.001
Aphididae	0.67(0.08)	1.00(0.01)	0.00(0.00)	0.33(0.08)	0.001
Cecidomyiidae	1.33(0.58)	3.00(0.61)	7.67(0.42)	0.67(0.08)	0.001
Tridactylidae	1.33(0.53)	0.33(0.08)	3.33(0.06)	0.33(0.08)	0.001
Noctuidae	3.33(0.15)	0.67(0.07)	1.33(0.53)	0.00(0.00)	0.001
Arctiidae	2.00(0.00)	1.00(0.03)	0.00(0.00)	2.00(0.65)	0.001
Agromyzidae	1.33(0.53)	0.33(0.08)	1.00(0.73)	0.67(0.15)	0.001
Total	75.33(3.99)	37.01(3.56)	48.00(3.61)	17.00(2.67)	0.013

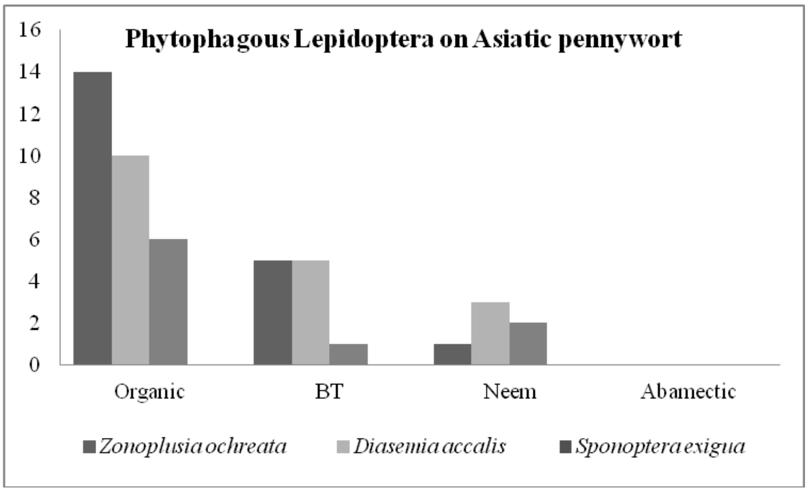

Figure 3. *Different composition of lepidopteran pest fauna on Asiatic pennywort farms. The data were obtained from three field pairs*

Composition of predator communities

Table 3 lists the main predators in Asiatic pennywort farms. In detail, a total of 34 predator specimens (18 in organic farming, 8 in Bt, 7 in neem and 1 in abamectin treatment) were recorded on Asiatic pennywort. At the farms treated with abamectin compared to the organic farming, Bt, and neem extract, a minimal abundance of total predators was detected. *Chrysomelidae* ($p < 0.005$), and *Coccinellidae* ($p < 0.001$) were significantly more abundant in organic farming than in Bt, neem extract and abamectin treatment. None of these predators were found to attack the larvae of *Lepidopteran* pests in the field.

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Table 3. *Mean presence per sampling point of predator on Asiatic pennywort in April to July 2016. Each value is the mean of three fields; SE was calculated using each sampling data in each farm as an independent data. Statistical significance based on ANOVA*

Predator taxa	Organic [Mean/3 sites (S.E.)]	Bt [Mean/3 sites (S.E.)]	Neem [Mean/3 sites (S.E.)]	Abamectin [Mean/3 sites (S.E.)]	*P*
Chrysomelidae	3.66(0.13)	1.00(0.03)	2.00(0.02)	0.33(0.08)	0.005
Agrionidae	1.33(0.08)	0.67(0.28)	0.33(0.08)	0.33(0.08)	0.115
Coccinellidae	0.33(0.10)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.001
Pentatomidae	0.66(0.18)	0.67(0.15)	0.33(0.08)	0.00(0.00)	0.172
Staphylinidae	0.33(0.18)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.203
Total	6.31(0.67)	2.34(0.46)	2.66(0.18)	0.66(0.16)	0.496

Composition of parasitoid communities

There were 14 parasitoids collected in Asiatic pennywort treated with organic farming, 10 collected from Bt treated areas, 5 collected from neem extract treated areas and none found in the abamectin treated areas (*Table 4*). The number of parasitoids were significantly higher in organic farms compared to Bt, neem extract and abamectin namely, *Cotesia flavipes* ($p < 0.001$) and *Chelonus* sp. ($p < 0.007$). Yet, the two parasitoids *Euplectrus* sp. nr. *bicolor* ($p < 0.001$) and *Cotesia* sp. ($p < 0.003$) were significantly more abundant in Bt and neem extract than in organic farms. *Euplectrus sp*. nr. *bicolor* is a larval ectoparasitoid of pennywort cutworm, *Zonoplusia ochreata*.

Table 4. *Mean presence per sampling point of parasitoids on Asiatic pennywort in April to July 2016. Each value was the mean of three fields; SE was calculated using each sampling data in each farm as an independent data. Statistical significance based on ANOVA*

Parasitoid	Organic [Mean/3 sites (S.E.)]	Bt [Mean/3 sites (S.E.)]	Neem [Mean/3 sites (S.E.)]	Abamectin [Mean/3 sites (S.E.)]	*P*
Euplectrus sp. nr. *bicolor*	1.00(0.00)	2.33(0.58)	0.33(0.08)	0.00(0.00)	0.001
Cotesia flavipes	2.00(0.03)	1.00(0.03)	0.00(0.00)	0.00(0.00)	0.001
Chelonus sp.	1.00(0.02)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.007
Cotesia sp.	0.67(0.08)	0.00(0.00)	1.33(0.03)	0.00(0.00)	0.003
Total	4.67(0.13)	3.33(0.61)	1.67(0.11)	0.00(0.00)	0.012

Discussion

The loss of biodiversity is related with more extensive agricultural areas (Froidevaux et al., 2017). Organic farming is used to alleviate the problems associated with biodiversity conservation in intensive agricultural practices. Our study showed a greater level of insect diversity (predator, parasitoid, and insect pest) in organic farms compared with conventional methods (Bt, neem extract, and abamectin) of Asiatic pennywort plantations. Organic crops increase the taxonomic richness and abundance of insects, therefore it conserves biodiversity (Montanez and Amarillo-Suárez, 2014). Tuck et al. (2014) state that organic farming promotes species richness and conserves biodiversity.

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Both Bt and neem extract treatment affect diversity indices. Lu et al. (2014) showed that there was no the consistent difference in abundance, diversity and species richness of beneficial arthropods in Bt spray and conventional cotton communities. Our studies showed neem extract has better insect control than Bt. Neem extract has insecticidal activity to a wide range of insects (George et al., 2014). Resende et al. (2016) indicated that the richness and diversity of insects depend on location and insecticide spray. Various control practices used in modern agriculture are related to the decline of biodiversity in production areas. To restore biodiversity is to implement crop production using biodiversity-based ecosystem services (Geiger, 2010)

Conclusions

This research is addressing various scales of lepidopterous management based on diversity measures. Diversity indices provide useful information for ecological structure, but they do not include species interaction. The sweep sampling could not detect all the insects in the areas and effect on the diversity index estimation. This is the first report on a survey of insect pests and natural enemies found in pennywort farming in Thailand. In the study it is proven that organic farming of Asiatic pennywort helps to conserve predators and parasites and enhances the ecology balance and insect diversity. A broad spectrum insecticide like abamectin had the greatest impact on biodiversity and abundance of insects. Bt and neem oil are more selective organic insecticides and had effect on predator and parasite diversity. However, critical questions remain including importance of parasitoid species and their relative abundance as a biological control agent. The impact of parasitoids on lepidopterous larvae should be investigated to develop alternatives for better control tactics in organic farming practice.

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IMPACTS OF MARINE RANCHING CONSTRUCTION ON SEDIMENT PORE WATER CHARACTERISTIC AND NUTRIENT FLUX ACROSS THE SEDIMENT–WATER INTERFACE IN A SUBTROPICAL MARINE RANCHING (ZHELIN BAY, CHINA)

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Abstract. Marine ranching is an effective way to restore depleted stocks and increase fisheries production. The effects of marine ranching construction on sediment pore water characteristics and nutrient flux across the sediment–water interface were determined by incubation experiments. Nine stations were selected along an inshore to offshore gradient of different sediment types representing different zones used for marine ranching in Zhelin Bay, China. The results showed that nutrient concentrations of overlying water and pore water varied by zone and were influenced by biological and physical characteristics. In the macroalgae zone (MA), macroalgae was cultured from September to May Decayed macroalgae in this zone during winter lead to pH variation (ranged from 8.05 to 8.16) and highest nitrite (range from 0.1719 to 0.9210 umol m^{-2} D^{-1}), ammonia (range from 1.778 to 4.448 umol m^{-2} D^{-1}) and total nitrogen (range from 23.43 to 140.6 umol m^{-2} D^{-1}). In the benthic molluscs' area, shellfish activities led to higher ammonia (range from 0.3312 to 7.725 umol L^{-1}) and total nitrogen (range from 59.67 to 447.7 umol L^{-1}) concentration in the overlying water during the summer season. In the artificial reef zone (depth 15-20 m), the deployment of artificial reef materials altered the sea floor which resulted in upwelling and a consequent increase in the flux of total phosphate. The results of this study will be useful to improve marine ranching efforts in China and worldwide.
Keywords: *fishery, macroalgae, benthic molluscs, artificial reef, water quality*

Introduction

Sustainable fisheries are a useful way to restore depleted stocks and increase production (Bell et al., 2008; Taylor et al., 2016). Stock enhancement and marine ranching have been proven to be an effective method to increase stocks (Camp et al., 2013; Hair et al., 2016; Leber et al., 2004; Lorenzen et al., 2010; Han et al., 2016). In recent years, marine ranching combined with artificial reef deployment has been used on a massive scale in China, Japan and other countries (Leber et al., 2004). A typical marine ranch in China is divided into four functional components including the artificial reef zone, macroalgae zone, mollusc zone and floating reef zone (Chen et al., 2015) and each zone has different ecological function. The artificial reef zone and floating reef zone provide shelter for fishery resources and enhance water nutrient; shellfish in the mollusc zone feed on microalgae; macroalgae absorb nutrients decrease red tide and provide food for human consumption.

The impacts of marine ranching on the physical, chemical and biological environments in each functional part would be significantly different (Gaertner-Mazouni et al., 2012; Layman et al., 2016; Raoux et al., 2017; Seaman, 2000; Wang et al., 2016; Wu et al., 2016). Thus, it is important to evaluate the biogeochemical changes associated with marine ranching construction. Models of mass fluxes and transformations of nutrients were used to describe the physical and biogeochemical process (Arndt et al., 2009; Denis and Grenz, 2003; Hu and Li, 2009; Miller, 1984; Rasheed et al., 2003). Dominant macrofauna enhanced benthic molecular diffusion and changed the biochemical processes in sediment (Zheng, 2011). Warnken et al. (2003) found shrimp trawling with removal of the upper oxic sediment layers could trend in benthic–pelagic coupling. Layman et al. (2016) proved that artificial reef deployment would enhance the primary production in seagrass ecosystem. Yet this rather general observation leaves many things unexplained about specific mechanistic links between marine ranching construction and nutrient supply.

The aim of this study was to assess nutrient fluxes across sediment–water interface and to evaluate the effects of marine ranching construction - artificial reef deployment, mollusc enhancement and macroalgae culture on sediment biogeochemistry. To help constrain the results, we have chosen a study site where benthic animal and fishery resources have been surveyed previously (Chen et al., 2015).

Materials and methods

Study area and sampling

Zhelin Bay is located in the north of Guangdong Province, China, with an area of 1320 km^2. It was an important fishing ground of the north South China Sea. However, increased fishing effort in this area has led to a substantial decline in its fishery resources. Thus, to enhance fishery resources the area was selected as a location for constructing a Zhelin marine ranching system (ZSR). There are four parts in ZSR; the artificial reef zone (AR), the benthic molluscs zone (BM), the macroalgea zone (MA) and the floating reef zone (cages) (FR). Sampling sites in ZSR were selected in AR, BM and MA respectively.

Sediment cores were collected at the selected sites by SCUBA in two seasons (summer and winter) in 2012 (*Table 1, Figure 1*).

Table 1. *Location of sample sites*

Stations	Description	Latitude (North)	Longtitude (East)
S		117°00′10.80″	23°32′27.60″
S	Macroalgae cultured area	117°03′32.40″	23°32′27.60″
S		117°06′05.40″	23°30′00.00″
AR		117°10′15.60″	23°31′01.20″
AR	Artificial reef deployed area	117°10′15.60″	23°29′34.80″
AR		117°11′24.00″	23°29′34.80″
M		117°05′60.00″	23°28′13.80″
M	Molluscs enhanced area	117°03′21.00″	23°28′46.80″
M		117°01′17.88″	23°26′56.40″

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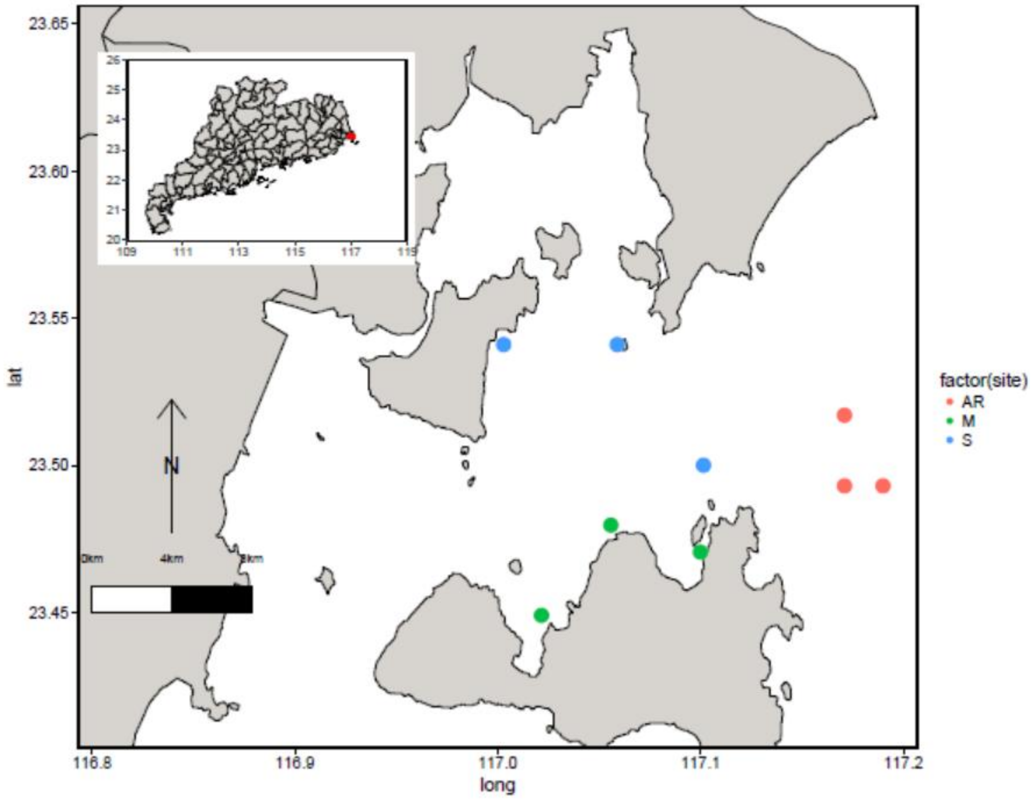

Figure 1. *Sample sites in Zhelin Bay marine ranching, China. AR: artificial reef deployed area; M: molluscs enhanced area; S: macroalgae cultured area*

Sediment cores of 0-30 cm were collected with stainless steel column sampler (5 cm in diameter). Then, sediment cores were transferred into transparent PVC wetland column without disturbance (30 cm in height and 5 cm in diameter), and immediately frozen (-20 °C) and preserved with $HgCl_2$ until analysis was performed.

Nutrient incubated fluxes

The incubation device used in this study was designed by Qin et al. (2012) (*Fig. 2*). Samples were collected as 15 cm-long sediment cores including 15 cm of the overlying water column. Each sediment core was sliced at 2 cm intervals over the first 10 cm with Rhizon to collect pore water (Seeberg-Elverfeldt et al., 2005) (*Fig. 3*). The temperature of the dark refrigerated cabinets was set at 10 °C and 25 °C to simulate the winter and summer temperature of ZSR, respectively. The overlying water was centrifuged at 50 r/min at AR samples, 40 r/min at BM samples, 30 r/min at MA with a motor rotating propeller which was positioned at the same distance (10 cm) above the sediment surface in all core tubes. The differences of revs among samples of different areas are according to the water current of the areas. All sea water samples were taken from ZSR before each experiment and returned to the lab with 4 °C cabinet. Sampling of the overlying water was done by a plastic outlet at 0 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h and pore water by means of Rhizon at 8 h and 48 h of the incubation period. The water volume removed during sampling was compensated by simultaneous input through the inlet, which retained the physical and chemical properties and total volume in the overlying water.

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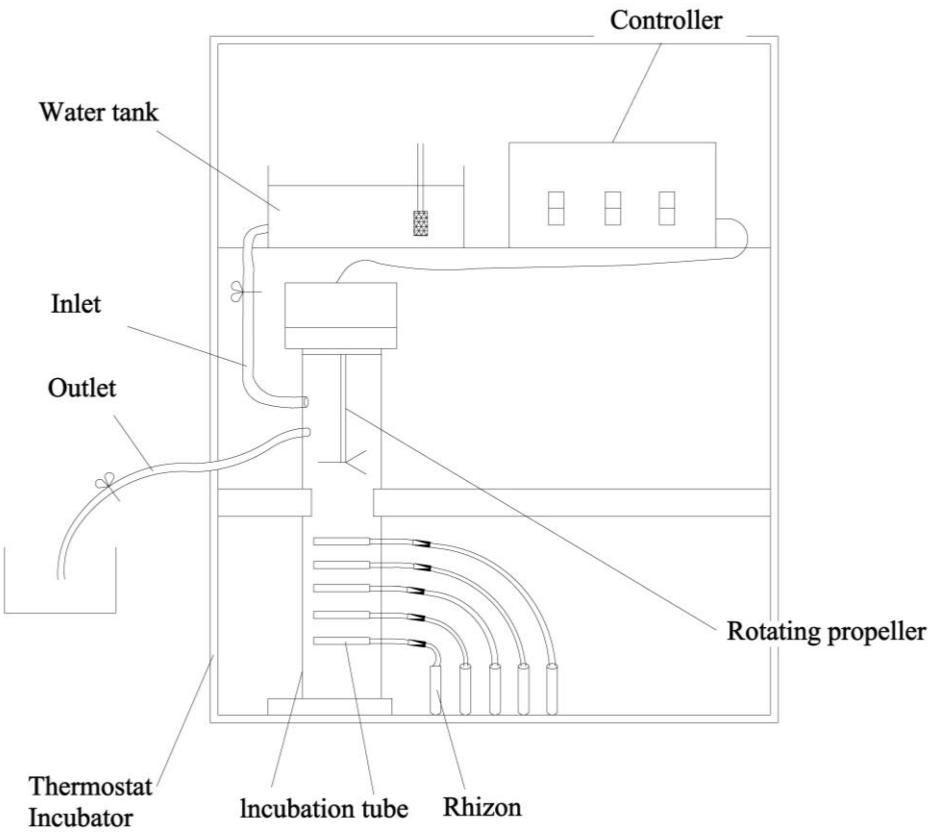

Figure 2. *Equipment used for measurements of benthic nutrient fluxes during incubations*

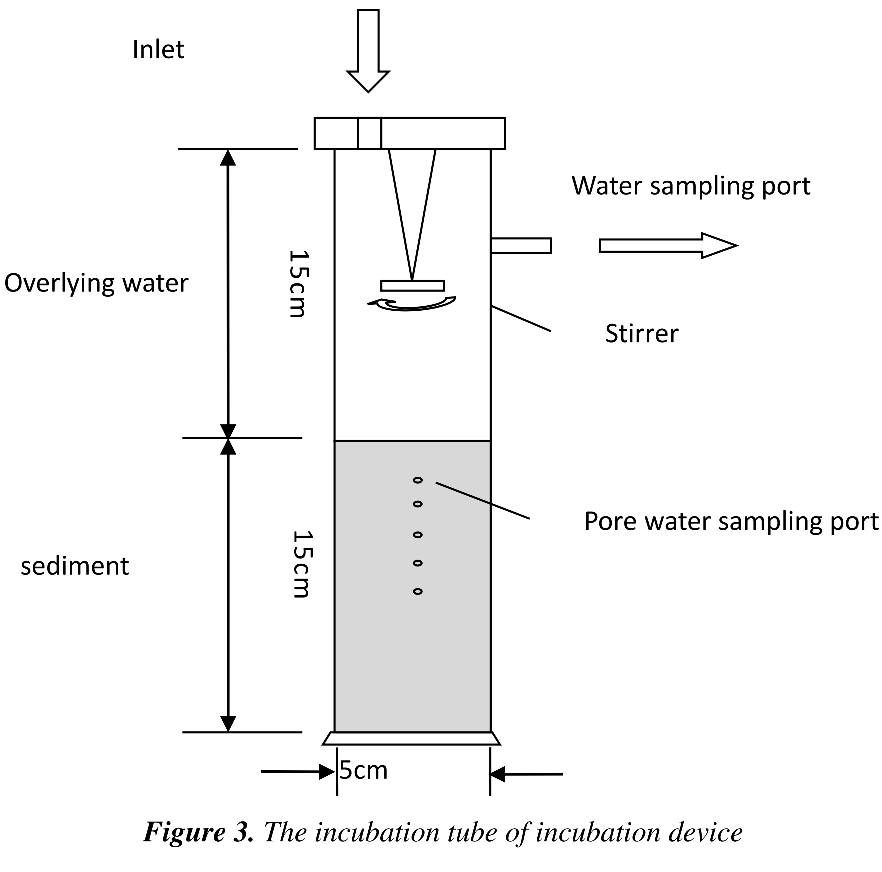

Figure 3. *The incubation tube of incubation device*

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DO and pH of overlying water were analyzed using a hand held YSI (YSI 650, YSI Incorporated). The overlying water and pore water samples were rapidly filtered using Whatman filter paper (Pore 0.45 um and diameter 47 mm; Whatman Inc., Florham Park, NJ) and analyzed for ammonia (NH_4^+), nitrite (NO_2^-), phosphate (PO_4^{3-}), total nitrogen (TN) and total phosphorus (TP). Concentration of NH_4^+, NO_2^-, PO_4^{3-}, TN and TP were determined spectrophotometerically using FIAstar5000 nutrient analyzer (FOSS Company) according to the methods described by Grasshoff et al. (2009).

Fluxes calculation and data analysis

The fluxes of NH_4^+, NO_2^-, PO_4^{3-}, TN and TP across sediment–water interface were calculated according to Fick's 1st law and using the following formula (*Eq. 1*) (Sakamaki et al., 2006):

$$F = \frac{[(C_s - C_i) - (C_s' - C_i')]}{(t_i - t_j) \times A} \times V \qquad \text{(Eq. 1)}$$

where *F* is the nutrient flux (mg m^{-2} h^{-1}), C_s and C_i are the nutrient concentration in the overlying water and pore water at the time of t_j, C_s' and C_i' are the nutrient concentration in the overlying water and pore water at the time of t_i, t_j and t_i is the time of beginning of incubation and end of incubation, individually, A is the sediment surface area in incubation tube and V is water volume.

Fluxes between the different pore water were calculated as follows (*Eq. 2*) (Zhang et al., 2013):

$$F = \frac{(C_s - C_i)}{(t_i - t_j)} \times H \qquad \text{(Eq. 2)}$$

where *H* was the height between the different pore.

A series of possible fits are compared through statistical *F* testing. Fluxes were calculated as slopes of linear regression of the changes in nutrient concentrations against time. Statistical significance of regression analysis was evaluated by a criterion of $p < 0.05$.

Results

Overlying water

DO and pH in the overlying water are illustrated in *Figure 4*. Dissolved oxygen in the overlying water decreased quickly in the first 4 h and then remained at a relatively stable concentration; no obvious differences were found among sites and seasons. The exception was site AR and MA in summer, where lower DO values were measured. Higher depleted rate of DO concentrations in site MA were probably due to decaying macroalgae (Wang et al., 2016). Otherwise, artificial reef construction will affect the local physical dynamics in the small-scale environments (Kim et al., 2016; Seaman, 2000). A large reef structure can create locally significant vertical upwelled current and cause resuspension or scouring of sediment around the reef bottom.

The concentration of NH_4^+, NO_2^- and PO_4^{3-} was highly variable (*Fig. 5*). Ammonium is higher than nitrite in site BM and MA during the incubated period. The highest

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concentration of ammonium was found in summer at site BM (11.99±2.01 umol L^{-1}, after 24 h incubation in summer). At site AR the concentration of ammonium in summer is lower than during winter. However, the trend of nitrite is reversed. At site BM, Nitrite levels are the lowest among all sites, though ammonium level was the highest. Phosphate concentrations were low at sites BM and MA (*Fig. 6*). Phosphate concentrations revealed no obvious differences among all sites and seasons (summer and winter, P>0.05). Highest phosphate levels were found in winter after a 4 h incubation period at site AR (5.360 umol L^{-1}).

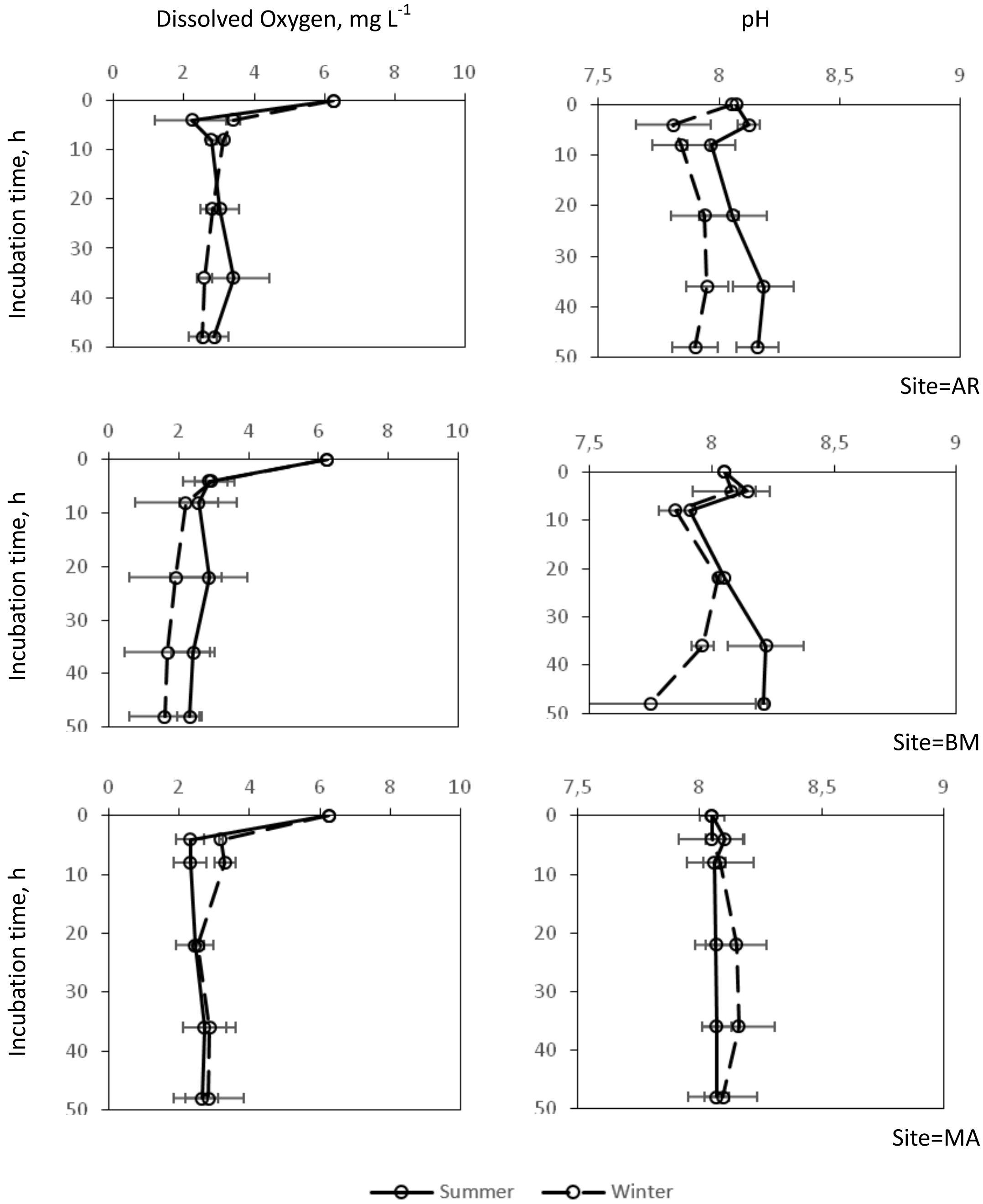

Figure 4. *Time course of DO and pH in the overlying water during the incubation in summer and winter*

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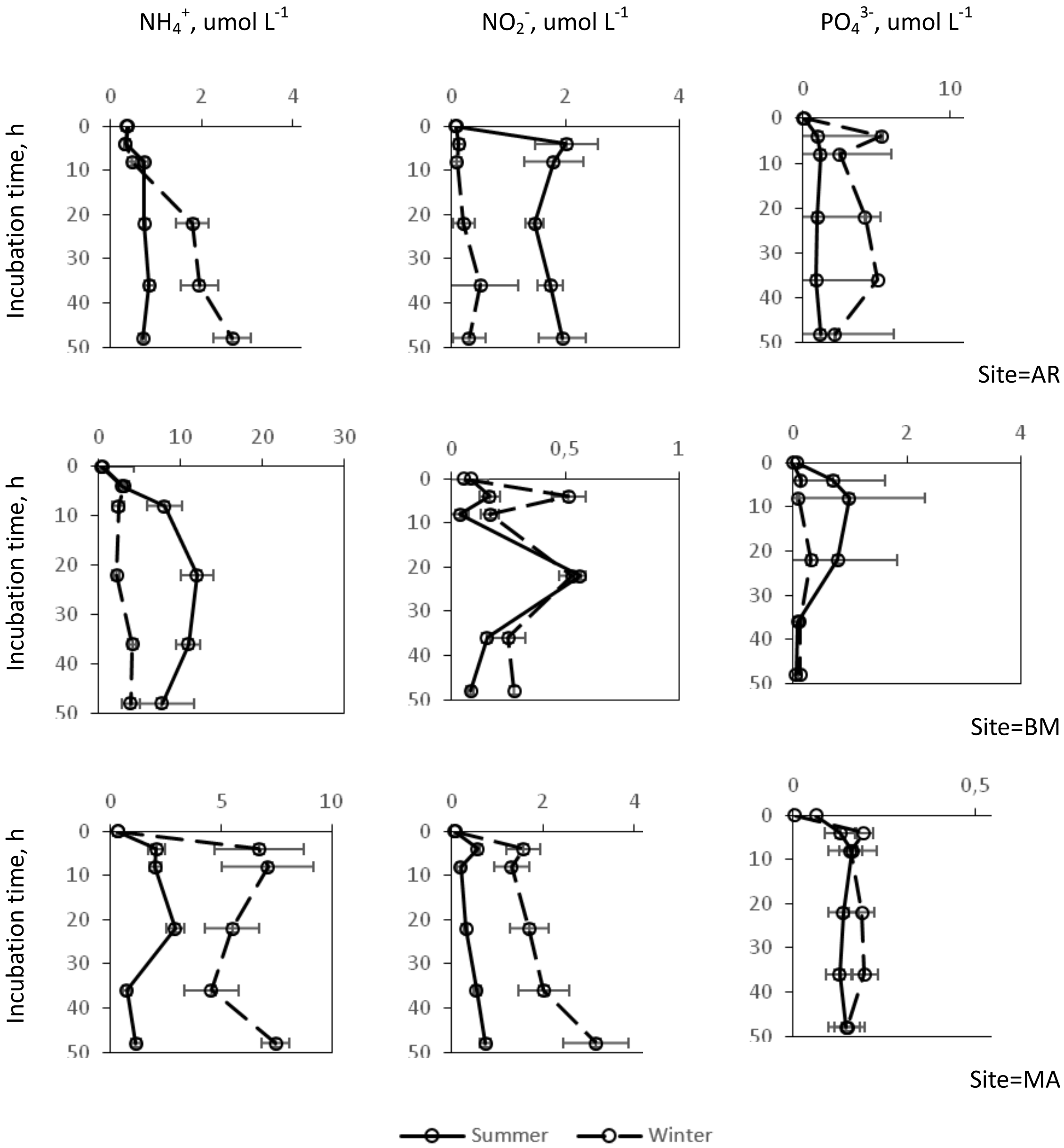

Figure 5. *Time course of ammonium, nitrite and phosphate during the incubation in spring and winter*

The range of TN and TP concentration in the overlying water throughout the incubation time was about one order of magnitude greater than TN and TP variations observed during the first 4-h incubations (*Fig. 5*). The TN levels were always higher than TP in both seasons and at all sites. The highest TN concentrations (447.1±63.32 umol L^{-1}) were found in summer at site BM. After incubation the higher TN concentration were found at site AR in winter (325.8±46.83 umol L^{-1}) and at site MA in both summer and winter (328.5±30.61 umol L^{-1} and 330.3±50.97 umol L^{-1}, respectively). TP concentrations were generally lower in summer and winter at site AR and in winter at site MA. These results mean that the high density of mollusc in site BM had bioturbation effects on benthic biogeochemistry.

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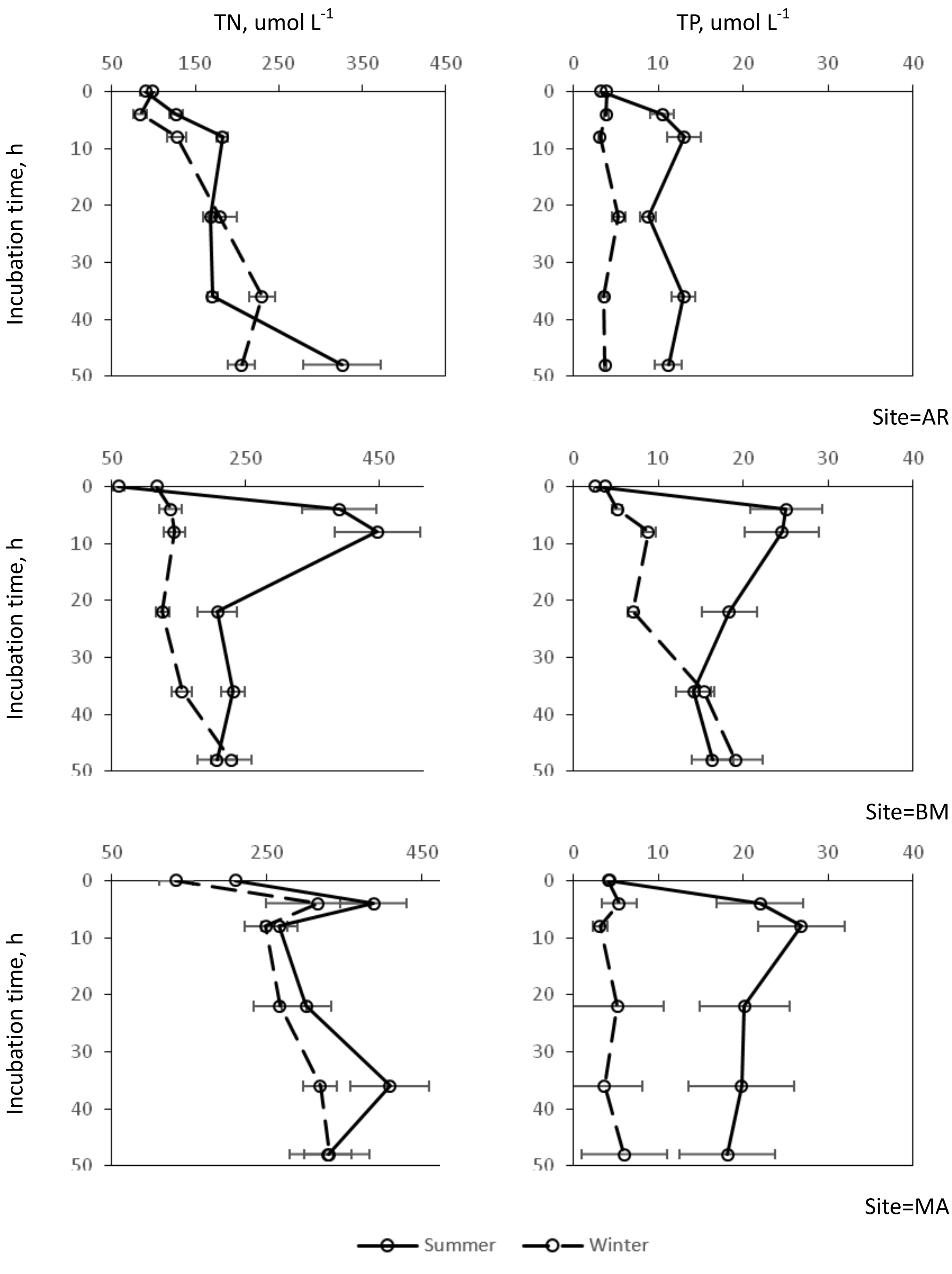

Figure 6. *TN and TP in the overlying water in summer and winter*

Pore water

For all sites, ammonium, nitrite and phosphate concentrations showed a sharp increase in the first 2 cm depth of the sediment and then demonstrated regular fluctuations with depth (*Fig. 7*). The pore water ammonium concentrations were approximately similar at both sites BM and MA for both seasons (varying from 2.428 to

55.68 umol L^{-1} and from 1.996 to 54.03 umol L^{-1} in sites BM and MA respectively). The highest ammonium concentrations were detected at site AR in winter (77.50 umol L^{-1}). Nitrite concentration showed a similar trend to ammonium, however the highest nitrite concentration 1182.14 umol L^{-1} was found at site MA in summer after 48 h of incubation. The pore water phosphate$^-$ concentration showed a gradual increase with depth and no conspicuous difference between the two seasons. However, phosphate concentration at site AR showed higher value than the other sites and with a strong positive gradient in overlying water. Significant variation ($p < 0.05$) of nitrite concentration with seasons were detected only in sites AR and BM. There are no significant differences between 8 h incubation and 48 h of incubation for ammonium, nitrite and phosphate concentrations ($p > 0.05$). TP and TN showed almost similar trend (*Fig. 8*). Total phosphate concentration at site BM was significantly lower than at site AR and MA (varying from 3.092 to 116.7 umol L^{-1}, from 8.802 to 167.00 umol L^{-1} and from 3.112 to 187.2 umol L^{-1} in site BM, AR and MA respectively).

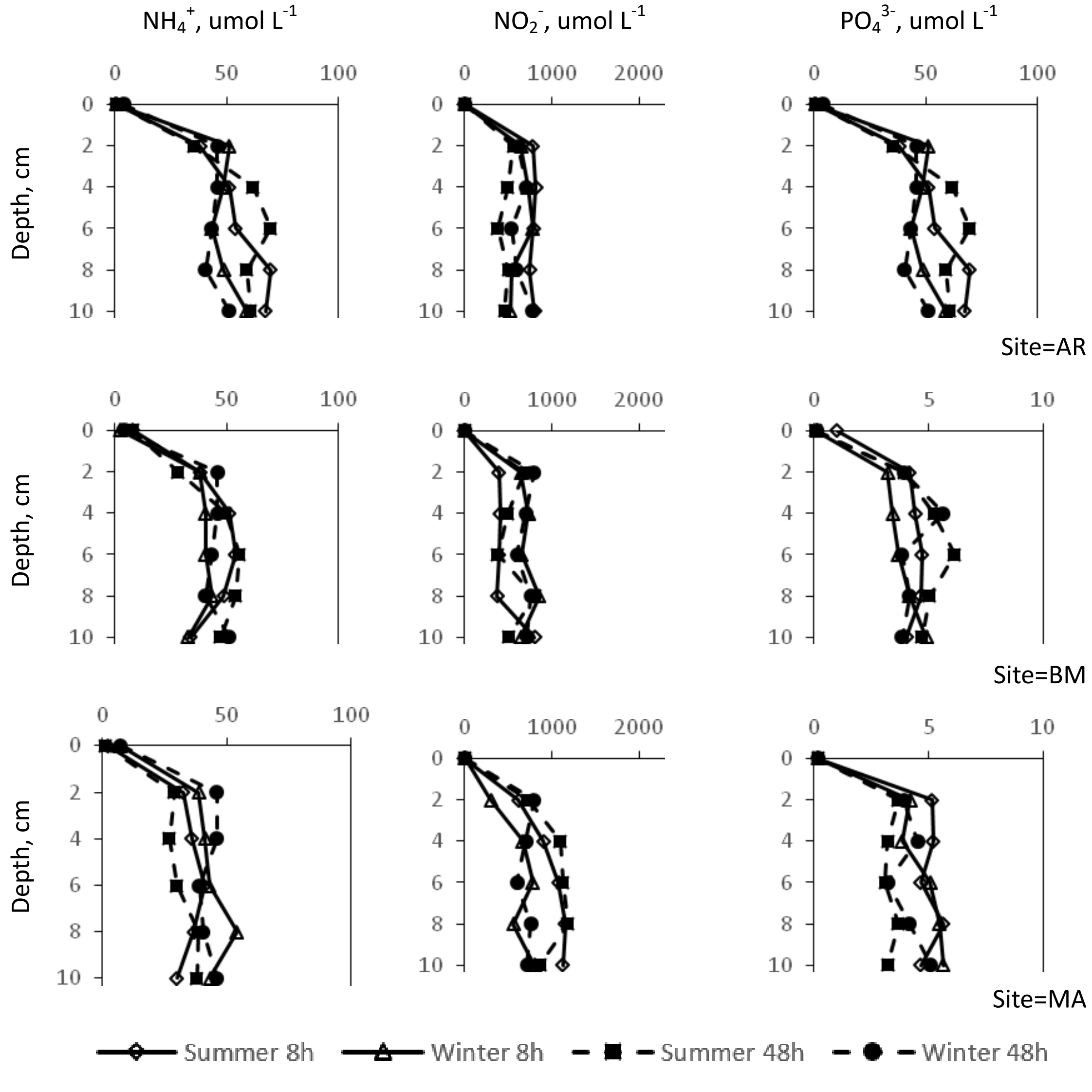

Figure 7. *Ammonium, nitrite and phosphate in the pore water after 8 and 48 h of incubation for spring and winter*

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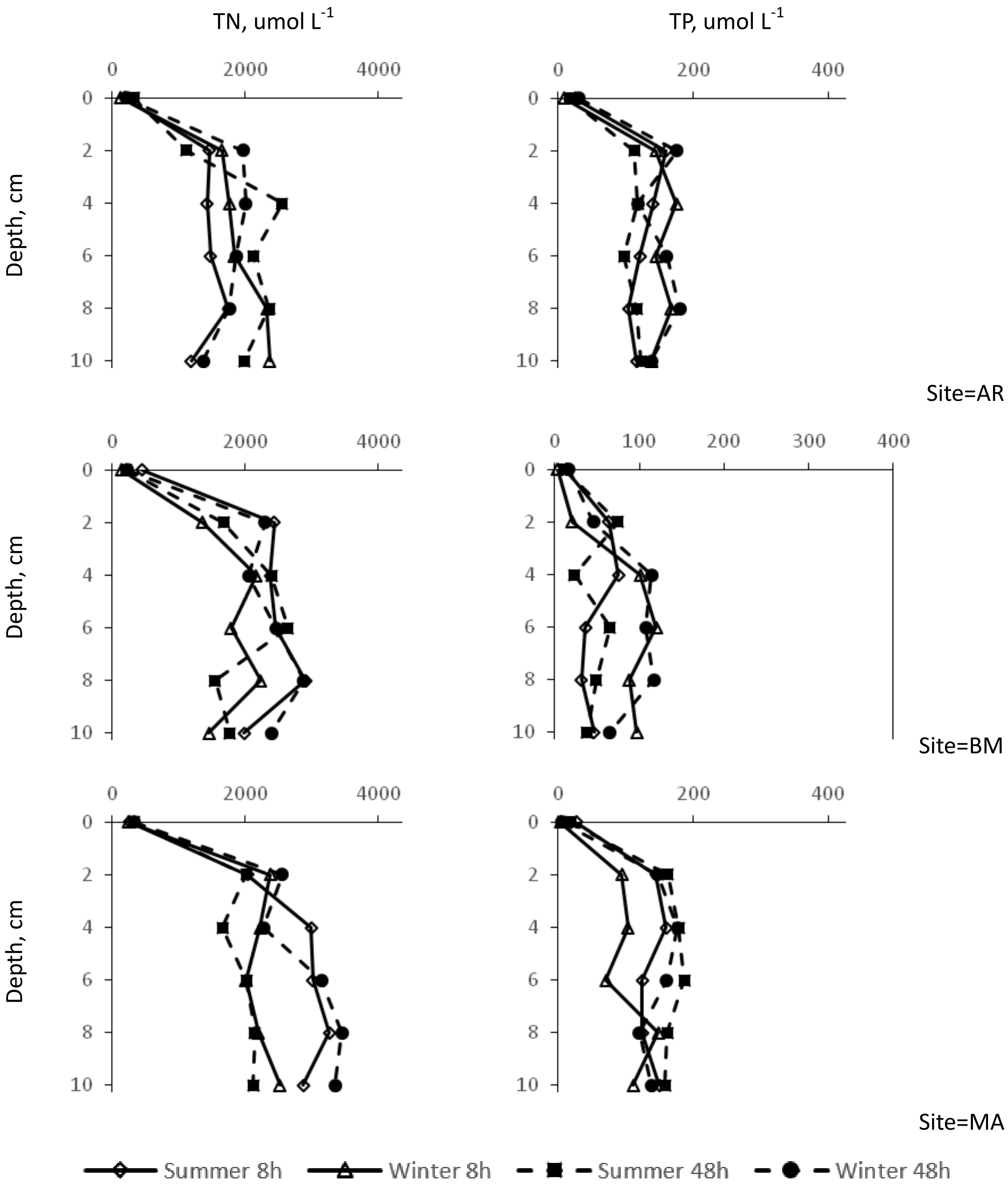

***Figure 8.** TN and TP in the pore water in the pore water after 8 and 48 h of incubation for spring and winter*

Nutrient flux

DO and nutrient flux at sediment–water interface varied widely from release to uptake within 4 h and two days of incubation (*Fig. 9*). Oxygen and nutrient fluxes after the first 4 h of incubation were almost ten times higher than after the total 48 h of incubation. Oxygen flux varies over similar ranges in summer and winter, from -2.54 to -2.09 mg m^{-2} D^{-1} and from -2.1888 to -1.8504 mg m^{-2} D^{-1} after the first 4 h incubation. Significant differences ($p < 0.05$) were found between seasons at site MA in the first 4 h

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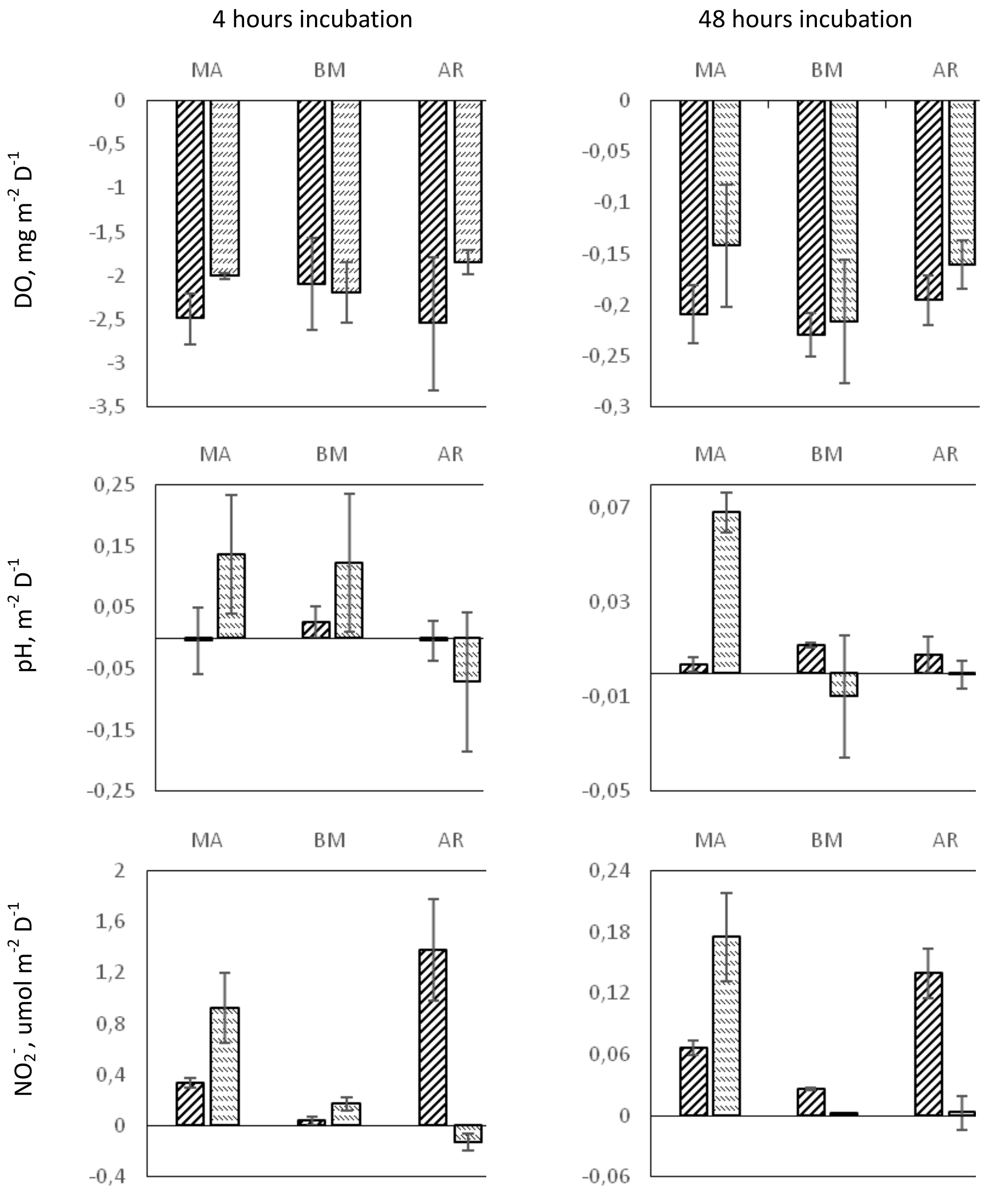
of incubation, where higher oxygen fluxes were found in summer. The difference determined during the two seasons suggests that the higher oxygen consumption coincided with higher macroagal decomposition, similar results were observed by Warnken et al. (2003). Nitrite fluxes were relatively low, in the range of 0.038 to 1.34 umol m^{-2} D^{-1} in summer and in the range of -0.1263 to 0.9210 umol m^{-2} D^{-1} in winter after the 4 h of incubation. Significant differences between seasons and among sites were observed. Nitrite uptake by the sediment after the first 4 h of incubation was demonstrated at site AR in winter. The maximum ammonium fluxes were recorded at site MA in winter after the first 4 h of incubation and at site BM in summer after the 48 h of incubation. Ammonium uptake by sediment was observed at site AR in both seasons after the 4 h of incubation. Phosphate exchange was generally very low, except for the high flux at site AR.



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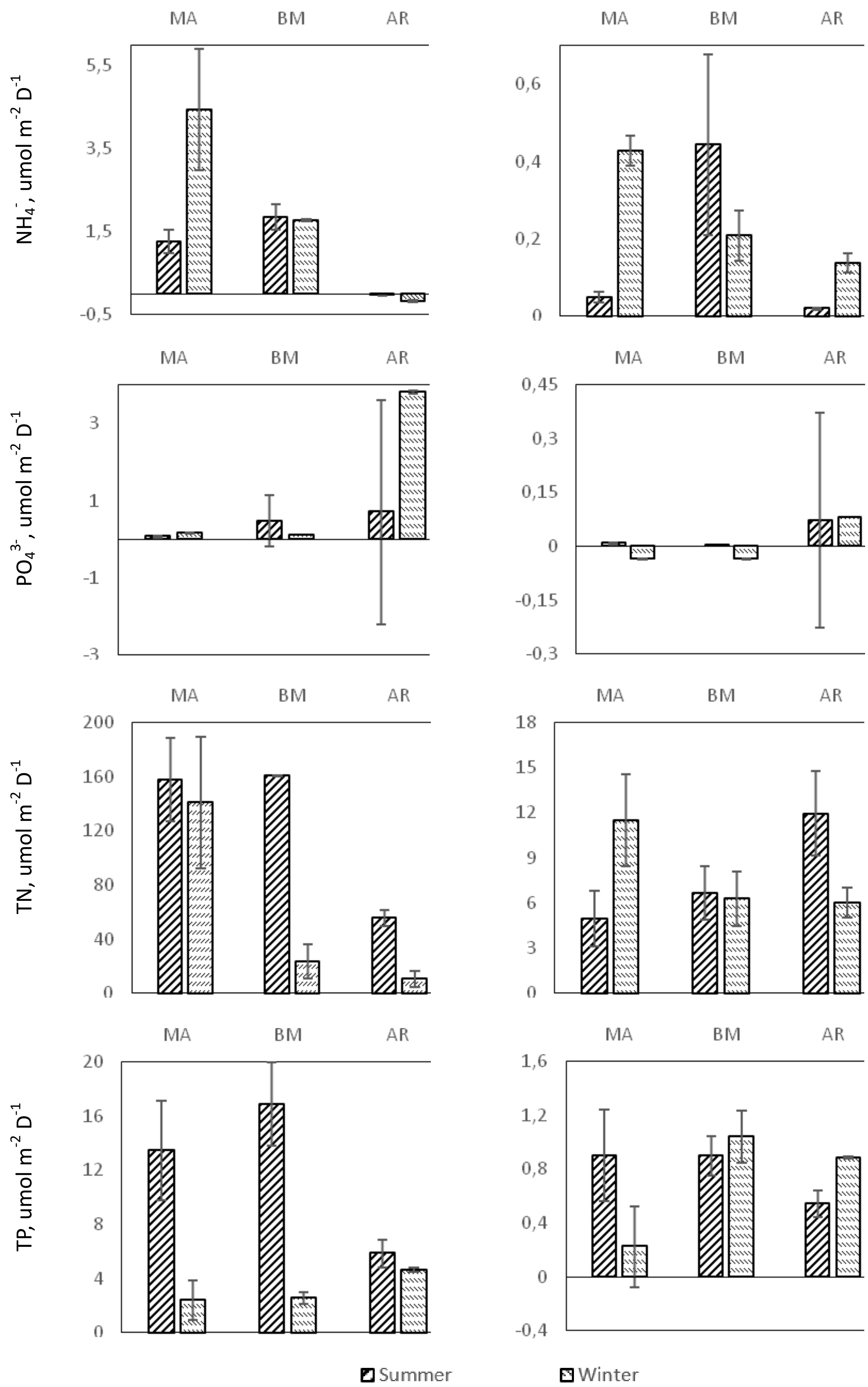

Figure 9. *Oxygen, pH and nutrient flux after 4 h and 48 h of incubation in summer and winter*

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Average fluxes were in the range -0.1743 to 4.4478 umol m^{-2} D^{-1}. TN and TP were generally released from the sediment to the overlying water. A significant difference was observed between seasons after the 4 h of incubation, except at site MA. After 48 h of incubation, the highest TN and TP fluxes were recorded at site AR in summer, at site MA in winter, at site MA in summer and at site BM in winter.

Discussion

Benthos activities such as microorganism (Hines et al., 1982), shellfish (Castel, 1984; Chen et al., 2016b; 2005; Zhang et al., 2011; Zheng 2011), crabs (Zheng, 2011), and worms (Chen et al., 2016a) elicited rapid changes to nutrient exchange at sediment–water interface. These burrowing organisms bioturbate the sediment effectively flushing the nutrient and oxygen-rich water to considerable depth (Chen et al., 2016a; Miller, 1984; Rakotomalala et al., 2015). Spatial distribution of marine organisms in Zhelin Bay marine ranching area could be crucial to nutrient flux. Shu et al. (2015) reported that the *k*-dominance curve of macro-benthic abundance in Zhelin bay which showed that the seasonal trend in species diversity was winter > spring ≈ autumn > summer. Otherwise, survey results showed that microbenthic in site BM was more than 2.0 times of site AR and 1.5 times of site MA (Chen et al., 2015), and highest molluscs density in site BM would be 200 ind. m^{-2}(Qin et al., 2016).

The pore water phosphate^{-} concentration showed a gradual increase with depth, and ammonium concentrations varied from 2.428 to 55.68 umol L^{-1} and from 1.996 to 54.03 umol L^{-1} in sites BM and MA respectively at both sites BM and MA for both seasons. Nitrite concentration showed a similar trend to ammonium. A similar trend was generally observed for nitrite concentration and phosphorus concentration (Denis and Grenz, 2003) and ammonia concentration (Sakamaki et al., 2006). Conversely, our results did not correspond with calculated in Bohai Bay coastal zone by Mu et al. (2016) who reported nitrite and DIN decreased with sediment depth.

Otherwise, decomposing organisms affected biogeochemical conditions of sediment (Chelsky et al., 2016; Wang et al., 2016). At site MA, higher nitrite, ammonia and phosphate fluxes were found in the winter, which was the same as the large scale macroalagae culture in this season. The high bloom of macroalgae would eventually become senescent and die. Decomposition of macroalgae stimulated sediment oxygen demand and an efflux of nitrogen and phosphorus (Wang et al., 2016). Welsh (2003) and Chelsky et al. (2016) study results showed that the physical barrier created by organism decomposition, reduces the transport of oxygen into the deeper sediment, however, our study did not show a similar trend. Benthic organisms, especially deposit feeders, will be present in this area and will be responsible for sediment reworking (Miller, 1984). This may explain the reason we found a similar trend in the MA and BM sites.

Moreover, hydrodynamics is an important factor that will influence sediment-surface water flux; a range of models has been used characteristics (Arndt et al., 2009; Bianchi et al., 2004; D’Itri, 1985; Gardner and Kjerfve, 2006; Hu and Li, 2009; Proctor et al., 2003). The phosphate flux at site AR in this experiment was higher than at the other sites. Also, if artificial reef deployment is to improve and increase fishery resources in the local area, then, it will affect the sediment-surface flux. In the early survey of Zhelin Bay ranching, the results showed higher fishery resources at site AR (Chen et al., 2015). Raoux et al. (2017) and Wu et al. (2016) also obtained higher

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fishery resources in artificial reef area. These biological and physical changes are the main reasons which influence nutrient fluxes.

Conclusion

This preliminary study of different ecological functional zones used in marine ranching is useful for explaining the ecological function of different zones. Macroalgae decomposition showed the effect of overlying water nutrient concentration and nutrient fluxes which led to highest pH variation and highest nitrite, ammonia and total nitrogen fluxes in winter. As a presumed function of bioturbation in site BM, nutrient concentration in surface water increased quickly, while pore water nutrient kept in a stable level. Furthermore, artificial reef deployment changed the physical environment of sea bed and created upwelling at that area, which increased the flux of total phosphate. Our research results provide a basis for more specific studies towards the impacts on biological geochemistry of marine ranching construction. Further investigation should consider the physical feature of the sediment and induce biological species to study the bioturbation and physical changes of ecosystems.

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METHYL JASMONATE AFFECTS POPULATION DENSITIES OF PHYTOPHAGOUS AND ENTOMOPHAGOUS INSECTS IN WHEAT

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Abstract. Methyl jasmonate (MEJA), a well-known herbivore-induced plant volatile, promotes plant defences against various stress factors. The aim of this study was to investigate the effects of different doses of exogenously applied MEJA on the population densities of wheat insect pests and their natural enemies by employing three sampling methods (direct visual sampling, sweep net, sticky traps) in 2012 and 2013 under semi-arid conditions. MEJA treatments had repellent effects on aphids (Hemiptera: Aphididae), phytophagous thrips species (Thysanoptera: Phlaeothripidae and Thripidae) and hoverfly species (Diptera: Syrphidae), whereas it was attractive to wheat stem sawflies (Hymenoptera: Cephidae), lady beetle species (Coleoptera: Coccinellidae) and *Collyria coxator* (Hymenoptera: Ichneumonidae). MEJA treatments had no effect on aphid parasitoids abundances (Hymenoptera: Braconidae). The experimental outcomes varied, depending on plant phenology and sampling method. MEJA treatment also led to reductions in wheat yield and plant height. MEJA treatment could be beneficial as a natural pest control tool when certain species are targeted.
Keywords: *semiochemical, predator, parasitoid, wheat insect pests, Triticum aestivum*

Introduction

Wheat, *Triticum aestivum* L., one of the most important food crops, confronts many stress factors, including insect pests and microbial diseases. Insect pests such as aphids (Hemiptera: Aphididae) and thrips species (Thysanoptera: Phlaeothripidae) reduce grain yield by piercing and sucking the contents of stems, leaves and spikes, while the larvae of wheat stem sawfly species cause decreased yield and grain number by disrupting sap flow as they tunnel into host stems. Therefore, these pests may cause substantial yield losses (Seddigh and Bandani, 2012; Larsson, 2005; Özberk et al., 2005). Extensive use of insecticides is the mainstay method to minimize yield losses, but these treatments are unsustainable and environmentally damaging. Moreover, insecticides are only partially effective against some of the pests e.g., wheat stem sawflies owing to their cryptic egg laying and life cycle. In addition, the natural enemies of these pests can also be harmed by insecticides, which is counterproductive to controlling the pest populations. Therefore, management of insect pests requires better methods.

Plants under herbivore attack release volatile organic plant compounds (VOCs) to minimize damage by settled attackers or to prevent impending dangers (Kessler and Baldwin, 2001; Tan et al., 2012). These herbivore-induced plant volatiles (HIPVs) are not only repellent to insect herbivores, but they also encourage the foraging behavior of natural enemies of the pests (Boughton et al., 2006; Moraes et al., 2009; Mandour et al., 2013). Among a large variety of HIPVs, the jasmonates seem to be key components of

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plant defense systems (Lackman et al., 2011). Even though jasmonic acid (JA) is the most abundant volatile among the jasmonates, exogenous application of methyl jasmonate (MEJA) is generally more effective because of its greater volatility (Beltrano et al., 1998). Previous studies have demonstrated that MEJA induces plant defenses by increasing the activity of lipoxygenase and the production of C6-aldehydes, thus suppressing larval feeding by the hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae) and the cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae) (Avdiuskho et al., 1997). MEJA applications were also found to decrease the population growth of two phloem feeders, the green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) on tomato plants (*Lycopersicon esculentum*) and the potato aphid *Macrosiphum euphorbiae* on potato plants (*Solanum tuberosum*) (Boughton et al., 2006; Brunissen et al., 2010). Population growth of the soybean aphid *Aphis glycines* Matsumura (Hemiptera: Aphididae) and the soybean thrips *Neohydatothrips variabilis* (Beach) (Thysanoptera: Thripidae) was reduced by MEJA application (Selig et al., 2016). In addition, MEJA treatment contributes to plant defenses by attracting natural enemies of pests. For example, MEJA-treated persimmon plants (*Diospyros kaki*) were found to be attractive to the lady beetle *Chilocorus kuwanae* (Coleoptera: Coccinellidae) (Zhang et al., 2009). Furthermore, more parasitic wasps of the family Braconidae were attracted to MEJA-baited traps than unbaited traps in field trials (James, 2005). Another braconid, *Cotesia plutellae*, a larval parasitoid of *Plutella xylostella* (Lepidoptera: Yponomeutidae), preferred MEJA in Y-tube olfactometer tests (Ibrahim et al., 2005). Based on either single-dose studies of jasmonates or the targeting of single organisms under controlled conditions, the majority-opinion in the literature is that MEJA and other jasmonates are repellent to herbivores and attractant to their natural enemies (Birkett et al., 2000; Bruce et al., 2003; El-Wakeil et al., 2010; Dewhirst et al., 2012; El-Wakeil and Volkmar, 2012; Egger and Koschier, 2014). However, more studies of the effects of plant defense elicitors under field conditions are needed to elucidate whether they are useful tools for integrated pest management strategies.

Here, we investigated the influence of different MEJA doses on the population densities of wheat insect pests (aphids, thrips and wheat stem sawflies) and their natural enemies (aphid parasitoids, lady beetles, hoverflies and *Collyria coxator*) in two different plant growth stages. Population fluctuations were monitored by employing three different sampling methods, direct visual sampling, sweep-net sampling and colored (yellow and blue) sticky traps.

Materials and methods

Experimental design

Two years (2012 and 2013) of field experiments were conducted in a wheat field (*Triticum aestivum* L., cv. Pehlivan, the most commonly sown cultivar) established on alluvial soil under semi-arid climate conditions (Diyarbakir, Turkey 37°54'16"N; 40°16'46"E). Standard agronomic practices (e.g., fertilization) were made in our experimental plots. No pesticides were used in our experimental plots throughout of the study. The experimental system consisted of wheat plots established in a randomized complete block design. Each plot was 3×3 m^2 (4 replications) and 4×2 m^2 (3 replications) in 2012 and 2013, respectively. The distance between experimental plots and other cultivated or non-cultivated areas was at least 2 m.

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Exogenous MEJA treatments

Plants were treated with MEJA (≥96% purity) (Sigma-Aldrich) twice in each year (2012 and 2013). The first and second treatments were performed at wheat growth stages GS-37th (flag leaf just visible, still rolled) and GS-59th (End of heading: inflorescence fully emerged) according to BBCH scale for cereals, respectively (Witzenberger et al., 1989; Lancashire et al., 1991). Three doses of MEJA (14.2 mM, 7.1 mM and 3.55 mM, of which the highest dose was applied by McEwen (2011) to cruciferous plants) were employed as the first treatment in year 1. However, we lowered the doses to 3.55 mM, 1.76 mM and 0.88 mM for all subsequent treatments (i.e. the second treatment of year 1 and both treatments of year 2) due to high phytotoxic effects observed one day after the first treatment of year 1. Silwet Gold (Chemtura) surfactant at a ratio of 10 ml per 100 L of water was added to each dose in all treatments. Plants in control plots were treated with distilled water and surfactant only. A hydraulic backpack sprayer was used to apply the treatments.

Sampling methods

Direct visual samplings

Presence of phytophagous and entomophagous insects was checked on all above-ground parts of ten plants. All biological forms, i.e. from egg to adult, were checked by using a 5× hand magnifier and recorded. Samplings were carried out 1 day prior to and 1, 3, 10 and 17 days after the MEJA treatments. There was a 4-day interval between the last sampling of the first treatment period and the first sampling of the second treatment period in both years.

Sweep-net samplings

A standard sampling net (R = 35 cm) was used to sweep samples from the experimental plots uniformly. The collected samples were put into clear plastic bags with a paper towel to reduce humidity and then labeled. The samples were transferred to the laboratory and put in a freezer (-20°C). All specimens were recorded afterward. Sweep-net sampling intervals were the same as those of the direct visual samplings.

Colored sticky trap samplings

Yellow and blue sticky traps (Kapar Organic, 20×25 cm) were placed in the middle of MEJA-treated and untreated control plots one week prior to the first samplings in both years to sample the population densities of flying wheat insect pests and their natural enemies. Data concerning the insects caught by the traps were recorded. The traps were cleaned with a wooden spatula and the insect-catching sticky substrate (Kapar Organic) was renewed when needed. The sampling intervals for the colored sticky traps were the same as those of the direct visual samplings, except that no samplings were conducted on day 1 after the treatments.

Effects of MEJA on agronomic properties

Seventy-five randomly chosen heads were collected from each plot in mid-June, at harvest time. Grains were extracted from the heads and separated from the glume by hand. Grains were kept under laboratory conditions until weighed. In addition, the heights of 10 wheat plants per plot were measured at the the GS 92nd (over-ripe: grain very hard, cannot be dented by thumbnail) according to BBCH scale.

Statistical analysis

All log (n + 1) tranformed experimental data were checked for normality (Shapiro–Wilk's test) and homogeneity of variances (Levene's test) before analysis. To reveal effects of MEJA treatments on wheat insect pests and their natural enemies, data of each year and treatment periods were analyzed by fitting generalized linear mixed models (GLMM, lme4 package) with poisson error distribution (log link function) (Diggle et al., 2002, Bolker et al., 2009, Bates et al., 2015). MEJA doses were fixed factor whereas sampling dates were random factor for all cases. The fixed factor effects were tested using likelihood-ratio (LR) tests ($p < 0.05$). Tukey's post hoc tests were used to compare the means at the 95% confidence level (with the glht function of the multcomp package). The effects of MEJA treatment on plant height and yield were analyzed with one-way ANOVA. Means of all data were separated using the Tukey's multiple comparison test at the 95% confidence level. Insect taxa which did not respond to MEJA treatment in any treatment period or experimental year are not presented in the figures, but their statistical results are presented in *Table 1*. All statistical analyses were performed with R software, version 3.4.0 (R Core Team, 2017).

Results

Effects of different methyl jasmonate doses on some insect pests

Aphid species (Hemiptera: Aphididae)

There were two aphid species in our experimental area, *Sitobion avenae* (F.) and *Rhopalosiphum padi* (L.).

Direct visual samplings of apterous aphids: Generalized linear mixed model analysis revealed that the aphid densities in MEJA (0.88 mM and 3.55 mM)-treated wheat plots were lower than in control plots for the second treatment period and pooled whole season of year 1 ($\chi^2_{2012\text{-ST}} = 89.94$, $P < 0.001$, $\chi^2_{2012\text{-whole}} = 87.10$, $P < 0.001$; *Fig. 1A*). Data of the first treatment period of year 1 showed no significant differences among MEJA dose treatments ($P > 0.05$, *Table 1*). The population densities of aphid species on MEJA-treated and untreated plants were not different in the pooled whole-season and data of both treatment periods of year 2 ($P > 0.05$, *Table 1*).

Sweep-net sampling for apterous aphids: The population densities of apterous aphids swept from MEJA-treated and untreated control plants were not significantly different throughout the study. No apterous aphids were sampled in the first treatment period of year 2 ($P > 0.05$, *Table 1*).

Direct visual sampling of alate aphids: Data of whole season of year 1 demonstrated significant differences between the mean densities of alate aphids in MEJA-treated and control plants while alate aphid densities did not differ between treatments for first and second treatment periods ($\chi^2_{2012\text{-FT}} = 1.83$, $P = 0.607$; $\chi^2_{2012\text{-ST}} = 4.15$, $P = 0.245$; $\chi^2_{2012\text{-whole}} = 18.66$, $P < 0.001$; *Fig. 1B*). However, there were no significant differences between alate aphids visually counted on plants between MEJA-treated plants and controls in year 2 ($P > 0.05$, *Table 1*). Furthermore, we did not encounter any alate aphid individuals on any experimental plants in the second treatment period of year 2.

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Table 1. *Summary of the data analysis (GLMM) indicating the influence of methyl jasmonate (MEJA) treatments on some insect species sampled by different sampling methods (data pooled over sampling intervals within treatment periods and whole season). NI: no insect recorded (df = 3 for all cases)*

Pest insects	**Year**	**Sampling method**	**First treatment**		**Second treatment**		**Whole season**	
			χ^2	***P***	χ^2	***P***	χ^2	***P***
Apterous aphids	2012	Visual Sampling	3.52	0.317	89.94	<0.001	87.10	<0.001
		Sweep-net	0.86	0.833	6.76	0.079	5.56	0.134
	2013	Visual sampling	3.02	0.387	6.24	0.100	2.57	0.460
		Sweep-net	NI		2.72	0.435	5.13	0.162
Alate aphids	2012	Visual sampling	1.83	0.607	4.15	0.245	18.66	<0.001
		Sweep-net	2.34	0.504	3.77	0.286	5.53	0.136
		Yellow traps	2.02	0.567	0.46	0.925	1.53	0.675
	2013	Visual sampling	3.02	0.387	NI		0.02	0.999
		Sweep-net	2.92	0.403	4.12	0.248	5.21	0.156
		Yellow traps	12.16	0.006	19.98	<0.001	15.16	0.001
Thrips	2012	Visual sampling	3.30	0.346	1.81	0.612	1.63	0.651
		Sweep-net	17.67	<0.001	19.22	<0.001	31.50	<0.001
	2013	Visual sampling	3.02	0.387	1.01	0.796	0.08	0.993
		Sweep-net	1.97	0.577	1.81	0.612	2.74	0.433
Wheat stem sawflies	2012	Sweep-net	2.41	0.490	2.64	0.450	2.32	0.508
		Yellow traps	16.55	<0.001	44.33	<0.001	47.34	<0.001
		Blue traps	1.45	0.693	5.31	0.150	2.63	0.451
	2013	Sweep-net	2.44	0.485	1.45	0.693	2.80	0.423
		Yellow traps	5.08	0.165	1.53	0.673	0.39	0.940
		Blue traps	0.20	0.649	4.28	0.232	4.43	0.218
Natural enemies								
Aphid parasitoids	2012	Visual Sampling	0.32	0.954	1.53	0.673	1.80	0.614
		Sweep-net	1.28	0.733	5.68	0.128	4.89	0.179
		Yellow traps	0.42	0.934	1.65	0.646	0.39	0.941
	2013	Visual Sampling	2.66	0.445	3.02	0.388	0.03	0.998
		Sweep-net	1.29	0.729	1.81	0.612	3.62	0.305
		Yellow traps	0.40	0.938	1.53	0.673	0.39	0.940
Ladybirds	2012	Visual sampling	NI		20.95	<0.001	20.22	<0.001
		Sweep-net	3.15	0.367	4.65	0.198	5.27	0.152
		Yellow traps	1.44	0.694	13.92	0.003	12.31	0.006
		Blue traps	NI		1.53	0.673	1.40	0.704
	2013	Visual sampling	2.02	0.567	1.45	0.693	0.29	0.960
		Sweep-net	3.38	0.336	1.37	0.710	2.10	0.551
		Yellow traps	2.66	0.445	1.81	0.612	2.90	0.406
		Blue traps	NI		3.56	0.312	3.44	0.327
Syrphid flies	2012	Sweep-net	3.15	0.367	2.19	0.533	3.22	0.357
		Yellow traps	1.44	0.694	6.13	0.105	4.73	0.192
		Blue traps	6.72	0.081	23.09	<0.001	24.80	<0.001
	2013	Sweep-net	4.98	0.172	1.61	0.655	1.45	0.693
		Yellow traps	6.02	0.110	0.57	0.901	2.86	0.412
		Blue traps	6.65	0.083	2.19	0.530	3.84	0.278
C. coxator	2012	Sweep-net	10.64	0.013	0.88	0.827	10.07	0.017
		Yellow traps	13.19	0.004	29.64	<0.001	41.11	<0.001
		Blue traps	2.94	0.400	1.47	0.688	3.89	0.273
	2013	Sweep-net	5.77	0.123	0.57	0.901	6.12	0.105
		Yellow traps	27.37	<0.001	12.11	0.006	37.31	<0.001
		Blue traps	0.48	0.92	2.93	0.402	1.74	0.627

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Sweep-net sampling for alate aphids: There were no significant effects of MEJA treatments on alate aphids collected by sweep-net sampling during the entire experiment ($P > 0.05$, *Table 1*).

Yellow sticky trap samplings for alate aphids: In both treatment periods and pooled whole-season data of year 1, the population densities of aphid species in MEJA-treated and untreated plants did not differ significantly ($P > 0.05$, *Table 1*). Evaluation of data of the first treatment period of year 2 demonstrated number of aphids caught on yellow traps were higher in 0.88 mM MEJA- treated plots than control plots ($\chi^2_{2013\text{-}FT} = 12.16$, $P = 0.006$). However, 1.76 mM MEJA- treated plots had lower number of alate aphids than control and 0.88mM MEJA- treated plants in both second treatment period and whole season of year 2 ($\chi^2_{2013\text{-}ST} = 19.98$, $P < 0.001$; $\chi^2_{2013\text{-whole}} = 15.16$, $P = 0.001$; *Fig. 1E*).

Phytophagous thrips species

Eleven phytophagous thrips species belonging to two families of Thysanoptera were recorded during the experiments. The numbers of *Haplanthrips tritici* (Kurdjumov), *H. aculeatus* (Fabricius) and *H. reuteri* (Karny) of family Phlaeothripidae were higher than those of *Frankliniella tenuicornis* (Uzel), *Thrips tabaci* (Lindeman), *Melanthrips pallidior* (Priesner), *T. angusticeps* (Uzel), *Kakothrips priesneri* (Pelikan), *Limothrips cerealium* (Haliday), *L. angulicornis* (Jablonowski) and *M. fuscus* (Sulzer) of family Thripidae.

Direct visual sampling of phytophagous thrips: There were no significant differences between the population densities of phytophagous thrips species sampled by direct counting on plants in pooled whole-season data and both treatment periods of both years ($P > 0.05$, *Table 1*).

Sweep-net sampling of phytophagous thrips: Evaluation of the first treatment period and whole season data of year 1 revealed that number of thrips in all MEJA treated plots were lower than control plots ($\chi^2_{2012\text{-}FT} = 17.67$, $P < 0.001$; $\chi^2_{2012\text{-whole}} = 31.50$, $P < 0.001$; *Fig 1C*). In addition, in year 1, MEJA-treated plants (0.88 mM) had lower population densities of pest thrips than control plants in the second treatment period ($\chi^2_{2012\text{-}ST} = 19.22$, $P < 0.001$; *Fig. 1C*). In contrast, in year 2, phytophagous thrips populations sampled by sweep-net from MEJA-treated and control plants did not differ significantly based on either pooled whole-season data or treatment periods ($P > 0.05$, *Table 1*).

Wheat stem sawfly species

Throughout the experiments, two wheat stem sawfly (Hymenoptera: Cephidae) species were collected, of which *Cephus pygmaeus* (L.) was more prevalent than *Trachelus tabidus* (F.).

Sweep-net samplings of wheat stem sawfly species: Wheat stem sawfly population densities determined by sweep-net of MEJA-treated and untreated plants were not significantly different for either whole-season pooled data or between treatment periods in both years ($P > 0.05$, *Table 1*).

Yellow sticky trap samplings of wheat stem sawfly species: Yellow sticky trap sampling analysis revealed that the mean densities of wheat stem sawflies in MEJA-treated plants were significantly higher than in control plants in whole-season pooled

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data, the first and the second treatment periods of year 1 ($\chi^2_{2012\text{-FT}} = 16.55$, $P < 0.001$; $\chi^2_{2012\text{-ST}} = 44.33$, $P < 0.001$; $\chi^2_{2012\text{-whole}} = 47.34$; $P < 0.001$; *Fig. 1D*). In year 2, there were no significant differences between or among MEJA treatments and/or control plants in both whole-season pooled data and the treatment periods ($P > 0.05$, *Table 1*).

Blue sticky trap sampling of wheat stem sawfly species: The population densities of wheat stem sawflies in MEJA-treated and untreated control plants, as observed by blue sticky traps, did not differ significantly in both whole-season pooled data and the treatment periods of both years ($P > 0.05$, *Table 1*).

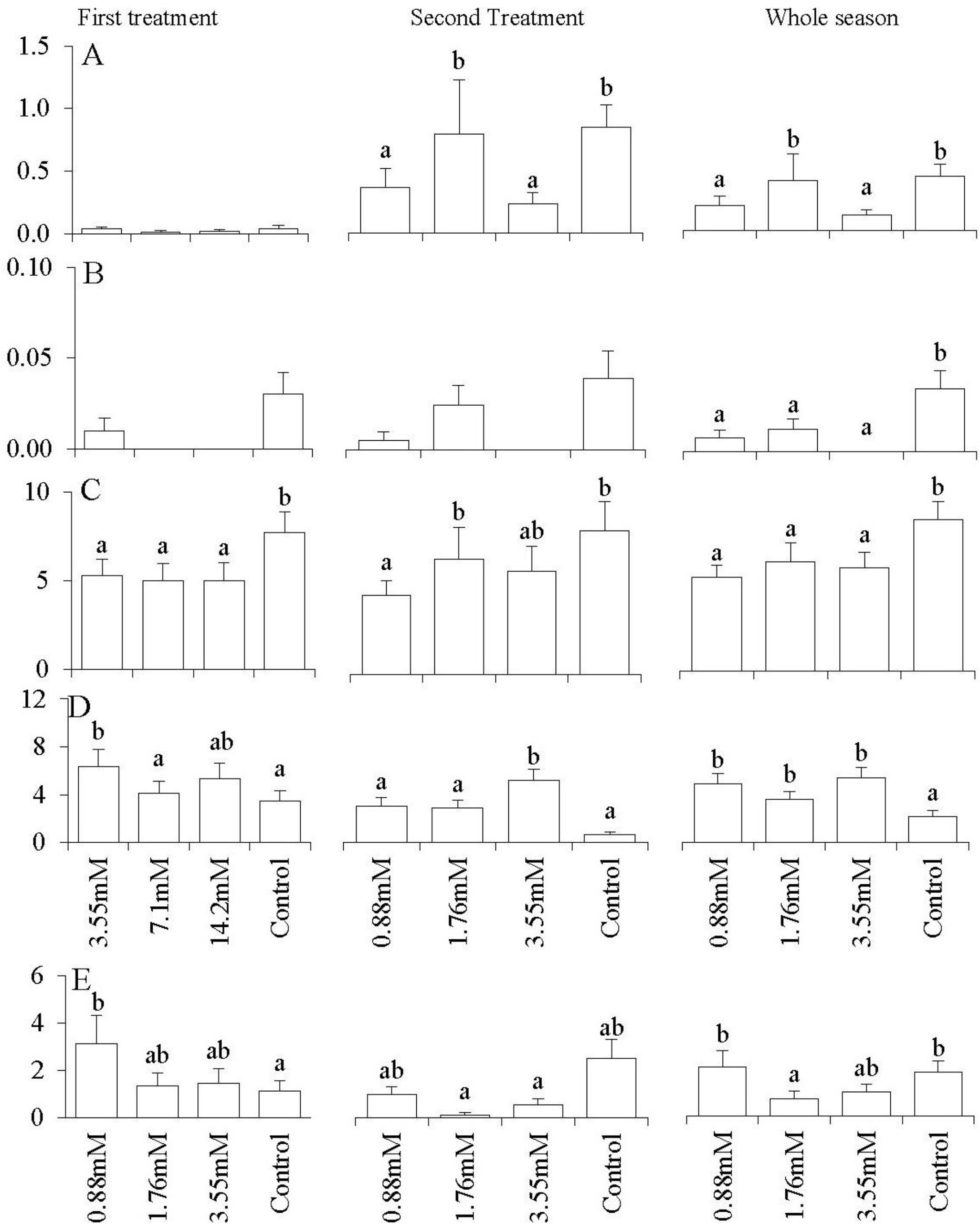

Figure 1. *Mean density (+SEM) of apterous aphids per plant in 2012 (A), alate aphids per plant in 2012 (B), pest thrips/ sweep-net in 2012 (C), wheat stem sawflies/ yellow traps in 2012 (D) and alate aphids/yellow traps in 2013 (E) in wheat plots treated with different methyl jasmonate doses for first-treatment and second-treatment periods and whole season (Means capped with different letters differ significantly, Tukey's multiple comparison test, P < 0.05)*

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Effects of methyl jasmonate treatments on parasitoids and predators of wheat pests

Aphid parasitoids

Three braconid aphid parasitoids, *Aphidius ervi*, *Praon gallicum* (Stary) and *Binodoxys acalephae* (Marshall) were collected during our experiments, and *A. ervi* was the most prevalent species.

Direct visual samplings for aphid parasitoids: There were no differences in the population densities of aphid parasitoids in MEJA- treated and control plants in whole-season pooled data and the treatment periods of year 1 and year 2 ($P > 0.05$, *Table 1*).

Sweep-net samplings for aphid parasitoids: The population densities of aphid parasitoids in MEJA- treated and untreated plants determined by sweep-net samplings were not significantly different in both pooled seasonal data and the treatment periods throughout the study ($P > 0.05$, *Table 1*).

Yellow sticky trap sampling of aphid parasitoids: Yellow sticky trap sampling results demonstrated that the population densities of aphid parasitoids in MEJA-treated and untreated plants were not significantly different in both pooled whole season data and the treatment periods ($P > 0.05$, *Table 1*).

Lady beetles (Coleoptera: Coccinellidae)

Three predatory lady beetle (Coleoptera: Coccinellidae) species were recorded throughout our study, namely *Coccinella septempunctata*, *Adalia bipunctata* (L.) and *C. undecimpunctata* (L.), of which *C. septempunctata* was the most abundant.

Direct visual samplings of lady beetles: The population density of lady beetles in plants treated with 3.55 mM MEJA was higher than those in the other treatments and the controls for the second treatment period. Accordingly, the density of lady beetles directly sampled on plants treated with highest dose were higher than other treatments and control plots in whole-season pooled data, while no individual lady beetle was recorded in the first treatment period ($\chi^2_{2012\text{-ST}} = 20.95$, $P < 0.001$; $\chi^2_{2012\text{-whole}} = 20.22$, $P < 0.001$; *Fig. 2A*). MEJA treatments did not have any significant effect on the population densities of lady beetles in year 2 ($P > 0.05$, *Table 1*).

Sweep-net sampling of lady beetles: Throughout the study, there were no significant differences in population densities of lady beetles determined by sweep-net sampling between MEJA-treated and untreated control plants ($P > 0.05$, *Table 1*).

Yellow sticky trap sampling of lady beetles: The population densities of lady beetles caught on yellow sticky traps in treated and untreated plants did not differ significantly from one another in first treatment period of year 1 and second treatment period and whole-season pooled data of year 2 ($P > 0.05$, *Table 1*). However, number of lady beetles caught on yellow traps in 3.55 mM MEJA-treated plants were higher than control plots in the second application period and whole season pooled data of year 1 ($\chi^2_{2012\text{-ST}} = 13.92$, $P = 0.003$; $\chi^2_{2012\text{-whole}} = 12.31$, $P = 0.006$; *Fig. 2B*).

Blue sticky trap samplings of lady beetles: In the first treatment periods of both years, we did not encounter any lady beetle individuals on blue sticky traps. In the second treatment periods and whole season data of both experimental years, the differences between MEJA-treated and untreated control plants were not significant ($P > 0.05$, *Table 1*).

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Hoverflies (Diptera: Syrphidae)

The hoverfly species collected consisted of four species, *Eupeodes corollae* (Fabricius), *Sphaerophoria scripta* (L.), *Melanostoma mellinum* (L.) and *S. turkmenica* (Bankowska). Of these species, *E. corollae* (F.) was the most prevalent.

Sweep-net samplings of hoverfly species: There were no significant differences in the numbers of hoverflies sampled from MEJA- treated and untreated control plants in both treatment periods and whole-season pooled data of both years ($P > 0.05$, *Table 1*).

Yellow sticky trap samplings of hoverflies: The population densities of hoverfly adults caught on yellow sticky traps were not significantly different between the MEJA-treated and control plants in both experimental years ($P > 0.05$, *Table 1*).

Blue sticky trap sampling of hoverflies: Data of the first treatment period of year 1 showed that there were no significant differences in the numbers of hoverfly adults caught on blue sticky traps between MEJA-treated and untreated plants ($P > 0.05$, *Table 1*). However, in the second application period, plants treated with 0.88 mM MEJA had a lower number of hoverflies compared to plants treated with other MEJA doses and control plants, which resulted in a significant reduction of the population density in 0.88 mM MEJA-treated plants over all treatments, including controls ($\chi^2_{2012\text{-}ST} = 23.09$, $P < 0.001$; $\chi^2_{2012\text{-whole}} = 24.80$, $P < 0.001$; *Fig. 2C*). In year 2, there were no differences between treatments in the whole-season data and the treatment periods ($P > 0.05$, *Table 1*).

A larval parasitoid of wheat stem sawfly species; Collyria coxator (Villers) (Hymenoptera: Ichneumonidae)

Sweep-net samplings of *C. coxator*: The population densities of *C. coxator* on the 14.2 mM MEJA-treated plants were higher than on the control plants in the first treatment period of year 1, which resulted in an increase of the *C. coxator* population density in plants treated with the highest MEJA dose over all treatments for the whole-season pooled data ($\chi^2_{2012\text{-}FT} = 10.64$, $P = 0.013$; $\chi^2_{2012\text{-whole}} = 10.07$, $P = 0.017$; *Fig. 2D*). However, there were no significant differences between the population densities of *C. coxator* in MEJA-treated and untreated control plants in the second treatment period of year 1 or in the pooled whole-season data and the treatment periods of year 2 ($P > 0.05$, *Table 1*).

Yellow sticky trap samplings of *C. coxator*: The population densities of *C. coxator*, as determined by yellow sticky traps, were significantly higher on MEJA-treated plants compared to control plants in both whole-season pooled data and the two treatment periods of both experimental years ($\chi^2_{2012\text{-}FT} = 13.19$, $P = 0.004$; $\chi^2_{2012\text{-}ST} = 29.64$, $P < 0.001$; $\chi^2_{2012\text{-whole}} = 41.11$, $P < 0.001$; $\chi^2_{2013\text{-}FT} = 27.37$, $P < 0.001$; $\chi^2_{2013\text{-}ST} = 12.11$, $P = 0.006$; $\chi^2_{2013\text{-whole}} = 37.31$, $P < 0.001$; *Fig. 2E* and *2F*).

Blue sticky trap samplings of *C. coxator*: *C. coxator* population densities, as determined by blue sticky traps, did not differ significantly between MEJA-treated and untreated control plants in both whole-season pooled data and the treatment periods of both years ($P > 0.05$, *Table 1*).

Effects of methyl jasmonate doses on wheat agronomic properties

The mean weights of 75 heads collected from MEJA-treated plants were significantly lower compared to untreated control plants in year 1, whereas the year-2 experiments

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showed no differences between or among treatments ($F_{2012} = 10.527$; d$f = 3$, 12; $P = 0.001$; $F_{2013} = 2.326$; d$f = 3$, 8; $P = 0.151$; *Fig. 3*).

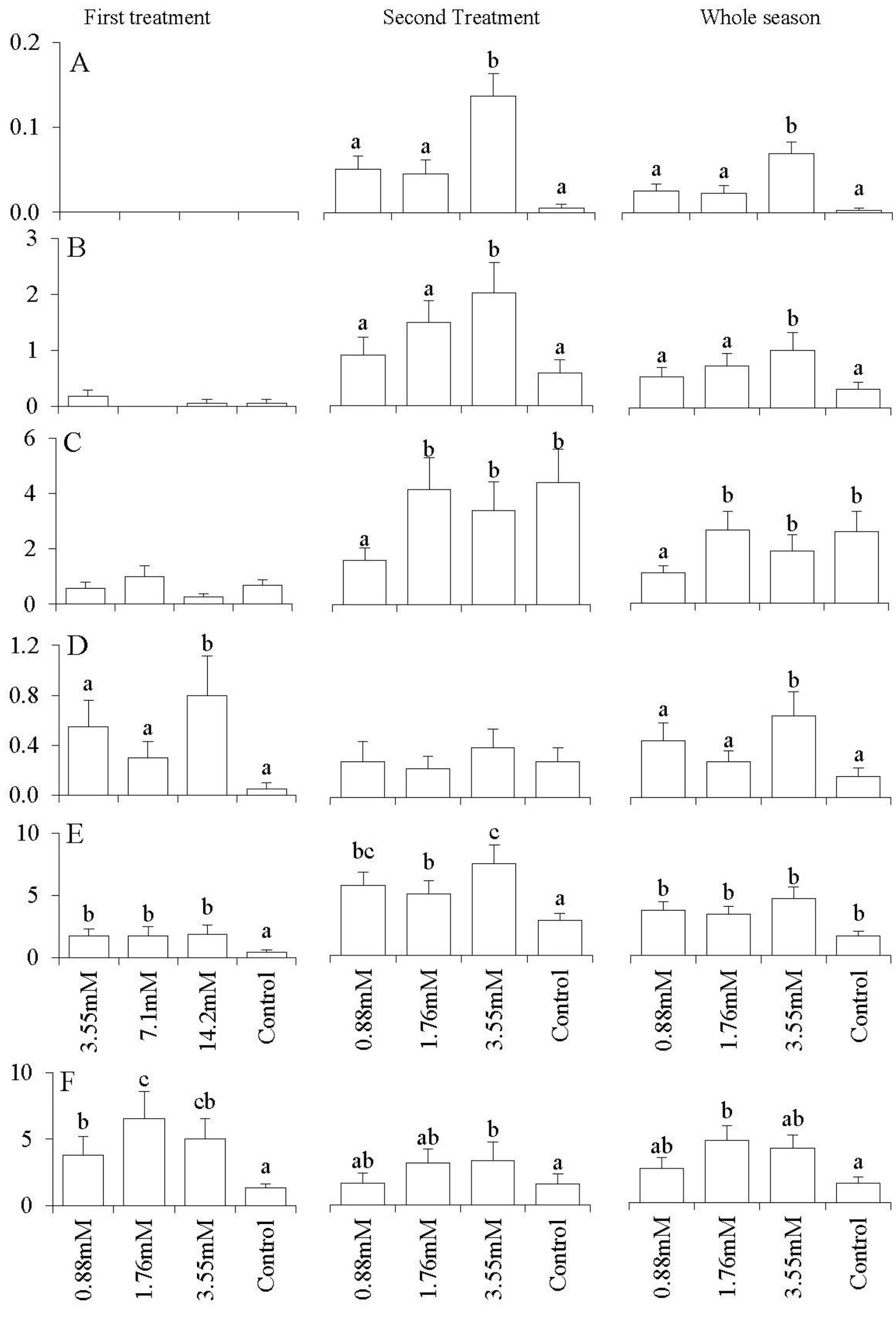

Figure 2. *Mean density (+SEM) of ladybeetles per plant in 2012 (A), ladybeetles/yellow traps in 2012 (B), hoverflies/blue traps in 2012 (C), C. coxator/sweep-net in 2012 (D), C. coxator/yellow traps in 2012 (E) and 2013 (F) in wheat plots treated with different methyl jasmonate doses for first-treatment and second-treatment periods and whole season (Means capped with different letters differ significantly, Tukey's multiple comparison test, P < 0.05)*

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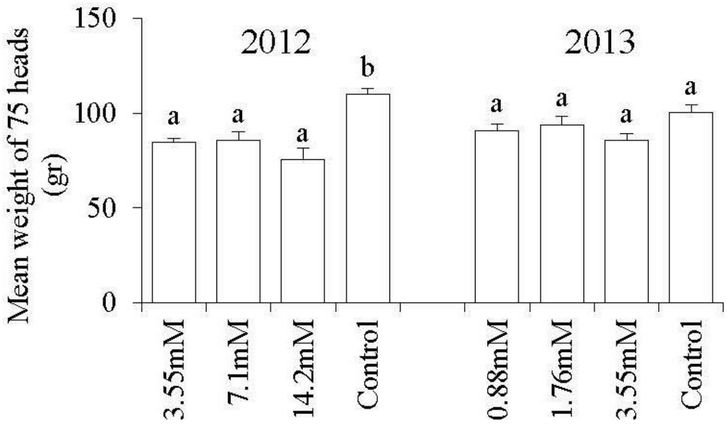

Figure 3. *Mean weight (+SEM) of 75 wheat heads from MEJA-treated and untreated control plants in 2012 (year 1) and 2013 (year 2). Means capped with different letters in the same year differ significantly (Tukey's test: P < 0.05)*

The mean heights of MEJA-treated plants were found to be significantly shorter than those of control plants in both years ($F_{2012} = 137.859$, df = 3, 156, $P < 0.001$; $F_{2013} = 106.402$, df = 3, 116, $P < 0.001$; *Fig. 4*).

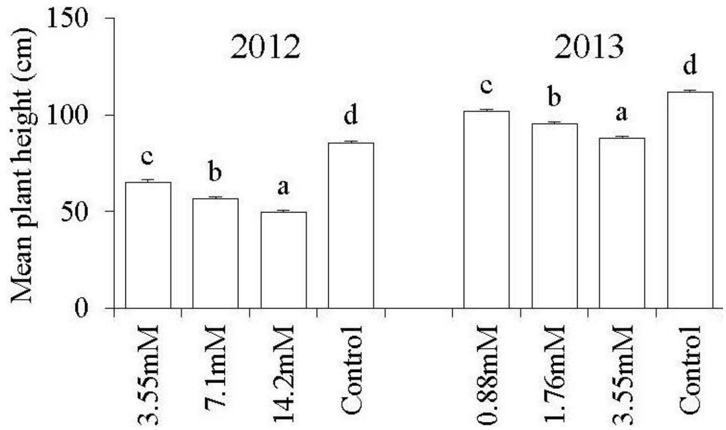

Figure 4. *Mean height (+SEM) of wheat plants from MEJA-treated and untreated plants in 2012 (year 1) and 2013 (year 2). Means capped with different letters in the same year differ significantly (Tukey's test: P < 0.05)*

Discussion

Results of the present study revealed that exogenous MEJA treatments had significant differential effects on both herbivores and their natural enemies in wheat.

Effects of different methyl jasmonate doses on some insect pests

Aphids

The population densities of apterous and alate aphids on MEJA-treated plants were significantly lower than those on control plants in both years, as assessed by direct visual counting, and the population densities of alate aphids determined by yellow sticky traps in MEJA-treated plants were lower than those of control plants in year 2.

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However, the sweep-net method registered no differences in aphid densities among wheat plants, treated and untreated. Our results of direct visual sampling and yellow trap sampling are in agreement with results of several previous investigations. For example, the population growth of the mustard aphid *Lipaphis erysimi* on Indian mustard plants (*Brassica juncea*) was reduced by MEJA treatments (Koramutla et al., 2014). In addition, electronic penetration recording data suggested reduced feeding of apterous *R. padi* on MEJA-treated wheat seedlings, which led to a reduction in the population growth of this pest (Slesak et al., 2001). Another population growth test revealed potential population growth- and performance-suppressing effects of MEJA-treated tomato plants on the phloem feeder *M. persicae* (Boughton et al., 2006). Also, the number of *R. padi* settling on MEJA-treated barley (cv. Kara) was found to be lower than for unexposed plants during natural daylight (Glinwood et al., 2007). Further tests revealed that reproductive performance of the Russian wheat aphid *Diuraphis noxia* was lower on barley plants (cv. Makoei) whose parent seeds had been treated with MEJA (Taami and Dolatti, 2013).

Three main jasmonate derivatives — jasmonic acid (JA), *cis*-jasmone (CJ) and MEJA — are known to have repellent, deterrent and/or antixenotic effects on aphid species attacking cereals and cotton (Omer et al., 2001, Bruce et al., 2003; El-Wakeil et al., 2010; El-Wakeil and Volkmar, 2012). The mechanism behind this aphid resistance is multifold and may be due to: increased hydroxamic acid content and inhibition of trypsin activity in wheat; activation of resistance-related enzymes such as polyphenol oxidase and peroxidase in wheat, tomato and Indian mustard; and up-regulation of jasmonate biosynthetic genes in these plants (Slesak et al., 2001; Boughton et al., 2006; Cao et al., 2014; Koramutla et al., 2014). Wild-type *Arabidopsis* and a constitutively active jasmonate signaling mutant both repelled *M. persicae* when treated with MEJA (Ellis et al., 2002). However, despite advances in understanding the molecular mechanisms (e.g. in terms of enzymes, non-enzyme proteins and genes) by which exogenous jasmonate treatment deters aphids, much less is known about the effects of MEJA on aphids under natural conditions.

The significant repellent effects of MEJA on aphids, as detected by visual counting (2012) and yellow traps (2013), occurred in different years. Therefore, whether HIPV treatments encourage apterous aphids to produce alate aphids for 'take-off' remains obscure. Factors like colonization, wind, temperature, day length, light density, moisture, host plant growth stage and its quality are known to play roles in aphid migration (Parry, 2013). Further detailed studies are needed to understand whether HIPVs are one of the factors that induce aphids to migrate.

Phytophagous thrips

The population densities of thrips species on MEJA-treated plants were significantly lower than on control plants in both treatment periods of year 1, according to sweep-net samplings, whereas visual samplings did not reveal any significant difference. To our knowledge, this is the first record of how thrips populations on wheat plants are affected by MEJA treatment, while there is a growing body of evidence on the effects of JA and CJ on thrips populations. For example, JA was found to reduce the population density of thrips species, likely consisting of *H. tritici*, *H. aculeatus*, *L. denticornis*, *F. teucornis* and *T. angusticeps*, on wheat plants (El-Wakeil et al., 2010; El-Wakeil and Volkmar, 2012). Another study indicated that performance and preference of *F. occidentalis* was

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restricted by JA application on *Arabidopsis* and *Brassica rapa* plants (Abe et al., 2009). MEJA and CJ were found to have deterrent effects on second instar larva of *F. occidentalis* on leaf dics of the bean *Faseolus vulgaris* in choice settlement assays (Egger and Koschier, 2014). In addition, the deterrent effects of jasmonates on feeding and oviposition by thrips were improved when combined with allylanisole (Egger et al., 2014). Jasmonates are also known to induce the formation of glandular trichomes, thus increasing the number of thrips entrapped by these plant-defense structures (Boughton et al., 2005). Furthermore, jasmonate-inducible defenses of tomato plants (i.e. proteinase inhibitors and polyphenol oxidase) were found to attenuate a community of herbivores, including a thrips species, *F. occidentalis* (Thaler et al., 2001). Apparently, jasmonate applications are responsible for inducing different defense mechanisms in plants and negatively affecting thrips performance and plant preference.

Wheat stem sawflies

MEJA may be a promising attractant for wheat stem sawfly adults, as their numbers were higher on MEJA-treated plants than on control plants, based on sampling by yellow sticky traps in our study, although data from the other sampling methods did not show significance. However, in our previous study, we found that the outcome of CJ on wheat stem sawflies is influenced by the sampling method, with more consistent results obtained from yellow sticky traps and indicating that CJ is repellent to wheat stem sawfly adults (Bayram and Tonğa, 2017). CJ-treated wheat plants release a group of HIPVs, one of which ((Z)-3-hexenyl acetate) repels the wheat stem sawfly *C. cinctus* at high doses while attracting it at lower concentrations (Piesik et al., 2008; Delaney et al., 2013). The same very high MEJA doses that we used in our study induced faster maturation of wheat plants in another study (Beltrano et al., 1998); therefore, in our field experiments, such faster growth may have limited the production of HIPVs like (Z)-3-hexenyl acetate, causing the compounds to remain at attractive (lower) concentrations after MEJA treatment. Nevertheless, such opposite results for chemicals belonging to the same biosynthetic pathway could be beneficial to “trap and kill” or “push-pull” pest management systems. In our studies, we did not assess oviposition-related parameters, e.g., eggs laid in each plant or the number of wheat stem sawfly females trapped. However, discovering the roles of HIPVs in the ovipositional behavior of wheat stem sawflies could help in developing these control strategies.

Effects of methyl jasmonate treatments on parasitoids and predators of wheat pests

Aphid parasitoids

The population densities of aphid parasitoids sampled from different MEJA-treated wheat plants in this study were not significantly different from those of control plants. In a previous field experiment, MEJA-baited traps were found to be attractive to parasitoids from the family Braconidae (James, 2005). Electroantennogram studies revealed that the parasitic braconid wasp *Microplitis croceipes* responds to MEJA (Park et al., 2001). Testing of CJ on aphid parasitoids showed it to be an attractant. For example, in wind tunnel studies, *A. ervi* was attracted to vapor exposure of CJ-treated *Vicia faba* plants (Birkett et al., 2000). Moreover, plant defenses induced by CJ in *Arabidopsis* attracted *A. ervi*, a generalist aphid parasitoid, but had no influence on *Diaeretiella rapae*, a specialist aphid parasitoid (Bruce et al., 2008). Dewhirst et al.

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(2012) found that HIPVs released from CJ-applied *Capsicum annum* plants were preferred by *A. ervi* and encouraged its foraging time. To our knowledge, the present study is the first report of aphid parasitoid responses to MEJA under field conditions. Our data are not in agreement with the above-mentioned CJ studies, but this could be due to the different jasmonate derivates investigated, the different species studied (host plant, insect pest, parasitoid), the phytotoxic effects of the MEJA doses, experiment type and/or ecological factors.

Lady beetles

Direct visual and yellow trap samplings showed that there were significant attractive effects of MEJA treatments on lady beetles, although these effects were not found based on sweep-net and blue sticky trap samplings. A previous study showed that persimmon plants infested with the herbivorous Japanese wax scale, *Ceroplastes japonicus* (Hemiptera: Coccidae), and persimmon plants treated with MEJA, released the terpenoid α-pinene, which was found to be a strong attractant to virgin female lady beetles of the species *Chilocorus kuwanae* (Coleoptera: Coccinellidae) (Zhang et al., 2009). In addition, an earlier study reported that CJ-impregrated filter papers were preferred over control arms by the seven-spotted lady beetle *C. septempunctata* in olfactometer studies (Birkett et al., 2000).

Hoverflies

The blue sticky trap sampling results in our study, but none of the other sampling methods we used, indicated an effect of MEJA on hoverflies (adults), suggesting a possible deterrent effect. In an earlier study, hoverflies did not positively respond to MEJA-baited traps in field tests (James, 2005). Thaler, (2002) found that JA-reduced aphid density was correlated with reduced numbers of hoverfly eggs, supporting the theory that hoverflies mostly employ visual cues to locate their hosts. Studies on the behavioral responses of *Episyrphus balteatus*, another hoverfly species, revealed that this hoverfly employs a composition of cues consisting of plant volatiles (i.e., green leaf volatiles and terpenoids) and a host pheromone(s) (Verheggen et al., 2008). Exogenous CJ application caused wheat plants to release volatiles such as (Z)-3-hexen-1-ol, which was found to contribute to host-plant locating and ovipositional behavior of the hoverflies (Verheggen et al., 2008; Delaney et al., 2013). A possible explanation is that the phytotoxic effects of MEJA may change both the chemical profile and the visual appearance of plants so that hoverflies may fail to locate their prey.

Collyria coxator

The population densities of *C. coxator*, a wheat stem sawfly larval parasitoid, were significantly higher in MEJA-treated wheat plants compared to control plants, according to both sweep-net and yellow sticky trap samplings. Our previous findings indicated that CJ is similarly attractive to *C. coxator* adults in field experiments (Bayram and Tonğa, 2017). The chemical ecology of *C. coxator* is not well-known. Thus, further investigations indicating how *C. coxator* adults respond either to jasmonates or to changes in the chemical profile of wheat after jasmonate treatment are highly suggested.

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Agronomic properties

Results of our experiments revealed that MEJA applications significantly reduced wheat yield. Our findings confirm previous results by Beltrano et al. (1998). They reported that MEJA treatments accelerated wheat senescence and shortened the grain-filling period, resulting in reduced yield. Similar yield-decreasing effects of MEJA were also observed in rice (Kim et al., 2009a). However, the inflence of MEJA on plants can vary; for example, MEJA-treated rice and wheat plants under drought conditions had higher yields (Kim et al., 2009b; Anjum et al., 2016). Therefore, the effects of MEJA might be dependent on environmental conditions such as drought or MEJA dosage, where high doses are phytotoxic (Boughton et al., 2006; Anjum et al., 2016). We observed such phytotoxic effects in the present study, in that the heights of plants (the above-ground parts) treated with MEJA grew shorter than those of untreated control plants. These results are concordant with previous investigations in which it was found that MEJA treatments stunted the height of Scots pine (*Pinus sylvestris* L., Pinaceae) seedlings (Heijari et al., 2005).

Our results indicate that exogenously applied MEJA could act as a repellent against some piercing-sucking wheat pests (aphids and thrips) and as an attractant for wheat stem sawfly adults. In addition, some natural enemies of insect herbivores (lady beetles and *C. coxator*) were attracted to MEJA-treated plants whereas hoverflies were repelled and braconid aphid parasitoids were not affected either way. These results could be exploited to benefit integrated pest management programs, especially in push-pull strategies, when a certain pest is targeted. The effects of MEJA doses on different species varied depending on the plant growth stage. MEJA doses tested in our study had adverse effects on some agronomic features of wheat. Therefore, our results suggest that the roles of lower doses of MEJA in plant defense induction should be taken into consideration in future studies. Our study lacks of phytochemical analysis of wheat volatile chemical changes after MEJA treatment, so that further studies providing such data are needed to support our field-experiment findings.

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IDENTIFYING LANDSCAPE VALUES AND STAKEHOLDER CONFLICTS FOR THE PROTECTION OF LANDSCAPE MULTIFUNCTIONALITY: THE CASE OF EKŞISU WETLANDS (TURKEY)

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Abstract. The aim of this study was to identify and define the multiple benefits that Ekşisu Wetlands (Upper-Euphrates Basin) provides to its local stakeholders, and the major problems and sectors/stakeholders in conflict that shape the area in order to inform the collaborative landscape planning process for the wetlands. Landscape value mapping and analysis of the stakeholders and conflicts between them were employed, as were statistical analysis of the linkages between the perceived benefits of the landscape and land use/land cover characteristics. Stakeholder analysis showed that the governmental institutions have more decision-making power than user group of the wetlands. Drainage control, over grazing, abstraction of sand-gravel from the river bed, environmental pollution and the lack of will to use the available legislative and administrative mechanisms are the primary factors that threaten the Ekşisu Wetlands and its multifunctionality. Among the landscape values examined, future value was considered the most important by the stakeholders. Two sets of landscape service bundles were identified; use and non-use landscape values; option landscape values- that are linked to Ekşisu Wetlands. Conflict between the nature conservation and agriculture sectors and, conflict between the stakeholders over provisioning, regulatory, and cultural services are two primary conflict issues that were identified in the drainage basin of the wetlands. Habitat protection and improvement, and protecting and improving the area's regulating and cultural functions were proposed as priorities for the collaborative management of the area.
Keywords: *landscape function; landscape services; collaborative landscape planning; wetland management; landscape service bundles*

Introduction

A landscape is a dynamic system–the result of diverse landscape functions–from which communities obtain multiple landscape services that are vital for human well-being and community sustainability (Kienast et al., 2009). Although landscape functions and their associated services are the primary elements necessary for the sustainability of individuals and communities, and quality of life, many of these functions are currently under threat (MEA, 2005; Carpenter et al., 2006; Daily et al., 2009).

Before the 1990s, landscape research and management practices focused primarily on a single function and landscape service (de Groot, 2006); however, relative to population growth, economics, environmental degradation, climate change, and sustainability, any landscape planning and management approach that focuses only on a single function and single benefit of a landscape is inadequate and cannot satisfy the varying needs and demands of all landscape stakeholders (Fry, 2001). The diverse and increasing demands on landscapes result in their degradation and conflicts between their stakeholders that vary in scale and context. A stakeholder is anybody who can affect or is affected by an organisation, strategy, project, change or usage. They can be internal or external and they can be at senior or junior levels (Anonymous, 2017). Stakeholder conflicts generally arise from the competing interests of a landscape's multiple stakeholders and are also related to top-down planning approaches that ignore local community and stakeholder needs. As such, since 2000 the importance given to and advocacy for landscape multifunctionality (Fry, 2001; de Groot, 2006; Potschin and Haines-Young, 2006), and inclusive participatory approaches to landscape planning have increased substantially (Luz, 2000; Buchecker, 2003; Selman, 2004).

In terms of multifunctionality and related landscape services, wetlands are among the most critical ecosystems on Earth. Covering 0.6% of Earth's surface, wetlands provide multiple landscape services to communities to a much greater degree than other types of landscapes (Mitsch and Gosselink, 2000; de Groot et al., 2006); however, due to the relative undesirability of wetlands, the competing and conflicting interests of wetland stakeholders, and ignorance concerning wetland ecological functions and public benefits, wetland ecosystems and their multifunctionality have been put under great pressure by human activity throughout history (Turner et al., 2000; Finlayson et al., 2005; de Groot et al., 2006). In order to protect the multifunctionality of wetlands via a collaborative decision-making process, the importance and value of the benefits provided by wetlands to local communities and stakeholders, as well as the stakeholders' conflicting interests must be identified (Brander et al., 2006; de Groot et al., 2006).

In Turkey, wetland landscapes are subjected to increasing threats and conflicts arising from short-term economic benefit-oriented land-use policies and local practices (Karadeniz et al., 2009; Uçak et al., 2014; Curebal et al., 2015). Ekşisu Wetlands in Turkey's northeast Anatolian Region is among the many facing such issues (Aslay and Kandemir 2009; Doğan et al., 2015). The aim of the present study was to identify the links between Ekşisu Wetlands' stakeholders and the links between those stakeholders and the wetlands, so as to inform a collaborative planning process aimed at protecting the multifunctionality of Ekşisu Wetlands. Based on the findings, we provide a discussion of the required tools and framework, with respect to their relevance to collaborative landscape planning, and recommendations for the collaborative stakeholder structure and possible strategies for ensuring the sustainability of Ekşisu Wetlands' multifunctionality.

Landscape multifunctionality

Based on the interlinked and multidimensional (temporal, biogeophysical, social, cultural, and economic) nature of today's landscapes, there is a growing consensus that landscapes are multifunctional systems (Brandt et al., 2000; Naveh, 2001; de Groot, 2006; Haines-Young and Potschin, 2010). In terms of sustainability, landscape multifunctionality is indicative of the actual or potential fulfillment of several functions, and the provision of multiple benefits (Wiggering et al., 2006). Recent research on landscape multifunctionality has focused on multiple related concepts, including landscape functions, landscape services, and ecosystem services.

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Landscape function refers to the capacity of a landscape to provide material and immaterial goods and services that meet the needs and demands of communities–directly or indirectly–and varies according to a landscape's characteristics. Landscape characteristics shape landscape functions, which are considered landscape services when people use and assign importance and value to them, according to the benefits they provide (Wiggering et al., 2006; Kienast et al., 2009; Selman, 2009; Termorshuizen and Opdam, 2009; Willemen et al., 2010; Hermann et al., 2011; Vallés-Planells, 2014). Landscape functions and their associated landscape services are categorized as follows: 1. Production functions: provision of natural products obtained from the ecosystem, such as nutrition, raw materials and energy; 2. Regulation and maintenance functions: regulation and maintenance services are the benefits provided by ecosystem processes, and include water purification, habitat and gene pool protection, and life-support systems; 3. Cultural functions: cultural services via physical, intellectual, spiritual, symbolic, and other interactions with biota, ecosystems, and land-/seascapes (MEA, 2005; CICES, 2017).

Landscape structure and function affect how individuals and communities regard the landscape, but are affected by human activity (Nassauer, 1995). A landscape and its assigned value informs individuals' types of land use, and choices, needs, and expectations related to where and how they live and work (Kaltenborn and Bjerke, 2002; Oliveira and Dineboska, 2004; Stewart et al., 2004; Oñate and Peco, 2005; Manzo and Perkins, 2006). Rolston and Coufal (1991), Brown (2006, 2005) and Brown and Raymond (2007) and defined 12 landscape values that focus on multiple landscape benefits, ranging from functional to symbolic (Zube,1987) such as aesthetic, heritage or intrinsic.

A comprehensive participatory approach that takes into consideration the multiple benefits of landscapes, and the multiple interests and opinions of all landscape stakeholders is needed to formulate optimal management strategies for the protection of the multifunctionality of landscapes. As such, there is an increasing emphasis on analysis and mapping of landscape services (MEA, 2005; Carpenter et al., 2006; Fisher, 2008; Daily et al., 2009; Bollinger and Kienast, 2010; Bollinger et al., 2011) and, identification of conflicts between stakeholders (Wondolleck and Yaffee, 2000; de Groot, 2006; Manzo and Perkins, 2006), and identification of the importance and assigned value of landscape benefits by multiple stakeholders, as powerful tools for use in a participatory landscape planning process (Brown, 2006; Brown and Raymond, 2007; Alessa et al., 2008; Fagerholm and Käyhkö, 2009; Raymond et al., 2009).

Addressing landscape values and conflicts through collaborative landscape planning

The limits of the traditional top-down landscape planning approach for effectively addressing the challenges of maintaining landscape multifunctionality highlight the need for participatory approaches. Consequently, the importance of a participatory planning process that facilitates stakeholder involvement, and consensus, negotiation, and collaboration between all relevant stakeholders has become widely recognized (Johnson et al., 2002; Mostert, 2003; Tippett et al., 2005; Giordana et al., 2007; Pahl-Wostl et al., 2007).

Collaborative planning is a promising new method for resolving conflicts between landscape stakeholders, based on shared values, and compromise and negotiation of joint goals for spatial planning and natural resource management. The process requires technical and social tools that facilitate equal and active involvement of all stakeholders

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in a joint decision-making process (Grimble and Wellard, 1997; Margerum, 2002; Overall, 2005; Innes and Booher, 2010); therefore, collaborative planning is both a technical and a social process. As a technical process, collaborative landscape planning deals with the sustainability of landscapes, whereas as a social process, it emphasizes consideration of the perspectives of multiple stakeholders, their interests/demands, and the conflicts between them in an effort to arrive at democratic solutions to conflicts via a bottom-up, active participatory process (Healey, 2003; Selman, 2004; de Groot, 2006; Termorshuizen and Opdam, 2009).

Via collaboration, negotiation, and conflict management based on trust, public responsibility for and a sense of ownership by stakeholders of landscape planning and management the decision-making process is greatly improved (Arnstein 1969; Selin and Chevez, 1995; Dijkstra et al., 2011). Collaborative planning is a comprehensive and strategic process for achieving joint formulation of shared goals by the multiple stakeholders of a planning area (Sabatier et al., 2005; Ferreyra and Beard, 2007; Margerum, 2002).

Materials and Methods

Study area

Ekşisu Wetlands is located 11 km east of Erzincan City Center in the northeastern Anatolia Region of Turkey. At an altitude of 1140-1160 m, it is bordered by the Keşiş Mountains to the north and the Erzincan-Erzurum Highway to the south (*Figure 1*).

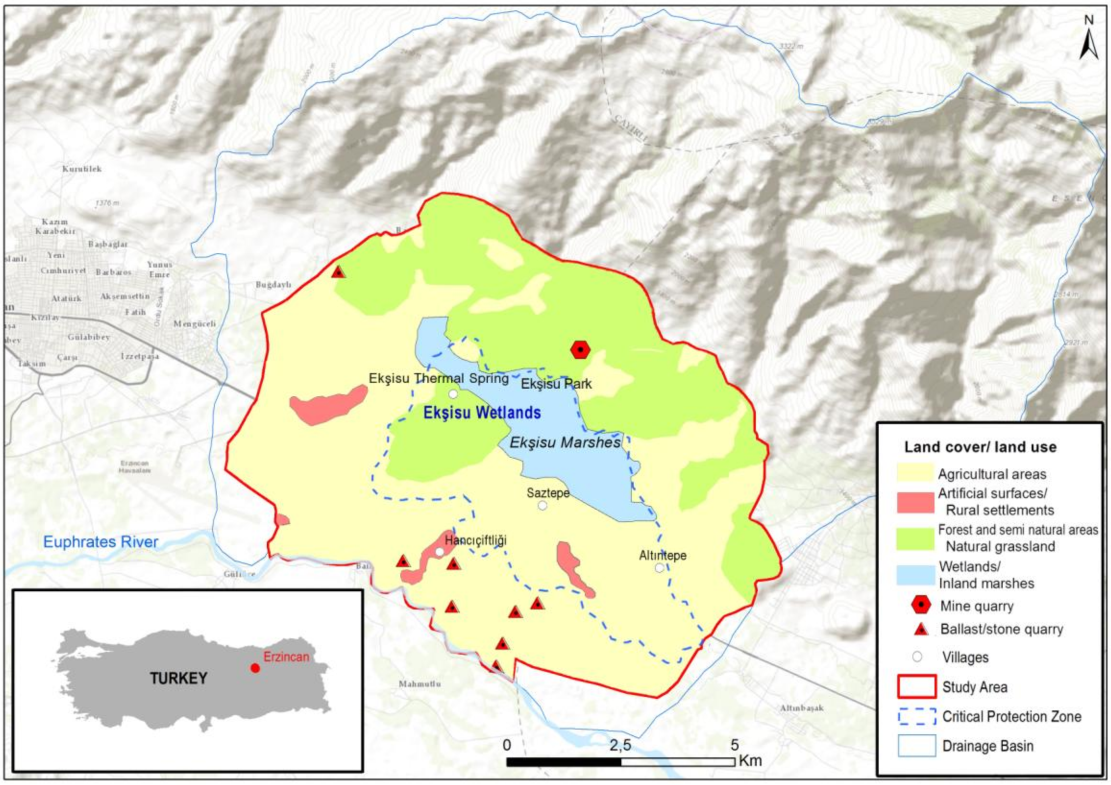

Figure 1. *Location of Ekşisu Wetlands and LULC types (source: ESRI Topographic Base Maps 2017 and developed from CORINE Land Cover, 2012)*

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Ekşisu Wetlands encompasses 8736 ha. Ekşisu Wetlands is under the administrative control of the towns Erzincan Central and Üzümlü. Ekşisu Wetlands is also approximately 5 km from the Euphrates River and lies on the North Anatolian Fault Line, making it part of Turkey's first-degree fault zone. The area, including the buffer zone, primarily contains saltwater marshes, meadows and agricultural fields. Alluvial soil is dominant in the study area (Akkan, 1964; Hayli, 2002; Akyıldız and Kılıç, 2006; Anonymous, 2014). Ownership of the land is both private and governmental. The area has been declared a 1st-Degree Natural Site area by the former Sivas Regional Board for Cultural and Natural Heritage Preservation. The Turkish Ministry of Forest and Water Affairs, General Directorate of Nature Conservation and National Parks and its local branch have primary responsibility for its management. Climatically, a typical east Anatolia continental climate prevails in the area and surroundings. The area has hot and dry weather during summer; the most hot month is August and the mean temperature in this month is 31,9 °C [1929-2016] and is cold and snowy during winter; the coldest month is January and the mean temperature in this month is -7,2°C [1929-2016] w. Based on observations since 1929, the annual mean temperature is 10,9 °C. Snowfall begins in late October and lasts until April. In general, precipitation is highest in spring (from April to June, but primarily in April and May) and lowest in summer (especially in August) (Kaya, 2011; Anonymous, 2017).

Ekşisu Wetlands is an ecologically valuable landscape that provides feeding, roosting, and nesting sites for thousands of migratory and resident birds, as well as plant genetic materials for research. In all, 85 bird, 13 butterfly, 8 reptile, and 13 mammal species have been recorded in Ekşisu Wetlands. Based on these numbers, the area is ranked number 1 of the 263 Important Nature Areas of Turkey and is among the 184 Important Bird Areas of Turkey Ekşisu Wetlands is the only habitat in the world for the globally threatened plant species *Soncus erzincanicus* .The study area is also where the nationally threatened species *Grus grus, Himantopus himantopus*, *Aythya nyroca,* and *Ixobrychus minutus* breed (Akyıldız and Kılıç, 2006; Yeniyurt et al., 2011).

The primary LULC types in the study area include such agricultural areas as non-irrigated and permanently irrigated arable lands, vineyards, and mixed orchards and pastures, as well as natural grasslands, inland marshes, rural settlements, and mineral extraction sites (*Figure 1*). Altıntepe and Saztepe tumuli, which are located in the south and southeast of the study area, include archeological sites from the Urartu Period (Akyıldız and Kılıç, 2006; Yeniyurt et al., 2011).

Landscape inventory, analysis, and evaluation

Baseline data on the current state of the natural and cultural processes, and assets of Ekşisu Wetlands, including its functional capacity, and present and foreseeable problems and conflicts related to land usage were inventoried and collated. To gain an overview of the landscape, the current state of its climate, topography, hydrology, soils, flora and fauna, and sociocultural and demographic characteristics were inventoried. Next, data obtained from government-sponsored research, academic studies (e.g. Akkan, 1964; Hayli, 2002; Akyıldız and Kılıç, 2006; Aslay and Kandemir, 2009), field visits, and community workshops conducted by the researchers were collated.

Stakeholder and problem analysis

Stakeholder and problem analyses were performed to identify groups and/or institutions that have a direct or indirect relationship with Ekşisu Wetlands that, directly

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or indirectly, influence, are in conflict with, and degrade Ekşisu Wetlands functions. Data from earlier studies (e.g. Hayli, 2002; Anonymous, 2005; Akyıldız and Kılıç, 2006; Aslay and Kandemir, 2009; Anonymous, 2010; Baylan, 2012; Anonymous, 2014) on Ekşisu Wetlands, and data of the stakeholders, problems related with the sustainability of Ekşisu Wetlands and conflicts between the stakeholders obtained from 1- day workshop on stakeholder and problem analysis and from field trips to the area were integrated. A 1-day workshop was conducted with 45 representatives and technical staff from the Erzincan Provincial Directorate of Forest and Water Affairs, State Water Affairs 82nd Branch Office, the Erzincan Provincial Directorate of Food, Agriculture, and Livestock, Erzincan University, Northeast Anatolia Development Agency, the Erzincan Governor's Office, mayors from the surrounding municipalities, village heads (muhtars), farmers, and agriculture and irrigation associations. The workshop participants were invited with the cooperation of the Erzincan Governor's Office. During the 1-day workshop, problems and conflicts associated with Ekşisu Wetlands were analyzed via root-cause analysis (Rooney and Heuvel, 2004), in an effort to identify the origins of the study area's degradation and threats. The analysis was conducted using brainstorming, joint thinking, and consensus, with the help of facilitators. The end result of the analysis was the creation of a problem tree.

Spatial data analysis, landscape value associations, and spatial multifunctionality

Mapping and analysis of the values Ekşisu Wetlands' stakeholders and communities assigned to the landscape were conducted. Initially, theoretical considerations, practical approaches, and implementations of landscape value mapping and analysis were examined in preparation for the study. The local Ekşisu Wetlands stakeholders chose 12 landscape values for the study. The values and analysis were based on research by Brown and Raymond (2007), Alessa et al. (2008) and Zhu et al. (2010). Value mapping consisted of preparation of a plain topographic map of the area (scale: 1:150,000) for each of the 12 landscape values. Statements and associated descriptions for each landscape value were written on each of the topographic maps.

The field study phase consisted of participatory mapping performed in a workshop format at the study area. First, the participants watched a short presentation on landscape values and the steps to be followed during the mapping process. Then, 45 representatives of national and local level governmental institutions, and municipalities responsible for management of the area, civil society organizations, researchers, farmers, and village heads scored each value on the maps using colored dots (yellow: 5 points; blue: 10 points; green: 20 points; orange: 50 points). Each landscape value map included 5 locations in and around Ekşisu Wetlands, and each participant could assign a maximum of 100 points per map. The surveyed landscape values are described in *Table 1*. Data collected during the workshop were inserted into digital data tables, and the locations of the mapped landscape value points were digitized using ArcView v.10.2.

In order to describe the spatial intensity of the landscape values, density surfaces were generated from point data layers using the kernel density function in ArcView Spatial Analyst. The kernel density output cell size was set to 100 m and the search radius was set to 1000 m. The identified point density and value importance clusters were converted into raster form. Point density clusters were categorized into 7 classes via natural breaks classification, which was used to visualize the distribution of importance by location per landscape value in the area.

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***Table 1.** Surveyed Ekşisu Wetlands landscape values (Brown and Raymond, 2007)*

Landscape Value	Description	Landscape Service Category	Abbre-viation
Aesthetic value	I value this area because of its pleasing natural beauty, scents, and sounds.	Cultural	**C**Aest
Economic value	I value this area because of its economic benefits, e.g. income-generating activities, such as agriculture and tourism.	Provisioning	**P**E
Recreation value	I value this area for outdoor activities and leisure.	Cultural	**C**R
Life-sustaining value	I value this area because it helps sustain human life and/or is important for air, water, and soil quality.	Regulating	**R**LS
Knowledge value	I value this area because of its role in environmental education.	Cultural	**C**K
Biological diversity value	I value this area because of its role in supporting biodiversity, e.g. plants, animals, and aquatic organisms.	Regulating	**R**B
Spiritual value	I value this area because it has spiritual significance and/or is a sacred place.	Cultural	**C**S
Intrinsic value	I value this area independent of any thoughts about it, and whether or not I use it; its value is its existence.	Cultural	**C**I
Heritage value	I value this area because of its relationship to natural and human history.	Cultural	**C**H
Future value	I value this area because it will allow future generations to benefit from it, as we do in the present.	Cultural	**C**F
Therapeutic value	I value this area because it improves physical and psychological wellbeing.	Cultural	**C**T
Wilderness value	I value this area because it is not man made.	Regulating	**R**W

Principal component analysis (PCA) and factor analysis were employed using IBM SPSS Statistics for Windows v.24.0 (IBM Corp., Armonk, NY, USA) to identify any associations between the mapped landscape values in the area. In addition, CORINE Land Cover (CLC) (2012) level 1 categories were used to analyze the spatial associations and co-occurrence of assigned landscape values in spatial scale to determine the links between LULC types and associated landscape values in an effort to understand the multifunctionality of the area. As such, a one-way ANOVA was conducted to compare the effects of primary LULC types on landscape value assignment in wetlands, natural grasslands, and agriculture categories in the study area. With this aim, factor scores of each location has been recorded as different variables and then based on the scores, the change between scores of LULC types has been analyzed.

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Conflict analysis

Conflict analysis was performed using data obtained from previous phases of the study, so as to identify the study area's conflicting interests, conflicting landscape services, and conflicting stakeholders. The conflict analysis considered and included the stakeholders, and their level of use/interest and influence on decisions regarding the management of the landscape, LULC types in Ekşisu Wetlands, and the values the stakeholders assigned to the landscape services, as well as the ongoing degradations and degradation risks on the landscape functions and services of the Ekşisu Wetlands (*Figure 2*). The conflicting stakeholders, uses of the landscape, and landscape service conflicts were visualized via symbols and indicated by zig-zag lines (Mason and Rychard, 2005) in the conflict figure (*Figure 7*).

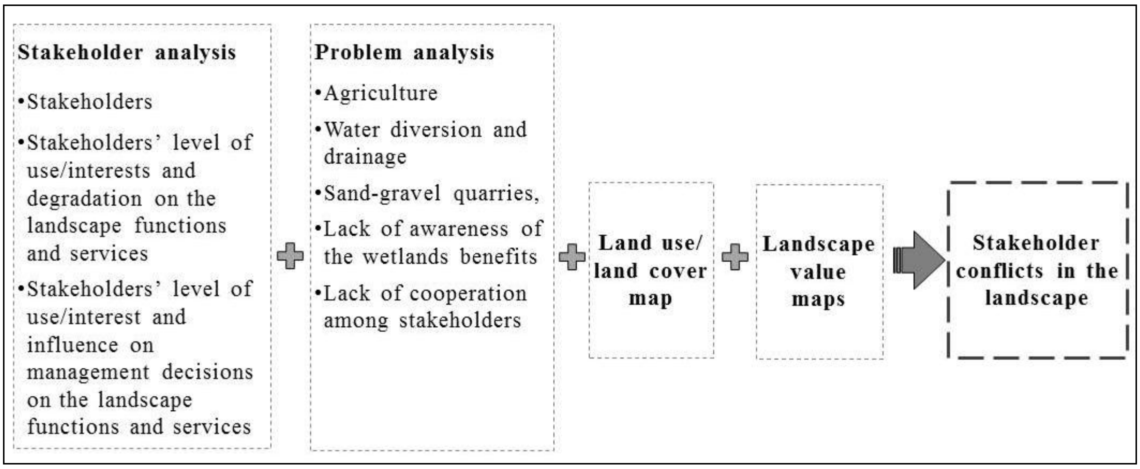

***Figure 2.** The conflict analysis framework*

Results

Stakeholders, pressures, and problems related to landscape function

Stakeholder analysis showed that there are multiple stakeholders with varying interests, influence, and power concerning the use and management of Ekşisu Wetlands (*Table 2*) and (*Figure 3*). Farmers/landowners, visitors, national and local level governmental institutions, the sand-gravel quarry sector, responsible for the management of water and agricultural resources, and natural habitats, and municipalities were the primary stakeholders with direct interests in and influence on the study area and its management.

Since the 1950s, the provincial branch of the General Directorate of State Water Affairs (SWA) has used drainage canals in the study area for drainage control which is the primary factor that threaten Ekşisu Wetlands' landscape functions, as a result of changes in the hydrological cycle, land degradation, and habitat fragmentation in the area and its surroundings. In 2011 local farmers burned 160 ha of reed field in order to obtain agricultural land, which also caused habitat destruction in the area. Another important problem in the area is pollution. Discharged domestic sewage from surrounding villages and industrial solid waste and sewage (from mines and stone quarries), and waste generated by recreational activity are the primary sources of pollution in the study area. Solid waste disposal was reported to be a factor threatening

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S*oncus erzincanicus*, in addition to overgrazing, which causes damage to habitat and dries vegetation (Akyıldız and Kılıç, 2006; Aslay and Kandemir, 2009; Yeniyurt et al., 2011; Baylan, 2012). Since 2009, sand and gravel quarry has become an important problem on the south part of the area where the Euphrates River borders the buffer zone of the wetlands.

Table 2. *Stakeholders of Ekşisu Wetlands*

<table>
<tr><th>Ekşisu Wetlands Stakeholders and their roles related with Ekşisu Wetlands</th><th colspan="2">Institutions</th></tr>
<tr><td rowspan="2">Management of Ekşisu Wetlands</td><td><u>Governmental Institutions - Central Level</u>
• Turkish Ministry of Forestry and Water Affairs (MFWA)
• Turkish General Directorate of State Water Affairs (SWA)
  • Provincial Directorate of the Turkish General Directorate of Nature Conservation and National Parks (GDNCNP)
  • Sensitive Ecosystems Branch Directorate
• Turkish Ministry of the Environment and Urbanism (MEU)
• Turkish Ministry of Culture and Tourism (MCT)
• Turkish Ministry of Food, Agriculture, and Livestock (MFAL)</td><td><u>Governmental Institutions - Local level</u>
• Erzincan Governor’s Office
• Provincial Branch of SWA: Regional Directorate 82
• Provincial Directorate of GDNCNP;
  • Wetlands Branch Directorate
  • Local Wetlands Commission
• Provincial Directorate of MEU
• Provincial Directorate of MCT
• North-Eastern Anatolia Development Agency (NEADA)
• Provincial Directorate of MFAL</td></tr>
<tr><td><u>Local municipalities/authorities</u>
• Erzincan Municipality
• Üzümlü Municipality
• Akyazı Municipality
• Village Heads (muhtars)</td><td><u>Civil Society Organizations</u>
• Turkish Chamber of Agriculture
• Nature conservation NGOs (national and local level)
• Union of Sheep Breeders
• Hunting Association</td></tr>
<tr><td>Stakeholders benefiting from the landscape functions of Ekşisu Wetlands (Users)</td><td colspan="2">• Local community
• Land owners/farmers and sheep and cattle breeders
• Hunters and illegal hunters
• Recreationists/Tourists
• Researchers
• Private sand- gravel quarry firms</td></tr>
</table>

The analysis quadrant for the influence (power) level on the management decisions for area and use/interest level of the stakeholders in the area shows that national and local governmental institutions have more decision-making power than user group of Ekşisu Wetlands (*Figure 3*); however, the current pressures and the threats to Ekşisu Wetlands indicate that those national and local governmental institutions that manage the area are not using the available legislative and administrative mechanisms or building a collaborative management process to ensure the sustainability of the wetlands.

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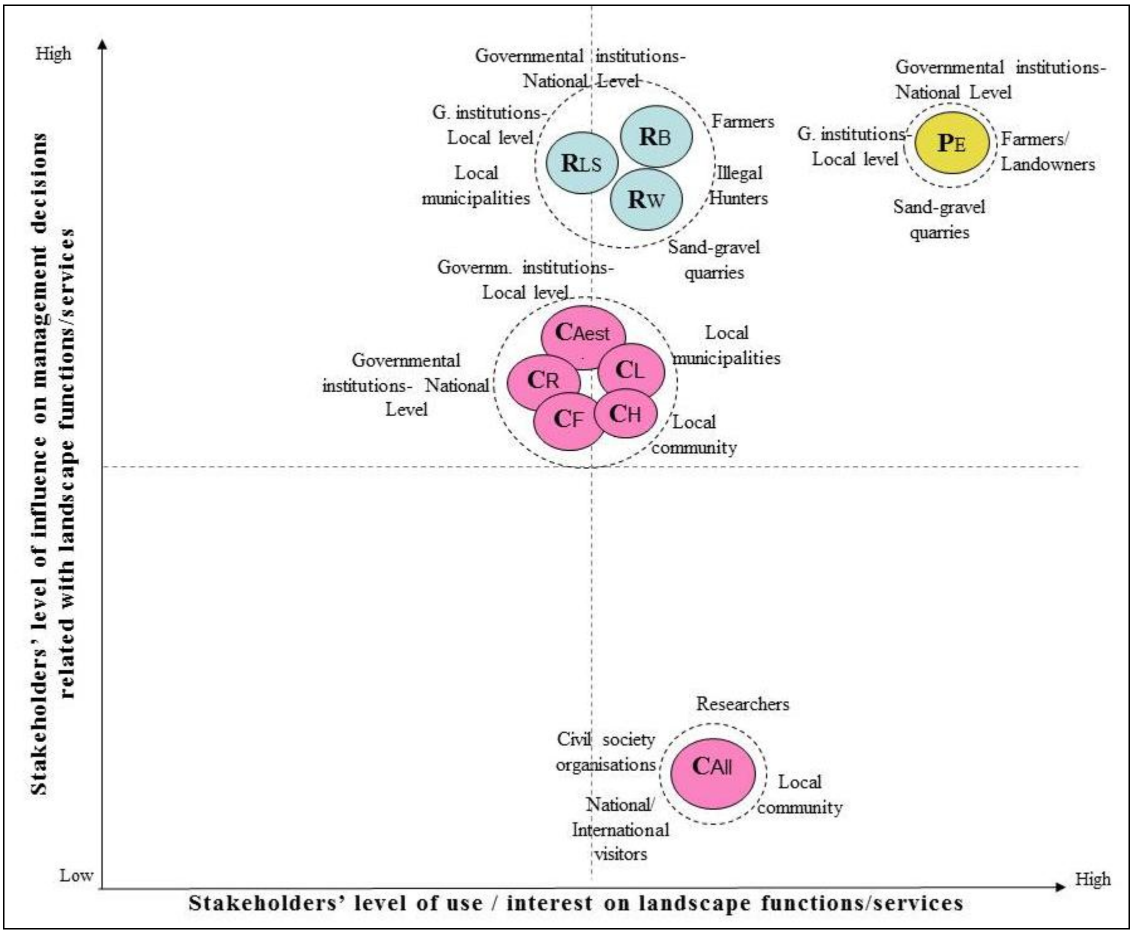

Figure 3. *Stakeholder levels of use/interest and influence on management decisions concerning the landscape functions and services of Ekşisu Wetlands*

One of the root causes of the problems identified in the study area is the lack of a wetland management plan. All of the above-mentioned factors such as over-grazing, drainage control and agricultural land expansion, abstraction of sand-gravel from the river bed and environmental pollutions and related degradation and degradation risks in the area as well as the lack of will to use the available legislative and administrative mechanisms directly and indirectly affect landscape characteristics, and as such, the landscape functions of Ekşisu Wetlands. Due to the negative effects on the study area's bioecological and physical characteristics associated with provisioning services, the regulation functions of the area are threatened. In addition, due to the interlinked relationships between landscape characteristics and functions, the socioeconomic and cultural characteristics of Ekşisu Wetlands (the ability to perform at capacity to fulfill its provisioning and cultural functions in the future) are at risk due to the threats to its bioecological characteristics.

Landscape values assigned to Ekşisu Wetlands

Among the 12 landscape values examined, future value was considered the most important (highest value score: 1475) by the area's stakeholders. Namely, heritage (1230), biodiversity (1145), and learning (1060) benefits are the next important values in the area. In contrast, spiritual (520) and therapeutic (630) values were the least important (lowest value scores) to the stakeholders (*Figure 4*). The stakeholders assigned importance to more than one place per landscape service, but more locations were marked for aesthetic and economic values than all the other landscape values that were investigated in the area.

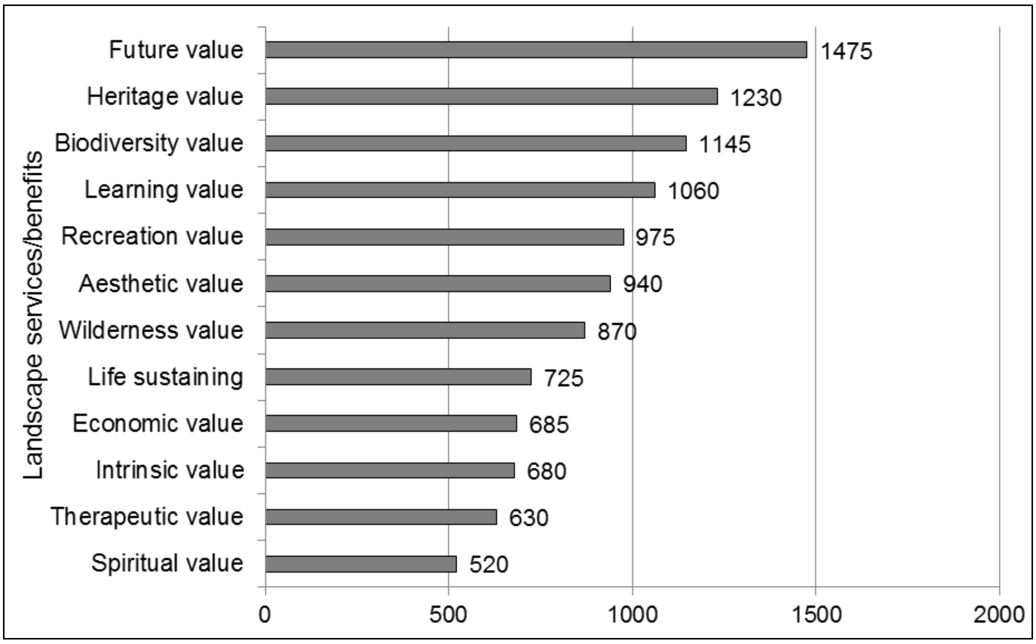

Figure 4. *Landscape value scores for Ekşisu Wetlands*

The area Ekşisu Park in the area that was designated for recreational purposes received high scores for all landscape values, except future and heritage values. The Altıntepe Archeological Site had the highest heritage (720) and future (720) value scores. Accordingly, Ekşisu Park and the Altıntepe Archeological Site were considered hotspots that are defined as the clusters of high values (Fagerholm and Niina Käyhkö, 2009) and areas that have functional diversity (Lavorel et al., 2010) because they provide multiple functions and benefits to the stakeholders. In addition, the Ekşisu Thermal Spring location was determined as the second important area for therapeutic benefits with the high therapeutic value score (225) after Ekşisu Park (370).

Ekşisu Wetlands landscape value mapping (based on stakeholder value scores) is shown in *Figures 5a-5c*. Locations with high scores for aesthetic value were higher in altitude than other areas and have an open view, along the banks of the Euphrates River, and Ekşisu marshes. These areas were also easier to access than other areas. Based on the observation of wetland birds, plants, and wild animals, the marshy area close to Ekşisu Park and Saztepe Archeological Site, and remote regions on the ridges of the Keşiş Mountains had high scores for biodiversity value. Areas with economic value were concentrated near rural settlements and agricultural areas, but also included locales with natural and cultural resources with the potential to generate tourism, including Ekşisu Thermal Spring, Ekşisu Park, and Altıntepe Archeological Site (*Figure 5a*).

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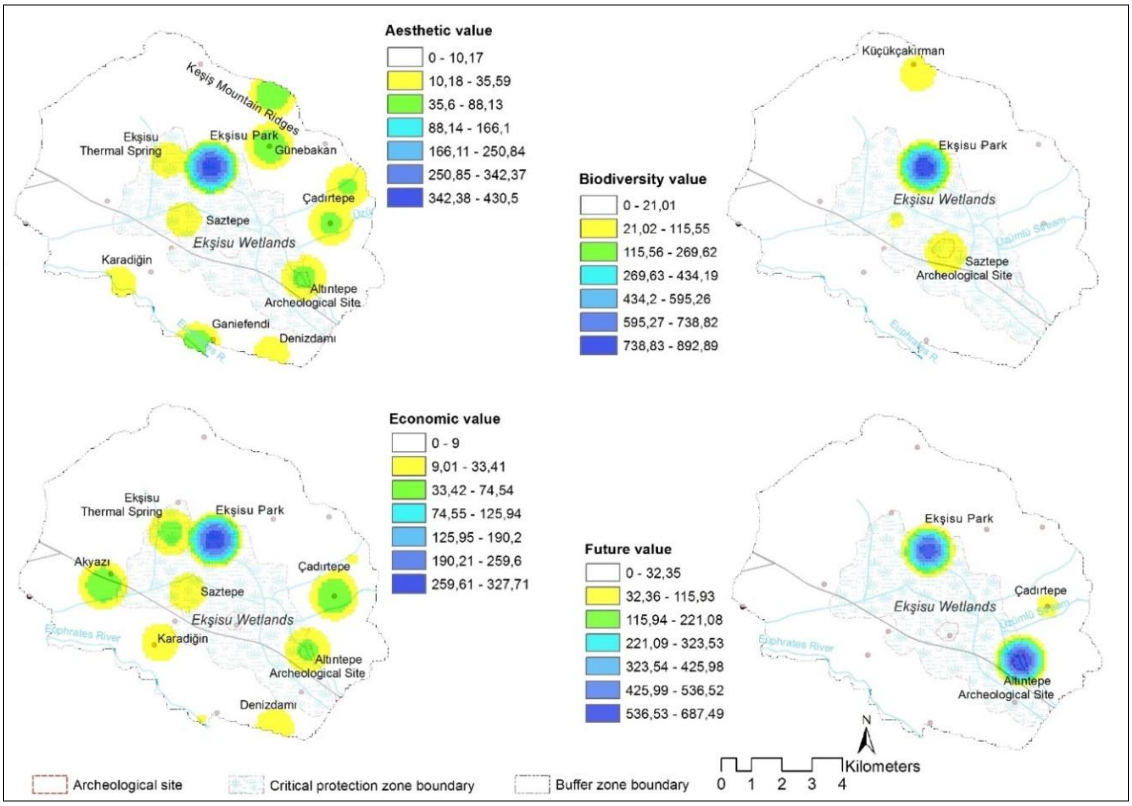

__Figure 5a.__ Landscape value mapping for Ekşisu Wetlands

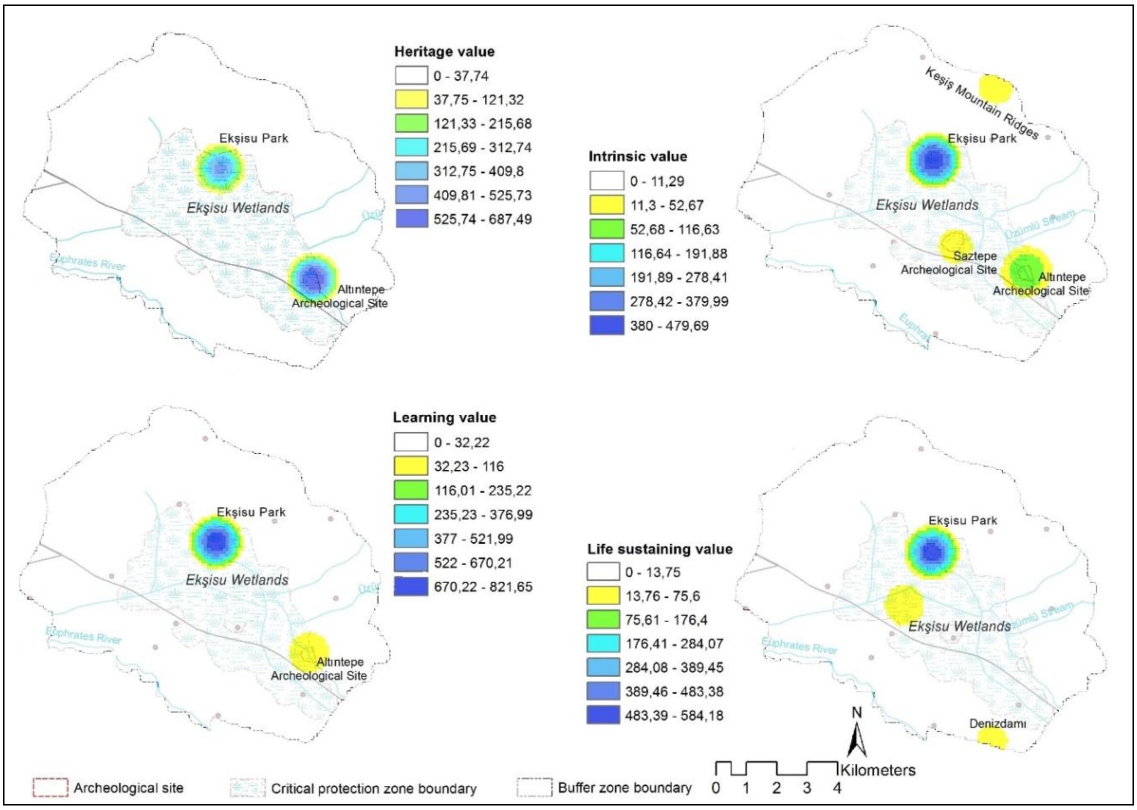

__Figure 5b.__ Landscape value mapping for Ekşisu Wetlands

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One of the least important landscape values, which followed therapeutic and spiritual values, was intrinsic value. Locations with intrinsic value were Ekşisu Park and ridges of the Keşiş Mountains, and locations with cultural heritage, such as Altıntepe and Saztepe archeological sites. Learning value was the fourth most important value, though there were few such locations rated by the stakeholders. The high learning value scores for Ekşisu Park and the Altıntepe Archeological Site are indicative of their importance for cultural purposes and environmental knowledge and research. The high life sustaining value scores for such places as the banks of the Euphrates River, Üzümlü Stream, and the lake near Ekşisu Park indicate the importance to the stakeholders of water for ecological cycles (*Figure 5b*).

Locations that had high scores for recreation value were those the stakeholders enjoyed via use of recreational facilities, and because of historical, aesthetic, and wilderness value. Landscape value mapping showed that spiritual value was the least important value for the stakeholders in the area of Ekşisu Park and an old grave of a religious person in Çadırtepe Village. The next least important value was therapeutic value, which was mostly associated with the Ekşisu Thermal Spring facilities and Ekşisu Park. The wilderness value score was 625 for Ekşisu Park and the surrounding marshy area, and the ridges of the Keşiş Mountains, where the ecosystem is completely natural and wild animals can be observed (*Figure 5c*).

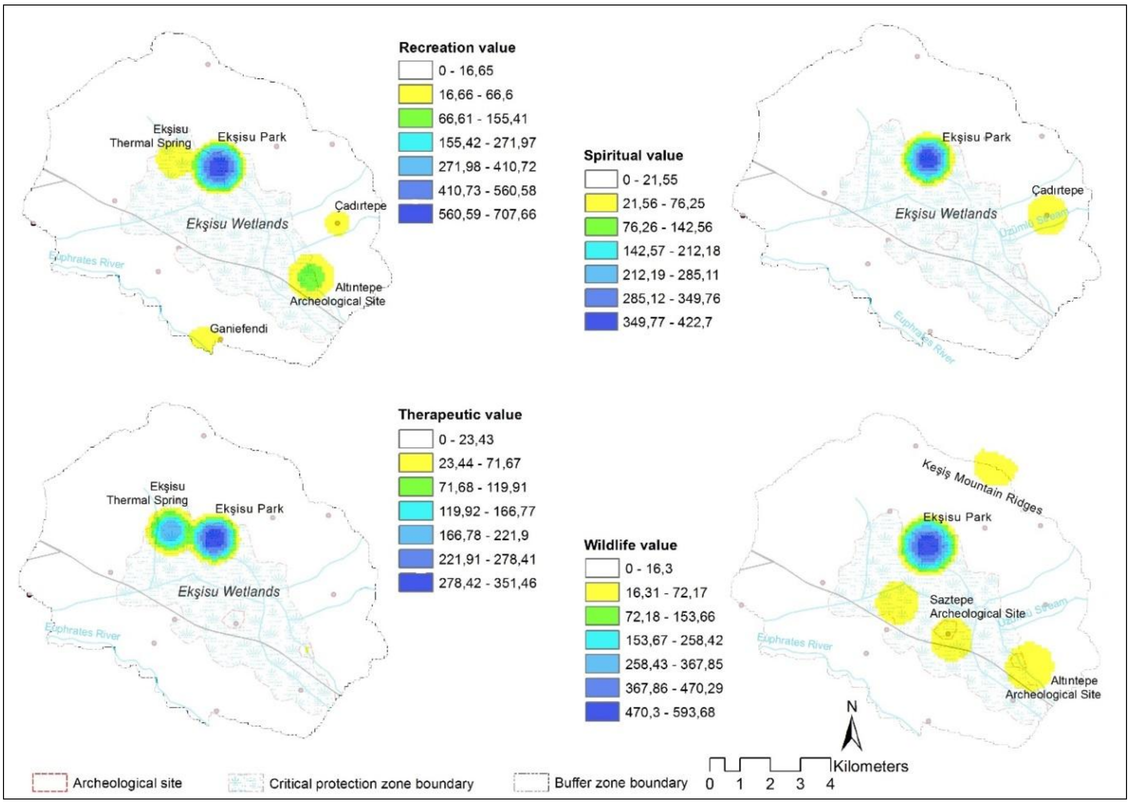

Figure 5c. *Landscape value mapping for Ekşisu Wetlands*

Associations between landscape values and multifunctionality

The 12 landscape values were measured in 16 locations within the study area and rendered as a 12 × 16 matrix using IBM SPSS statistics for Windows v.24.0. Factor

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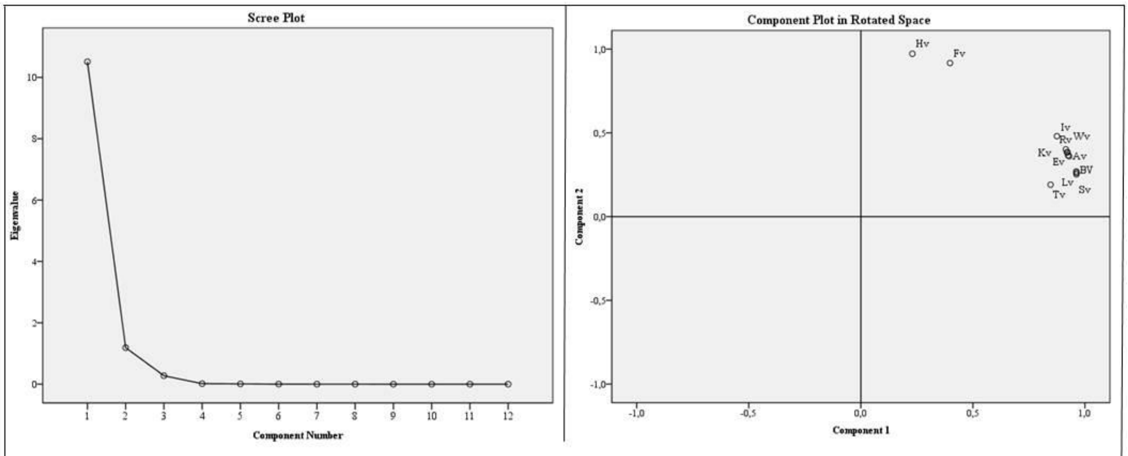
analysis via PCA, following factor rotation, showed that the measured landscape values had a 2-factor structure, which means there are 2 sets of associated landscape services-landscape service bundles that are linked to Ekşisu Wetlands. The structure of the determined factors with an eigen value >1 are shown in the scree plot and the component plot in rotated space (KMO measure of sampling adequacy = 0.794 and Bartlett's test of sphericity = 502.599; df = 66; P < 0.000) (*Figure 6*). Factor analysis results showed that the 2 factors explain 96% of the total variance; 1 factor explained 72% and 1 factor explained 25% of the variance. It was observed that the commonalities of the variables of those 2 factors ranged from 0.767 to 0.999.



Av: Aesthetic Value Bv: Biodiversity Value Ev: Economic Value Fv: Future Value Hv: Heritage Value Iv: Intrinsic Value Kv:Knowledge Value Lv: Life Sustaining V. Rv: Recreation Value Sv:Spiritual Value Tv:Therapeutic Value Wv:Wilderness Value

Figure 6. *Scree plot with eigen values based on PCA and factor analysis*

The first factor included 10 landscape values- life sustaining, biodiversity, spiritual, aesthetic, learning, wilderness, recreation, economic, intrinsic, therapeutic- that are supplied by the landscape via actual (direct) and passive (indirect) use. Based on Hein et al. (2006), this factor was labeled *Use and Non-Use Landscape Values* (the first landscape service bundle [F1]). The second factor included heritage and future values, which are associated with the stakeholders' wish that future generations will benefit from Ekşisu Wetlands. This second factor was labeled *Option Landscape Values* (the second landscape service bundle [F2]). The factor loadings for the value items are given in *Table 3*.

The results of one-way ANOVA analysis of the links between the primary LULC types in the study area and landscape values performed to define the multifunctionality of the area showed that the importance assigned to the landscape value variables by the stakeholders for the F1 factor differed according to LULC categories [F(2-13) = 5.377, P < 0.05]. Post-hoc comparison using Tukey's HSD test showed that the mean factor score for Wetlands ($\bar{x}$ =1.7082) was significantly high than the natural grasslands ($\bar{x}$ = –0.1194) and agriculture ($\bar{x}$ = –0.3133) type between LULC categories in F1's Factor scores (*Tables 4* and *5*).

Table 3. *Variable loadings for landscape services based on PCA*

Landscape service variables	Use and Non-Use Values	Option Values (F2)	Commonalitie
Life sustaining value	0.956		0.986
Biodiversity value	0.956		0.984
Spiritual value	0.950		0.976
Aesthetic value	0.919		0.945
Learning value	0.918		0.995
Wilderness value	0.916		0.980
Recreation value	0.914		0.996
Economic value	0.906		0.932
Intrinsic value	0.865		0.991
Therapeutic value	0.846		0.767
Heritage value		0.971	0.999
Future value		0.916	0.999
Eigenvalues	10.38	1.16	
Variance explained (%)	71.6	24.6	96.2

Table 4. *Differences in mean landscape value scores for F1 according to LULC categories, based on one-way ANOVA*

Variable	LULC categories	n	Mean	sd	df	F	P
Use and Non-Use Landscape Values (F1)	Wetlands	2	1.7082[a]	2.65	2-13	5.377	0.020
	Natural grassland	5	–0.1194[b]	0.17			
	Agricultural areas	9	–0.3133[b]	0.36			

[a,b]The most important groups according to Tukey's test.

Table 5. *Mean landscape value scores for LULC categories based on one-way ANOVA*

The Source of Change	Sum of Squares	df	Mean Square	F	P
Between Groups	6.791	2	3.395	5.377	0.020
Within Groups	8.209	13	0.631		
Total	15.000	15			

Additionally, the findings indicated that there was a non-significant effect of LULC type on the *Option Values* factor [F (2-13) = 0.482, P > 0.05].

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Conflicting landscape services and stakeholders

Data obtained via problem and stakeholder analyses, landscape value mapping, and LULC categories showed that 2 primary conflicts were generated by competing uses of the landscape functions and services in the drainage basin of Ekşisu Wetlands:

- Conflict between the nature conservation and agriculture sectors due to, the hydrologic regime employed, and degradation of habitats;
- Conflict between the stakeholders over provisioning, regulatory, and cultural services due to a high level of pressure on regulatory and cultural services resulting from the destructive effects of provisioning services (e.g. irrigation, drainage control for expand agricultural land, over grazing, sand and gravel quarrying).

Accordingly, local government institutions with primary responsibility for nature conservation are in conflict with farmers/landowners, illegal hunters, the local branch of State Water Affairs, and sand and gravel quarrying firms (*Figure 7*). Moreover, local communities are in conflict with local municipalities due to the lack of services and infrastructure necessary to benefit from the cultural functions of Ekşisu Wetlands.

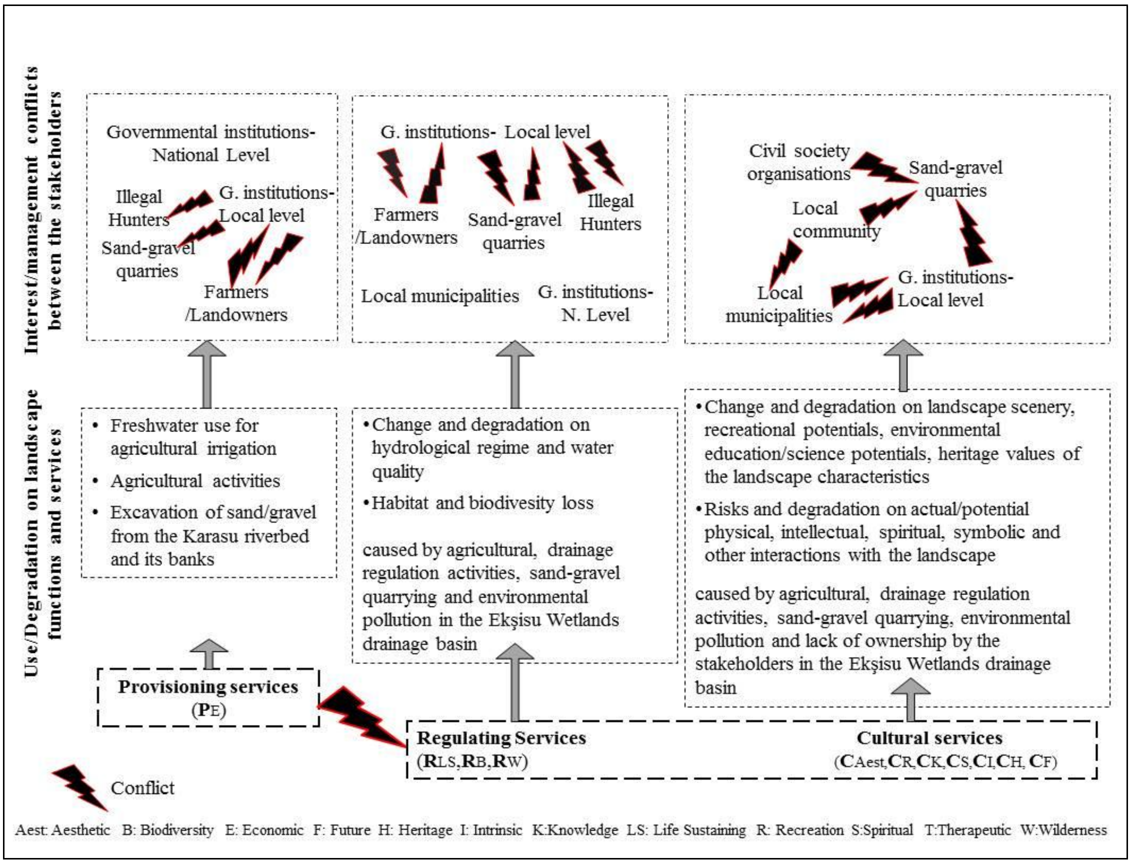

Figure 7. *Use/interest and management conflicts between stakeholders concerning Ekşisu Wetlands' landscape services*

Discussion

The present study analyzed the social and spatial dimensions of Ekşisu Wetlands, as well as their interactions, in an effort to identify the links in the Ekşisu Wetlands system

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in order to inform the collaborative landscape planning process for the wetlands. The results are presented as stakeholder interest/influence diagrams, value maps, conflict diagrams, and statistical figures. The findings show that there are strong associations between the Ekşisu Wetlands' landscape services, stakeholders, and LULC categories.

The present study's problem analysis show that Ekşisu Wetlands is under pressure from and the natural hydrological regime, habitat functions and cultural landscape service potentials are strongly negatively affected and degraded by various human activities. Agriculture, water diversion and drainage, and sand-gravel quarrying, lack of awareness of the wetlands' benefits, and lack of cooperation between stakeholders are some of the major factors that pose the greatest threat to Ekşisu Wetlands. Based on the poor state of the vegetation observed in the study area and surrounding mountains, soil erosion is another factor that threatens the area. Over grazing, clearance of natural vegetation cover, lack of enforcement of laws designed to protect pasture land, and alterations in drainage control hydrology to increase the amount of land suitable for agriculture have damaged the wetlands' plant and bird species populations and habitats.

In terms of landscape values, the present study area's stakeholders gave higher scores to cultural and regulation values than provisioning values, and considered the cultural and regulation value of the study area to be important for future generations. The Ekşisu Park area was identified as one of the few hotspots in the study area, with the maximum score for all landscape values, except heritage value and future value. The Altıntepe Archeological Site was identified as the hotspot for heritage and future values as it is an archeological site with Bronze Age (B.C. 3200-1900), Urartu (B.C. 900-650), Byzantine (B.C. 74- A.D.629) ruins that the stakeholders want to exist for future generations. Value mapping analysis shows that the stakeholders consider multiple locations valuable because they provide benefits to daily life, including aesthetic, economic, and recreation value, due to Ekşisu Wetlands' material (tangible) elements and capacities.

As reported in earlier studies on landscape values (Beverly et al., 2008; Kaplan et al., 1998), the visual features of the landscape and its recreational facilities are of value to the Ekşisu Wetlands stakeholders, indicating that the value assigned to the landscape services by the stakeholders and their perceptions of landscape importance based on usage (land use) and attractive landscapes characterized by natural and cultural landscape elements, and land cover have a positive effect on their relationship with the landscape. These findings suggest that the stakeholders, including local municipalities responsible for the management of Ekşisu Wetlands, should consider the importance of the development of facilities and activities that strengthen the connection between the stakeholders and the study area.

The low intrinsic, therapeutic and spiritual value score might have been related to the difficulty assigning importance to such intangible landscape values or very low-level landscape-related intangible meaning to the stakeholders. As an example; the low score for spiritual value might have been due to the absence of or minimal spiritual interactions with nature and with the study area among the stakeholders. The low score for therapeutic value might be indicative of the fact that the stakeholders derived little physical and psychological healing which might have been related to a lack of needed facilities at Ekşisu Wetlands, with the exception of a newly established Ekşisu Thermal Spring facility. The low intrinsic value score for Ekşisu Wetlands might also be related to a low level of knowledge and/or interest in ecological and social issues related to the area, both locally and nationally; if true, this indicates that educational programs

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designed to increase such knowledge and interest among the stakeholders should be incorporated into future collaborative planning processes for the area.

Employing PCA and factor analysis facilitated identification of the 12 landscape values in 2 general categories that are consistent with ecosystem service value type categorization, and wetland functions and value types (Hein et al., 2006). The results of the analysis also confirm the interlinkage and multifunctionality of Ekşisu Wetlands with 2 sets of landscape value bundles (F1 and F2). The factor analysis results show that there are strong positive associations between the use and non-use values of Ekşisu Wetlands that might be a result of the stakeholders' valuation of particular areas not only for a specific benefit, but also for different benefits, as reported earlier (Zhu et al., 2010). The observed positive associations between the use and non-use values might also have resulted from the synergy between landscape values, which if true, would require additional research and landscape services trade-off analysis to better inform actions designed to protect the multifunctionality of the study area. The observation of significant associations between landscape values and LULC types in Ekşisu Wetlands also confirm the existence of interlinkages between landscape functions and LULC types, as reported by Plieninger et al. (2013).

Analysis of the spatial associations between the landscape values show that the associated services–use and non-use landscape value bundle–also spatially co-occur in specific LULC types in Ekşisu Wetlands. One-way ANOVA of landscape values according to LULC type indicate that wetlands are of greater importance to the Ekşisu Wetlands stakeholders than expected based on the proportion of the study area categorized according to LULC agricultural land and grasslands. This result is similar to that reported by Kaltenborn and Bjerke (2002) and Brown et al. (2012), who observed that landscapes with lake elements were perceived to be more attractive than flat and open agricultural areas. Their findings and those of the present study indicate that LULC type affects stakeholder perception and valuation of landscape services.

Relevance for collaborative landscape management

Recent natural resource management and landscape policies and related research in developed countries have focused on landscape multifunctionality. In accordance with the need and demand for participatory planning approaches, the multifunctionality perspective requires that the landscape planning process considers and includes all relevant landscape stakeholders, as well as takes into consideration landscape functions and services to ensure sustainable landscape development (Albert et al., 2014; Sitas et al., 2014).

According to problem and stakeholder analysis, and conflict analysis findings in the present study, Ekşisu Wetlands is under pressure, has been damaged, and suffers due to conflicts between its stakeholders. The pressures and problems that threaten its landscape characteristics–and as such, its multifunctionality and landscape services–are a result of competing demands, unequal power relationships, and policy makers' lack of understanding of local stakeholders' relationship with the landscape. Worldwide, a wide range of landscapes are negatively impacted by similar issues and problems (MEA, 2005; Pinto-Correia et al., 2006; Selman, 2009; Willemen et al., 2010 and Hermann et al., 2011). In terms of wetlands damage and loss, Ekşisu Wetlands is similar to many other wetlands throughout the world, based on reports by Turner et al. (2000), Dixon and Wood (2003), Finlayson et al. (2005) and de Groot et al. (2006); therefore, the

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present findings indicate that the responsible national and local government bodies must create an Ekşisu Wetlands management plan in collaboration with all stakeholders.

As they can help delineate a landscape's various stakeholders' views and evaluations of a landscape, which are often ignored by conventional landscape research methodologies (Mander et al., 2007), the landscape values concept and landscape value mapping process are instrumental to collaborative landscape planning, and can inform the decision-making processes. Accordingly, in the present study a workshop-based landscape value mapping process was used to determine the Ekşisu Wetlands' stakeholders' opinions and evaluations of the landscape's benefits. As reported by Brown (2006), in addition to other communication tools that can be used with landscape stakeholders, landscape value mapping and the data it generates provide location-specific information that can be used by all relevant stakeholders for planning the development of landscapes. The present findings show that sociocultural valuation via landscape value mapping can be used to assess the perceived benefits of simultaneously performed multiple functions of a landscape, as well as for sociocultural assessment of wetlands.

Landscape value mapping can also help planners and decision makers formulate priorities and alternatives during the landscape planning process as stated by Brown (2005), Daily et al. (2009), Raymond et al. (2009) and Zhu et al. (2010), as well as during the management process; therefore, as reported by Soini (2001), Fagerholm et al. (2012), Brown and Donovan (2014), landscape value mapping remains a valuable method for landscape research and landscape planning. Yet, as observed during the mapping workshop performed during the present study, the value of the data obtained is limited due to the limited number of stakeholders that participated, their limited knowledge about the area, and their limited ability to express their opinions and perceptions about the study area's intangible values and other landscape values.

According to the present study's conflict analysis findings, the stakeholders' benefits from provisioning services result conflicts for the sustainability and protection of regulation and cultural landscape services, Stakeholders are in conflict over many wetland landscapes worldwide (Eppink et al., 2004; Hansson et al., 2005; Hein et al., 2006; Posthumus et al., 2010) and similar dynamics are occurring in the context of Ekşisu Wetland; therefore, as a multifunctional landscape, the various functions of and benefits provided by Ekşisu Wetlands are under threat.

The present study shows that problems related to sustainability of landscape functions, landscape values, and stakeholder conflicts are 3 concepts that can be used to form the basis of a collaborative planning and management processes that aim to preserve the multifunctionality of Ekşisu Wetlands. The stakeholders' shared values, which were identified via a collaborative process and can be used to inform the collaborative planning process for Ekşisu Wetlands, included heritage value, biological diversity value, future value, knowledge value, recreation value, and wilderness value. Accordingly, habitat protection and improvement, and protecting and improving the area's regulating and cultural functions were defined as priorities for the collaborative management of the area. According to stakeholder analysis findings, the stakeholders with the most power and those in conflict with each other must collaborate during the planning and management of the Ekşisu Wetlands, and those with little power and that not in conflict must be informed of the process.

According to the power of influence in decision making processes for the area, national and local governmental institutions, together with local municipalities, are the

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stakeholders best able to protect and improve the multifunctionality of Ekşisu Wetlands. Improvement of the natural hydrological cycle and erosion control measures, and enforcement of all laws regarding the protection of pasture land and biodiversity in the study area should be considered primary goals of the collaborative planning process. Farmers/landowners, hunters, and sand-gravel quarry firms, together with civil society organizations and representatives of the local community, must be encouraged to participate and/or support the activities of the Ekşisu Wetlands' other more powerful stakeholders. Participatory decision-making tools and methods, including facilitation, negotiation, conflict management, and wetland management, should be the focus of capacity building activities for the stakeholders both in the national and local level.

Conclusion

As a result of their multifunctionality, landscape degradation and stakeholder conflicts on wetland landscapes generally arise due to the competing interests of the multiple stakeholders. Turkey is as rich in terms of its wetlands, their multifuncionality and conflicts between the wetlands' stakeholders as it is poor in terms of their participatory planning and management. Therefore, Turkey has to give emphasis to protect the multifunctionality of her wetlands via identifying and understanding the importance and value of the benefits provided by these landscapes to local communities and stakeholders, as well as the stakeholders' conflicting interests. This case study on Ekşisu Wetlands emphases the need for understanding the social and spatial dimensions of wetlands, as well as their interactions, in an effort to identify the links in wetland systems so as to inform a collaborative planning process aimed at protecting the multifunctionality of these critical landscapes.

Although assessing multifunctionality is more easily addressed on a small wetland landscape, this case study has demonstrated the pressures and degradations through provisioning services that result conflicts for the sustainability and protection of regulation and cultural landscape services that are also faced in many wider wetland landscapes. Sustainability of landscape functions, improving the perceived and experienced benefits of local people, and conflict resolution between its stakeholders are the main concepts that can be used to form the basis of a collaborative planning and management processes that aim to preserve the multifunctionality of wetlands and other landscapes. Although the current and dominating top-down planning culture in the country and in the case area, national and local governmental institutions, together with local municipalities, are the stakeholders best able to protect the multifunctionality of Ekşisu Wetlands.

It is clear that the collaborative landscape planning process can play an important role in protecting the multiple functions of today's contested landscapes–if various stakeholders with multiple interests can commit to a shared vision and common goals. As such, common values and conflicts related to landscapes need to be identified via consensus and a negotiation-based joint working processes in order to foster shared responsibility for and assignment of value to the multidimensional character of landscapes.

While landscape value mapping methodology enables to identify various perceived benefits of and shared values for the landscape it is subject to some limitations in mapping the intangible landscape values such as spiritual and intrinsic. Additional work with qualitative methods can help to minimize these limitations of landscape value

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mapping. Employing PCA and factor analysis has demonstrated the interlinkages between landscape capacities and experienced benefits. Therefore, these analyses also enabled to understand the Ekşisu's multifunctionality. However, further research is needed on the synergies and trade-offs between the use and non-use landscape values and on the socio-cultural landscape characteristics to better inform collaborative actions designed to protect the multifunctionality of landscapes.

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BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS BY ENVIRONMENT-FRIENDLY PRETREATMENT METHODS: A REVIEW

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Abstract. Lignocellulosic biomass is one of the abundant renewable bioresources on earth. Its chemical composition, i.e., lignin hinders ethanol production and commercialization. Pretreatment processes are vital for efficient separation of the complex interlinked components and enhance the availability of every component, i.e., cellulose and hemicellulose. However, for the bioethanol production, a major barrier is the removal of strong lignin component which is highly resistant to solubilization and a major inhibitor for hydrolysis of cellulose and hemicellulose. Pretreatment of biomass is necessary to make it susceptible to microorganisms, enzymes, and pathogens. Consequently, for the ethanol production, pretreatment of lignocellulosic biomass process is very costly. The initial pretreatment approaches include physical, physicochemical and biological methods. It found out that; pretreatment methods have a significant impact on efficient production of ethanol from biomass. However, extensive research is still necessary for the development of new and more efficient pretreatment processes for conversion of lignocellulosic biomass to ethanol. Present review article presents recent development on lignocellulose biomass pretreatment. We discussed the different pretreatment methods along advantages, disadvantages, and challenges for bioethanol production. This review includes benefits and drawbacks and chemical, physical, physiochemical and biological pretreatment along with existing problems. For the production of ethanol, this review will help researchers regarding selection, development and further planning of pretreatment for different lignocellulosic residues.
Keywords: *Asia, crop residue, cellulose, energy resource, environmental safety, hemicellulose, lignin*

Introduction

Lignocellulosic biomass is one of the most suitable alternative energy sources which can be harnessed to meet up the challenges of energy security. Biomass has been a significant contributor in achieving sustainable development goals (Triwahyuni et al., 2015). Over the last few decades, researchers have thoroughly practiced multiple techniques to generate energy from biomass and its related materials (Ravindran et al., 2015; Yadav et al., 2017; Zhang et al., 2015). This achievement can be attributed to the main reasons like low cost and abundance e.g. ethanol production (Ruiz et al., 2013). Biodiesel is an energy conversion product made from animal fat and vegetable oil through the trans-esterification process. Several researchers have used plants and their products to generate energy such as rubber seed (Jose et al., 2011), Rubber seed oil (Dhawane et al., 2017), oil palm biomass (Bhatia et al., 2017), Wilson's Dogwood (Li et al., 2017), *Brassica napus* seed oil (Anwar et al., 2017), *Koelreuteria integrifolia* oil (Zhang et al., 2017), jatropha oil (Nisar et al., 2017), castor oil (Baskar and Soumiya, 2016), *Eruca sativa* (Mumtaz et al., 2016) and Pongamia biodiesel as fuel (Perumal and Ilangkumaran, 2017). But, the production of bio-diesel from lignocellulosic biomass is not suitable due to low oil contents. Several issues are involved in biomass gasification of the plant for operation and designing reasons. Most of these causes may end in malfunctioning of the plant (Ruiz et al., 2013).

In EU, the target has been set to replace 10% for their automobile fuel with biofuels by 2020 (Porzio et al., 2012). It has been unanimously planned to consume 93.67 10^6 USD to support 2nd generation biofuels on industrial demo schemes through seventh framework program (Balan et al., 2013). The United States is targeting to produce 60.5 10^9L of 2nd generation bio-ethanol by the year of 2022 (Langholtz et al., 2012). By 2020, China is also planning to consume 12.7 10^9 L non-grain fuel ethanol (Chang et al., 2012). India has also announced a goal to replace 20% fossil fuel consumption with bioethanol and biodiesel by this year (Ravindranath et al., 2011).

In Pakistan, Sugar Mills Association (PSMA) is the agency responsible for producing bioethanol in the country. (Asif, 2009) The industry is entirely dependent upon fermentation of sugarcane molasses. Currently, 21 distilleries are trying to produce different grade ethanol in the country with an annual capacity of $500 10^6$ L. One kg of molasses is estimated to yield 0.240-0.270 L ethanol depending upon the molasses quality. In 2011-2012, Pakistan generated revenue of $260 10^6$ USD from $460 10^6$ L ethanol export at the trading price of 0.65 USD/L (Bhutto et al., 2015). Production of ethanol from molasses is not a feasible strategy due to increasing molasses cost (Arshad et al., 2008). The decline in agricultural land for sugarcane cultivation is also a chief reason of sugarcane shortage in future (Bhutto et al., 2015). In future, this will decrease the bioethanol production in the country, so it is essential for Pakistan to diversify its ethanol production to attain the national ethanol requirement and foreign export. For bioethanol production, biomass in the form of crop residues such as wheat straw, rice straw, maize stalk, cotton stalk and sugarcane tops, etc. are available in Pakistan (*Table 1*) (Yasin, 2012).

Pakistan produces field-based crop residue (69 million tons per year) which are commonly considered useless. It is estimated that four major crops produce 50 million tons of residue annually including 6.88 million tons of sugarcane bagasse. It is estimated that 10.942 million tons resource potential is available from four crops (wheat, rice, corn and cotton) without commercial and domestic consumption (*Table 2*) (Mirza et al., 2017). Bhutto et al. (2015) forecasted five lignocellulosic feedstocks such as cotton straws, sugarcane tops, rice straw, maize stalks and wheat straw yearly yield from 2013-2030 in Pakistan (*Fig. 1*). They also predicted the increasing potential to produce 2nd generation bioethanol up to 11.897 109 L by 2030 *(Fig. 2*) (Bhutto et al., 2015). This review critically appraises chemical, physical, physiochemical and biological pretreatment methods for bioethanol production. This review will be helpful for researchers in selecting, developing and planning for pretreatment strategies related to the different lignocellulosic residue.

Table 1. *Seasonal available different crop residue in Pakistan (Yasin, 2012)*

Residue Availability	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Wheat Residue												
Rice Residue												
Sugarcane top												
Cotton stalk												
Corn stalk												

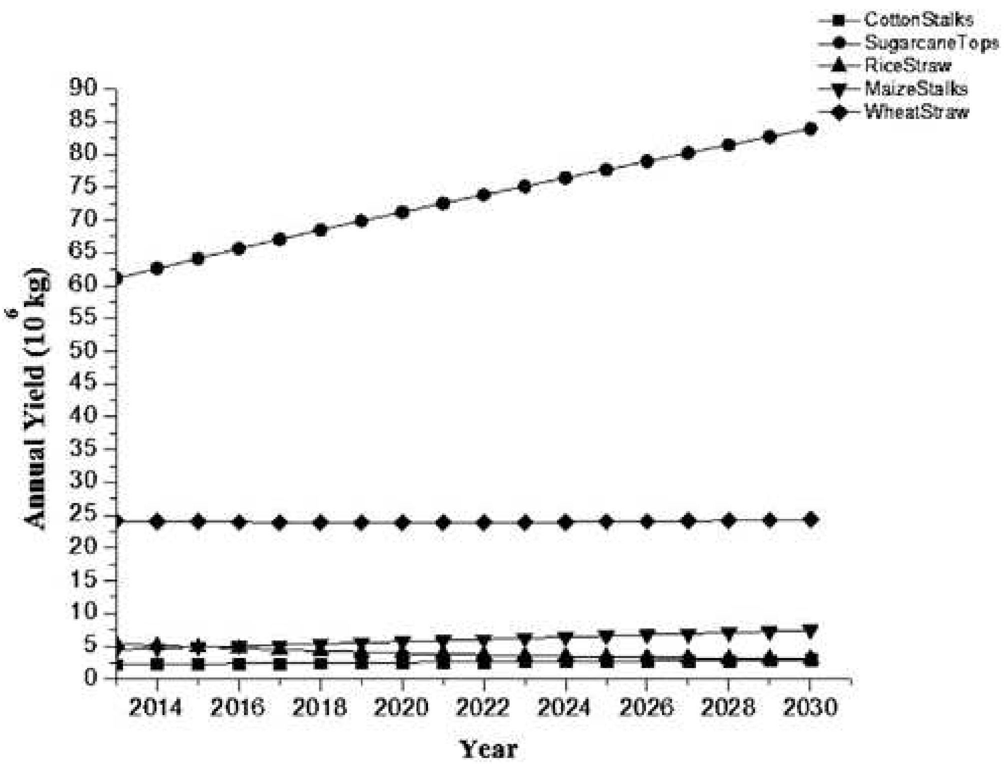

Figure 1. *Feedstock's potential from 2013-2030 based upon the available of crop residue such as wheat straw, rice straw, sugarcane tops, cotton stalks, and maize stalks and in Pakistan (Bhutto et al., 2015)*

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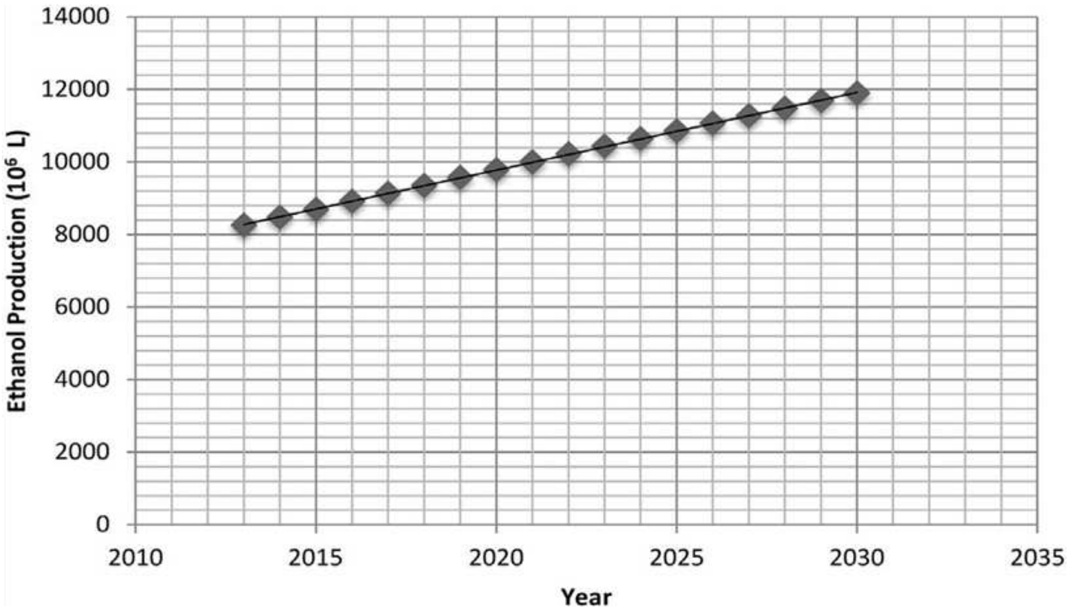

Figure 2. *Ethanol production potential from 2013-2030 based upon the available of crop residues such as wheat straw, rice straw, sugarcane tops, cotton stalks, and maize stalks and in Pakistan (Bhutto et al., 2015)*

Table 2. *Market price of different crops residue and ethanol potential after various pretreatment in Pakistan*

Lignocellulosic residue	Purchase price USD/10^3 kg	Treatment	Ethanol recovery	References
Wheat straw	25-35	H_2SO_4	19 g/l	(Bhutto et al., 2015; Saha et al., 2005a, 2005b)
Rice Straw	24	Dilute H_2SO_4	6.5–11.35 g/l	(Bhutto et al., 2015; Karimi et al., 2006)
Corn stalk	20	H_2SO_4	196 kg/t	(Bhutto et al., 2015; Demirbaş, 2004)
Sugarcane tops	8.5	H_2SO_4	3.35 g/l	(Bhutto et al., 2015; Dawson and Boopathy, 2007)
Cotton stalks	20	H_2SO_4	14.2 g/l	(Bhutto et al., 2015; Yu and Zhang, 2004)

Lignocelluloses

Lignocellulosic biomass refers to dry matter of plant (Collard and Blin, 2014) mainly composed of 25–30% hemicellulose, 40–50% cellulose, 15–20% lignin, and traces of pectin, nitrogen compounds, and inorganic ingredients (*Fig. 3*) (*Table 3*) (Knauf and Moniruzzaman, 2004; Mori et al., 2015). Cellulose is a linear syndiotactic (alternate spatial arrangement of side chain) polymer of glucose linked together by β-l,4-glycosidic bonds. Cellulose is the most abundant compound present on the earth with important features like biocompatibility, stereoregularity and hydrophobicity. Its distinct polymer chains have highly crystalline structure, and orderly bundled arrangements

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cause its stable properties. Its structure determines the framework of the cell wall. Lignin inhibits the process of hydrolysis because it is a complex hydrophobic polymer. Lignin is 3-D heterogeneous polycrystalline reticulated polymer which belongs to the polyphenol compound. This kind of polymers composed of phenylpropane structural units via ether linkages and carbon-carbon bonds. It has no proper regularity and orderliness of the repeating units. Hemicellulose is a mixture consists of different polysaccharides. Polysaccharides in hemicellulose are straight and branched as well. This polysaccharide has a low degree of polymerization, and without crystalline regions, so it can be easily degraded into monosaccharides such as fructose, xylose, galactose, dextrose, arabinose, and mannose (Balat, 2011; Chundawat et al., 2011; Karimi and Taherzadeh, 2016). Different factors determine the cell wall stability and extent of difficulty for its degradation. These factors include high crystallization zone along with several binding forces presents between cell wall constituents. The level of polymerization is also crucial in assessing wall degradation. The degradation of any class of ingredients will be subjected to the constraint of other ingredients. Thus, it is compulsory to degrade the network structure of lignin and efficient utilization of cellulose, lignocellulose by pretreatment. (Behera et al., 2014; Chaula et al., 2014; Liggenstoffer et al., 2014). Lignocellulosic biomass chemical structure causes key challenges to development and commercialization for ethanol because lignin surrounds the hemicellulose and cellulose. Pretreatment of biomass is necessary to make the biomass susceptible to microorganisms, enzymes, and pathogens. Consequently, for the bioethanol production, pretreatment of lignocellulosic biomass process is very expensive (Studer et al., 2011) (Mupondwa et al., 2016). Hence, for the selection of suitable pretreatment method should cover following points; the selected method should preserve the hemicellulose fraction; avoid the size reduction of biomass particles; reduce the energy demands; minimise the formation of degradation products; contain cheap catalyst recycle; contain cheap catalyst for pretreatment; and high-value co-product should produce from lignin (Wyman, 1999).

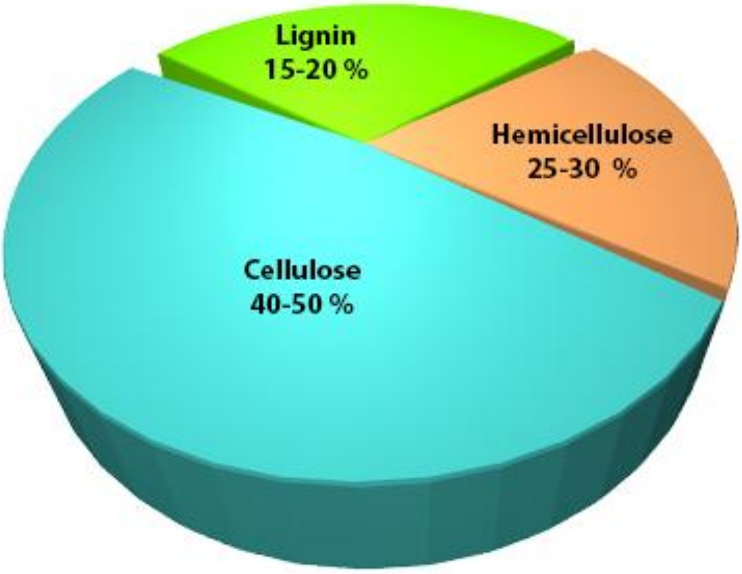

Figure 3. *General composition of lignocellulosic biomass*

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Table 3. *Composition of various lignocellulosic biomass*

Agricultural and herbaceous biomass	Lignin (%)	Hemicellulose (%)	Cellulose (%)	Ash (%)	References
Wheat straw	17–19	26–32	33–38	3.74	(Rabemanolontsoa and Saka, 2013; Saini et al., 2015)
Rice straw	12–14	23–28	28–36	19.8	(Qu et al., 2011; Saini et al., 2015)
Barley straw	14–19	27–38	31–45	-	(Saini et al., 2015)
Rye straw	16–19	27–30	33–35	-	(Sánchez, 2009)
Oat straw	16–19	27–38	31–37	-	(Sánchez, 2009)
Rice husk	26–31	18–21	25–35	17.27	(Ludueña et al., 2011; Rabemanolontsoa and Saka, 2013)
Sugarcane bagasse	20–42	19–25	42–48	-	(Kim and Day, 2011; Saini et al., 2015)
Sweet sorghum bagasse	14–21	18–27	34–45	-	(Saini et al., 2015)
Corn stover	7–19	24–26	38–40	6.8	(Qu et al., 2011; Saini et al., 2015; Zhu et al., 2005)
Corn cobs	14–15	35–39	42–45	3.53	(Kuhad and Singh, 1993; Prasad et al., 2007; Rabemanolontsoa and Saka, 2013)
Corn leaves	15.18	13.27	26.93	10.95	(Rabemanolontsoa and Saka, 2013)
Bamboo	20.81	19.49	39.80	1.21	(Rabemanolontsoa and Saka, 2013)
Switchgrass	10–40	30–50	5–20	5-6	(Lynel et al., 1999; McKendry, 2002)
Hazelnut shell	42.1	28.2	25.2	1.4	(Demirbaş, 2005)
Miscanthus	24–25	18–24	38–40	5.5	(Brosse et al., 2010; Rabemanolontsoa and Saka, 2013)
Hardwood biomass					
Beech	20	33	45	0.2	(Di Blasi et al., 2010)
Poplar	20	24	49	1	(Di Blasi et al., 2010)
Aspen	19.5	21.7	52.7	0.3	(Taherzadeh et al., 1997)
Cherry wood	18	29	46	0.5	(Di Blasi et al., 2010)
Willow	29.3	16.7	41.7	2.5	(Taherzadeh et al., 1997)
softwood biomass					
Pine	27.3	20.3	46.9	0.3	(Taherzadeh et al., 1997)
P.armandii Franch	24.1	17.8	48.4	0.2	(Wang et al., 2016)
Spruce	27.6	29.4	43.0	0.6	(Demirbaş, 2005)
Japanese cedar	33.8	23.1	38.6	0.3	(Rabemanolontsoa and Saka, 2013)
Fir	30	22	45	0.5	(Di Blasi et al., 2010)

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Pretreatment techniques of lignocellulosic biomass

The main aim of pretreatment method is to change the chemical composition, macrostructure and microstructure of lignocellulose. It also makes the natural lignocellulosic macromolecule susceptible to microbial degradation (*Fig. 4*) (An et al., 2015). Hence, a pretreatment process is necessary to decrease the cellulose crystallinity, remove lignin and enhance the porosity of the material. An effective pretreatment should be cost-effective, produce fewer inhibitors and produce significant percent of cellulose support (Sun and Cheng, 2002).

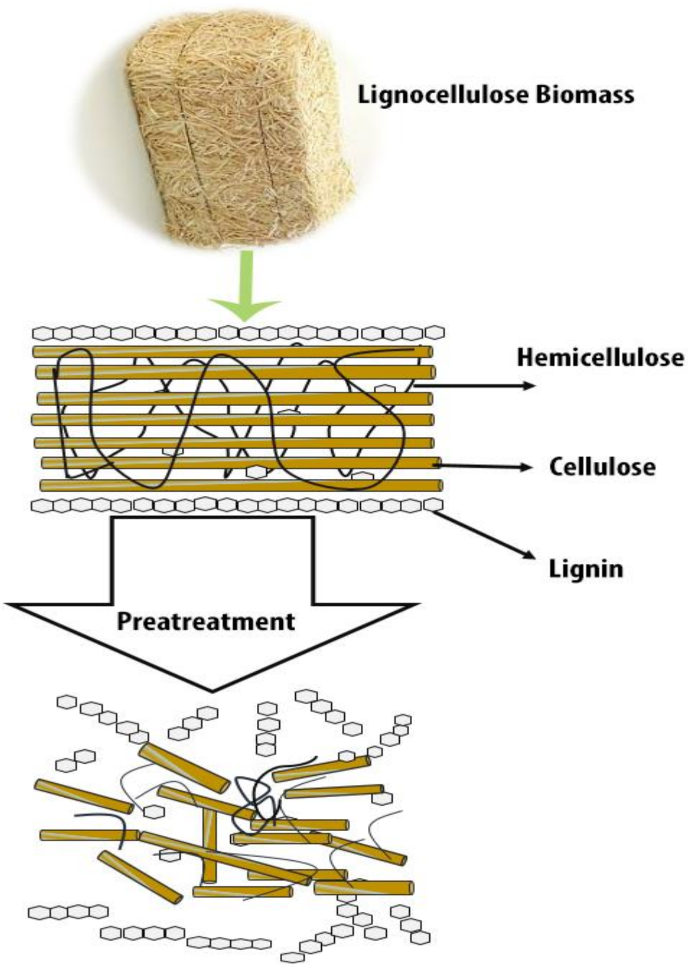

Figure 4. *Lignocellulosic biomass after pretreatment*

Common physical, chemical and biological methods or their combination are involved in this process (*Fig. 5*) (Taherzadeh and Karimi, 2008). Mostly physical pretreatment process include the biomass size reduction to increase the available surface area and reduce the cellulose crystallinity or degree of polymerization (Sanchez and Cardona, 2008). In chemical pretreatment process, various chemicals including acids are used for biomass pretreatment e.g. ionic liquids, an organic solvent, alkali and oxidizing agents (Fu et al., 2010; Fu and Mazza, 2011a, 2011b). Physio-chemical pretreatment is the combination of mechanical and chemical processes. The verity of methods contains catalyzed steam explosion such as carbon

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dioxide, ammonia fiber expansion, sulfur dioxide explosion, ammonia recycle percolation and liquid hot water (Sun and Cheng, 2002). In biological pretreatment, natural microorganisms (e.g. bacteria, soft-rot and brown-white fungi) having enzymes are used to destruct the cell wall of lignocellulosic biomass (Sánchez, 2009). Chemical and physical pretreatment have relatively good results as compared to other pretreatment, but apparatus requirement is very strict and causes severe pollution. Biological pretreatment process is environment-friendly and consumes less energy as compared to other pretreatment methods. However, biological pretreatment is expensive and enzymes activity during biomass decomposition is very slow requiring long duration (Taherzadeh and Karimi, 2008).

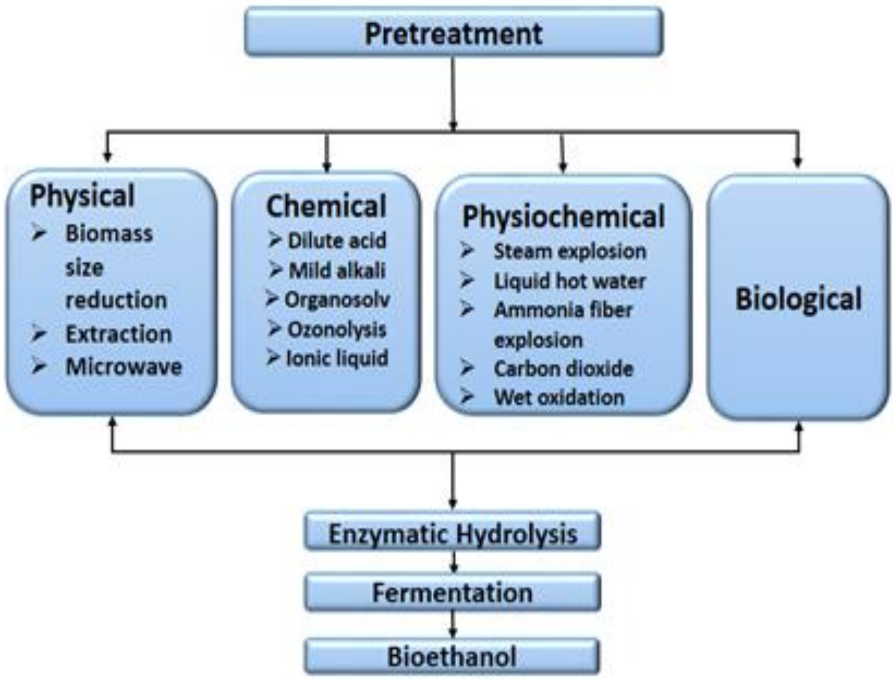

Figure 5. *Various types of pretreatment methods*

Physical pretreatment

Biomass size reduction

Various mechanical size reduction methods are employed to increase the digestibility of lignocellulosic biomass such as chipping, shredding, grinding, coarse size reduction and milling (Laser et al., 2002). These pretreatment methods decrease the cellulose crystallinity and the degree of polymerization as well as increase the specific surface area (Sanchez and Cardona, 2008). Menegol et al. (2016) also revealed that physical pretreatment leads to higher ethanol production after delignification of elephant grass. However, studies demonstrated that more reduction of biomass particles i.e. smaller than 0.4 mm has no significant effect on yield and rate of hydrolysis (Chang et al., 1997). The energy demand for mechanical comminution of lignocellulosic material depends on agricultural biomass features and final particle size (Cadoche and López, 1989). Higher energy demand for milling procedure is the main drawback of this technique (Hideno et al., 2009). Studies suggest that milling before pretreatment have

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several benefits like low consumption of milling energy and no production of fermentation inhibitors. It also reduces the cost of separating solids from liquids by decreasing the solids to liquids ratio (Zhu et al., 2010). Kim et al. (2013) compared three different milling method such as planetary, attrition and ball milling. Ball milling was less effective in reducing the size of biomass as compared to attrition milling and planetary milling. The highest amount of galactose and glucose were produced by planetary mill method rather than other tested milling methods. The point to be noted is that all the mill pretreatment methods do not produce any toxic compounds e.g. levulinic acid and hydroxymethyl furfuraldehyde (HMF). It makes mill pretreatment a good choice of initial pretreatment for a broad range of lignocellulosic feedstocks. Application of wet disk milling and ball milling used to estimate the effectiveness of milling on sugarcane bagasse. According to assessment, hydrolysis yield of xylose and glucose under optimum condition of mill treated bagasse were 72.1% and 78.7% respectively. Whereas maximum xylose and glucose yielded for bagasse wet milling-treated were 36.7% and 49.3% respectively (da Silva et al., 2010; Hideno et al., 2009).

Extrusion

Extrusion is a developed method of mechanical comminution in which the access of enzyme is widened to strike well-exposed carbohydrates (Zhan et al., 2006). It includes rapid mixing, less residence period, high shear, moderate barrel temperature, no furfural, no washing, and conditioning. Moreover, most possibilities of continuous operation are considered among the benefits of this method (Karunanithy et al., 2008; Karunanithy and Muthukumarappan, 2011). Various factors should be handled such as compression ratio, temperature, and speed of screw to obtain the extrusion pretreatment efficiency (Karunanithy and Muthukumarappan, 2010). In another experiment, Yoo et al. (2011) obtained 94.8% glucose conversion after enzymatic hydrolysis (glucose yield of 0.37 g/g biomass). Lee et al. revealed that Douglas fir was extruded before hydrolysis and it showed 62.4% conversion of the feedstock into glucose. They reported its ability to run continuously with zero effluent waste. Extrusion pretreatment method is feasible to apply on an industrial level and also don’t cause an environmental problem (Lee et al., 2009a).

Microwave

Microwave irradiation is broadly used for the pretreatment of lignocellulosic feedstock because of numerous reasons such as lower energy demand, less production of inhibitors, easy operation, degrades structural organization of cellulose fraction and high heating capacity in short duration (Hu and Wen, 2008). In microwave irradiation process electromagnetic field is employed. For the bioethanol production, this approach is applied for the pretreated biomass structural disruption. Microwave attacks the polar bond by vibrating the structure until material inside becomes heated. As a result, complex lignocellulosic structure fractures and enlarge the surface area for the subsequent enzymatic attack. Microwave treatment is reckoned as an improvement effort through assisting other treatment methods. Recently, the microwave was used to assist hydrothermal hydrolysis of sago pith waste (Ma et al., 2009). The microwave assisted hydrothermal hydrolysis consumed only 33 kJ and 69 kJ per every gram production of glucose and ethanol respectively, which graded as energy efficient. Moreover, for more actual breakdown the mild alkali reagents addition is preferred. Additionally, it has been revealed microwave based alkali pretreatment of switchgrass produced approximately 70-90% sugars (Hu and Wen, 2008).

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Zhu et al. (2015a, 2015b, 2016) checked the effects of microwave on chemically pretreated *Miscanthus*. As compared to conventional heating, sulfuric acid and sodium hydroxide pretreatment generated 12 times higher yield in half of the time consumed for heating. Primarily, this was due to lignin solubilization and pre-disruption of crystalline cellulose with the chemical pretreatment. When pretreated with 0.2 M sulfuric acid at 180 °C temperature for 20 minutes, the maximum glucose yield obtained was 46.7%, and sugar yield was 75.3% (Zhu et al., 2015a, 2015b). Likewise, Zhu et al. (2016) established an orthogonal design to improve the microwave pretreatment of wheat straw and also enhance the ethanol production from 2.678-14.8%.

Physio-chemical pretreatment

Steam explosion

Previously, it was known as steam explosion; steam pretreatment is a physicochemical strategy commonly used for lignocellulose biomass hydrolysis (Agbor et al., 2011). This method exposes the biomass at high-pressure steam for a few seconds initially at the range 160 °C to 260 °C (Sun and Cheng, 2002). The hydrolysis of hemicellulose into glucose and xylose monomers is carried out by the acetic acid formed from the hemicellulose acetyl groups during this pretreatment. Hence this procedure is also known as auto-hydrolysis (Mosier et al., 2005). Several factors such as resistance time, the size of biomass, moisture content and temperature affect the pretreatment (Rabemanolontsoa and Saka, 2016). The presence of H_2SO_4, CO_2 or SO_2 as a catalyst can enhance the actual efficiency of this process. Without these catalysts, the acidic catalyst has been found most effective for minimized the production of inhibitor compounds, recovery of hemicellulose sugar and better enzymatic hydrolysis. This pretreatment has been found effective for agricultural residue and hardwoods pretreatment. Steam pretreatment has some advantages such as low energy requirements, limited chemicals use, no recycling cost and environment-friendly. On the other side, the risk of high-temperature formation of fermentation inhibitory compounds, lignin-carbohydrate matrix incomplete digestion and the need to wash the hydrolysate (Agbor et al., 2011). After 72 hours of enzymatic hydrolysis, corn stover pretreated with steam pretreatment under catalyst (SO_2) has been shown to give high yields of sugar such as almost 80% overall xylose yield and nearly 90% overall glucose yield (Öhgren et al., 2005). Ethanol can be produced from lignocellulosic biomass employing steam pretreatment followed by fermentation and enzymatic hydrolysis. Sugar yield of both cellulose and hemicellulose are critical parameters for an economically reasonable for ethanol production process (Öhgren et al., 2007).

Liquid hot water

Liquid hot water, also known as hot compressed water is same like stream pretreatment method. However, as its name indicates, water is used at high pressure up to 5 Mpa and high temperature 170–230 °C instead of steam. This is also variously mentioned as hydro thermolysis, aquasolv, hydrothermal pretreatment and aqueous fractionation. It removes lignin offering extra accessible cellulose and hemicellulose (Agbor et al., 2011; Laser et al., 2002; Yang and Wyman, 2004). Liquid hot water is an effective method to treat different biomass types including softwoods (Rabemanolontsoa and Saka, 2016). Water-insoluble material, solids enriched with cellulose and liquid fraction such as water, inhibitors, solubilized hemicellulose are

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generated as slurry through the pretreatment procedure. In liquid hot water control pH between 4–7 is appropriate to avoid the sugar degradation and inhibitors formation.

Laser et al. (2002) achieved maximum solubilized hemicellulose with minimum inhibitors production and pH control during hot water pretreatment of corn stover at 190^{o}C for 15 minutes. They obtained conversion of cellulose to glucose at the rate of 90% through the enzymatic hydrolysis. This pretreatment method is economically and environmentally attractive because chemical catalyst or acid is not used in this process (Mosier et al., 2005). Its advantages include low-temperature requirement, no inhibitory compounds formation at high temperature, and low-priced solvent of liquid hot water process (Agbor et al., 2011; Yang and Wyman, 2004).

Ammonia fiber explosion

Soaking aqueous ammonia, ammonia fiber explosion and ammonia recycle percolation are the methods employed for the lignocellulosic biomass pretreatment in which liquid ammonia is used (Agbor et al., 2011). In soaking ammonia, biomass is treated with aqueous ammonia in a batch reactor at temperature (30–60 °C) which decrease the through putting during the process of pretreatment (Kim and Lee, 2005a). Ammonia fiber explosion pretreatment is similar to a steam explosion, which treats lignocellulosic biomass with ammonia on specific temperature (60–100 °C) and high pressure (250–300 psi) for a short period. The operational parameters involved in the ammonia fibers explosion are temperature, blowdown pressure, water loading, and ammonia loading (Holtzapple et al., 1991). It causes the swelling and phase change of biomass cellulose crystallinity leading to increasing the reactivity of leftover carbohydrates after pretreatment. As compared to other pretreatment methods, ammonia fiber explosion does not produce inhibitors which are extremely desirable for downstream processing. The lack of extra steps such as detoxification, water washing, reuse of large quantity of water and recovery make the overall cost significantly low. If ammonia fiber explosion pretreatment is employed under optimized condition more than 90% hemicellulose and cellulose can be converted into fermentable sugar (Uppugundla et al., 2014). Ammonia recycle percolation is a powerful method as compared to any other conventional pretreatment methods to remove lignin and without any contamination e.g. sulfur and sodium (Yoon et al., 1995).

Ammonia recycle percolation process does not affect the cellulose but makes hemicellulose soluble. This method needs high amount of energy to maintain temperature. Ammonia fiber explosion and ammonia recycle percolation more efficient for agriculture residues and herbaceous plant (Alvira et al., 2010; Kim and Lee, 2005b). Belkacemi et al. (1998) produced ethanol from agriculture residue and forages through the pretreatment of ammonia fiber explosion. They pretreated the forage-based lignocellulosic biomass such as barley straw, corn stalk, alfalfa, reed canarygrass and timothy grass and obtained 60-70% sugar yield by using ammonia fiber explosion pretreatment. There are several advantages include ammonia recovery, no inhibitors production, lignin redistribution and ability to accomplish theoretical yield level (Sun and Cheng, 2002). This pretreatment has major challenge of total energy consumption cost (Banerjee et al., 2009).

Carbon dioxide (CO_2)

For bioethanol production, the use of carbon dioxide (CO_2) as supercritical liquid technology is a feasible strategy. It provides the modification of possible pretreatment

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process and usually used for coffee decaffeination technique (Zheng et al., 1998). Supercritical CO_2 pretreat the lignocellulose biomass which means that the gas behaves like a solvent. At high temperature, supercritical carbon dioxide passes through a vessel having biomass. The vessel temperature is kept high for several minutes. At high-temperature CO_2 enter the biomass and forms carbonic acid which causes hemicellulose hydrolysis. High-pressure gas disorders the biomass structure and increase the accessible surface area (Hendriks and Zeeman, 2009; Kim and Hong, 2001; Zheng et al., 1995). Biomass without sufficient moisture is not suitable for this pretreatment process (Kim and Hong, 2001). However, supercritical liquid technology is considered beneficial because it contains both features liquid insolvent and gas transfer in mass. As compared to steam ammonia and steam explosion, this process is favorable for the production of ethanol,because it produces low inhibitory products and removes lignin at more feasible way (non-acidic and non-corrosive). Moreover, CO_2 as the supercritical fluid pierce the crystalline easy and also not degrade the required sugar monomers because of its mild environment (Zheng et al., 1998).

Wet oxidation

In wet oxidation pretreatment method, biomass is subjected to high temperature i.e. 170–200 °C and high pressure i.e. 500–2000 kPa for 10–15 minutes. Wet oxidation is a simple and easy method. This had earlier been employed for soil remediation and wastewater treatment. This approach degrades the lignocellulosic material and produces fewer inhibitors, eliminates lignin as well as lower cellulose. Hence it provides a suitable condition for the procedures like fermentation and enzymatic hydrolysis to work on the pretreated product. However, common challenges like energy efficiency and capital cost are associated with this method (Banerjee et al., 2009; Chaturvedi and Verma, 2013; Martín et al., 2008). Wet oxidation method is suitable for the lignin enriched biomass residue. The efficiency of this approach depends upon three factors i.e. temperature, reaction time and oxygen pressure. In this process, water behaves like an acid and catalyzes hydrolytic reactions when the temperature is above 170 °C. Small pentose monomers are formed after breaking down of hemicellulose. The lignin undergoes oxidation, while the cellulose is least affected by wet oxidation pretreatment. Besides these, in wet oxidation additional chemical agents such as sodium carbonate and alkaline peroxide decrease the reaction temperature and improved hemicellulose degradation along with decreased production of inhibitors e.g. furfurals and furfurl aldehydes (Banerjee et al., 2011).

Banerjee et al. (2009) have maximized the wet oxidation condition for the production of ethanol from rice husk. Reducing sugar yields higher than 70% was recorded after this pretreatment method. Rice husk pretreated with Alkaline Peroxide Assisted Wet Air indicated result solubilization of 88 and 67% lignin and hemicellulose, respectively. The glucose amount improved 13 folds as compared to rice husk without treatment.

Chemical pretreatment

Dilute acid pretreatment

Diluted acid pretreatment is a conventional method and forms inhibitory products. However, it is commonly used for bioethanol production from lignocellulosic feedstock on industrial scale (Saha et al., 2005a; Sassner et al., 2008; Sun and Cheng, 2005). Based on the type of end application, two kinds of acid pretreatment are established;

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temperature less than 120 °C for long duration 30 to 90 minutes and high temperature more than 180 °C for 1 to 5 minutes respectively. Various kinds of reactors are also employed such as plug flow, shrinking-bed, percolation and flow-through. However, before further processing, inhibitors generated in acid pretreatment should be removed. To make the process economically feasible, the concentrated acid should be recovered after hydrolysis (Digman et al., 2010; Sassner et al., 2008). For the pretreatment of lignocellulosic biomass, different acids have been applied on industrial scale such as phosphoric acid, hydrochloric acid, nitric acid, and dilute sulphuric acid. Various organic acids such as acetic acid, lactic acid, maleic acid and peracetic acid are being used in the acid pretreatment of lignocellulosic biomass (Balat et al., 2008; Digman et al., 2010; Refaat, 2012). In acid pretreatment, rye straw and bermudagrass were pretreated with 1.5% sulphuric acid resulted in production of 22.93% reducing sugars, while hydrolysis yielded 19.71 reducing sugars respectively (Sun and Cheng, 2005). Another study revealed 204.1 mg/g of reducing sugars from Bermuda grass pretreated with 1.2 % dilute sulfuric acid for 1 hour at 121 °C (Sun and Cheng, 2005). In another study, bioethanol was produced through dilute acid pretreatment and silver grass using as feedstock (Guo et al., 2008). The silver grass produced 64.3% ethanol and 70–75% xylan sugars after diluted acid pretreatment at 121 °C for 30 minutes (Aditiya et al., 2015). Di-carboxylic acid: Oxalic acid, maleic acids and other kinds of are known as dicarboxylic acids are being utilized by the researchers in order to overcome the disadvantages of sulfuric acid. Dicarboxylic organic acid show high pKa values, which make this type of acids more effective for carrying out the hydrolysis of substrate over variety of pH and temperature (Lee and Jeffries, 2011)**.** Lee and coworkers (2011) pretreated the corn cobs with oxalic acid and heated for 26 minutes at 168 °C temperature. A total 13% sugar yield was found by the oxalic acid pretreatment (Lee and Jeffries, 2011). Maleic acid is another dicarboxylic acid which is commonly employed for the acid pretreatment. (Mosier et al., 2002). Lee and Jeffries (2011) examined the effect of sulfuric, maleic and oxalic acid on lignocellulosic biomass degradation and hydrolysis at same combined severity factor (CSF) during hydrolysis. Glucose and xylose concentration was found to be highest in maleic acid as compared to oxalic and sulfuric acid at low combined severity factor (CSF). When the maleic acid was employed for the pretreatment of biomass, the subsequent fermentation with biomass which was pretreated yielded maximum ethanol (19.2 g/L) at CSF 1.9 (Lee and Jeffries, 2011).

Pretreatment of lignocellulosic biomass with sulfuric acid is a conventional method because of its low cost. Hence, it has certain disadvantages such as corrosion of reaction vessel and production of inhibitory compounds (Lee and Jeffries, 2011). The toxic and corrosive nature of various acid needs appropriate material for constructing the reactor which can sustain the corrosive nature of acid and experimental conditions (Saha et al., 2005a). Whereas, dilute acid pretreatment acid does not need acid recovery with the insignificant acid loss (Refaat, 2012). Less amount of inhibitors compounds are produced by the Oxalic acid (Lee and Jeffries, 2011). The advantages discussed above, the maleic acid in specific has khyd/kdeg which helps cellulose hydrolysis to glucose and also over glucose degradation (Mosier et al., 2002).

Mid alkali pretreatment

As compared to acid treatment, alkali pretreatment methods commonly employed at ambient pressure and temperature. This breaks down extra lignin content and

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converts into a better available condition for hydrolysis afterward (Kumar and Wyman, 2009; Refaat, 2012). Alkali reagents are used that are hydroxyl derivatives of sodium, calcium, potassium and ammonium salts. Sodium hydroxide was found to be effective as compared to these hydroxyl derivatives (Kumar and Wyman, 2009). However, with the alkali pretreatment, the solubility of hemicellulose and cellulose is little as compared to acid pretreatment. Disrupted lignin structure, decreased the degree of polymerization and high internal cellulose surface area improves the solubility (Taherzadeh and Karimi, 2008).

Sun et al. (1995) improved the temperature and pretreatment utilizing 1.5% sodium hydroxide released 80% hemicellulose and 60 % lignin at 20 °C for 144 hours. Zhao et al. (2008) used different biomass such as switchgrass, softwoods, wheat straw and hardwoods to check the effect of sodium hydroxide containing less than 26% lignin. However, no effect on softwood of dilute sodium hydroxide was observed with lignin content larger than 26% (Kumar and Wyman, 2009).

As compared to other pretreatments, e.g. Acid pretreatment, mild alkali pretreatment is less harsh and can also be effectively carried out at ambient condition. However, higher temperature is required if the pretreatment is needed to be conducted for an extended time. Further to remove the inhibitors like lignin, a neutralizing step is necessary (Brodeur et al., 2011). Lime pretreatment is cheap as compared to the alkaline agents (Brodeur et al., 2011). Park et al., (2010 a,b) adjusted the lime pretreatment method through neutralizing lime with carbon dioxide before hydrolysis and removed the solid-liquid separation step resulting in 89% glucose recovery from leaf star rice straw. They also applied the alteration to study the fermentation (SSF) and simultaneous scarification by using *Pichia stipites* and *Saccharomyces cerevisae*. After 79 hours fermentation at 30 °C found 74% increase in ethanol yield.

Organosolv pretreatment

Organosolv is applied to dissolve lignin from lignocellulose biomass (Zhao et al., 2009). This method is also considered as one of chemical pretreatment (Zhao et al., 2009), that includes the application of organic solvents e.g. acetone, ethanol, ethylene glycol, methanol, and tetrahydro furfuryl alcohol (Mesa et al., 2011) Usually, acid, salt or base are the catalysts of this process (Bajpai, 2016). Organic or inorganic acids (sulfuric acid and hydrochloric acids) and bases (lime, sodium hydroxide, ammonia) are used as catalysts (Zhao et al., 2009). Lignin is a value-added product which is separated from biomass by this process that reveals enzymatic hydrolysis of cellulose fiber which leads to higher conversion of biomass. Apart from lignin, during organosolv pretreatment hemicellulose syrup C5 and C6 and cellulose fraction is also produced (Agbor et al., 2011). A study by Li et al. (Li et al., 2009) applied phosphoric acid and acetone and reported 93% quality bioethanol production from lignocellulosic biomass by using the organosolv method. High-quality bioethanol is the main advantage of this approach (Zhao et al., 2009). The disadvantages are the low-boiling point of organic solvents, high risk of high-pressure operation, high risk of volatility of harsh solvents (Agbor et al., 2011; Sun and Chen, 2008). A high flame of harsh solvents and large fire explosions can damage without the lack of particular safety measures. A method that treats the biomass at high temperature 100 °C and pressures 1 atm with the mixture of phenol, water, and HCl is termed, Battelle is the type of Organosolv (Villaverde et al., 2010). The Organosolv pretreatment process is shown in (*Fig. 6*)

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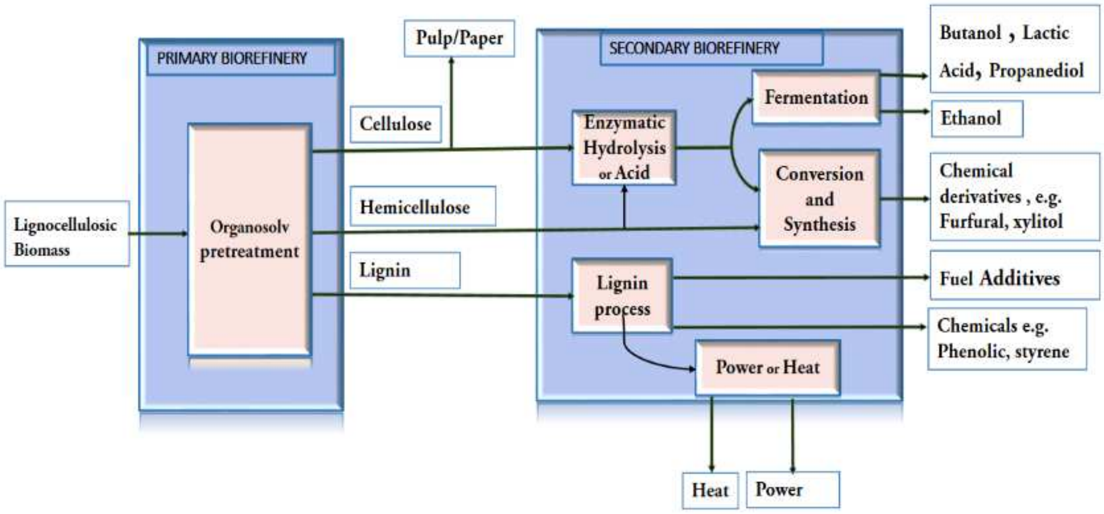

Figure 6. *Organosolv pretreatment process (Sidiras and Salapa, 2015; Zhang et al., 2016)*

Ozonolysis

In Ozonolysis pretreatment ozone gas used as an oxidant to break up lignin, hemicellulose and enhance the biodegradation of cellulose (Balat, 2011). It has been used to eliminate the lignin in a different kind of biomass e.g. poplar sawdust (Vidal and Molinier, 1988), green hay, bagasse, peanut and pine (Neely, 1984). After the removal of 60% lignin, cellulose enzymolysis rate can rise fivefold by the pretreatment. The lignin content can also be decreased from 29% to 8%, and the quality of the enzymatic hydrolysis yield can be increased by 57% (Sun and Cheng, 2002). Ozonolysis can also eradicate the lignin efficiently at normal pressure and room temperature similar to another chemical pretreatment method. This pretreatment method has several advantages. It is environmentally friendly because toxic inhibitors are not produced and have no effect on post-pretreatment processes e.g. yeast fermentation and enzymatic hydrolysis (Quesada et al., 1999; Sun and Cheng, 2002). Lack of degradation by-products which leads to less complication in the subsequent hydrolysis steps is considered most beneficial (García-Cubero et al., 2009). On the other hand, an enormous amount of expensive ozone is used to treat lignocellulosic material which is the main disadvantage (Sun and Cheng, 2002).

Ionic liquid pretreatment

Ionic liquid pretreatment is considered as a green and sustainable method using ionic liquid fractures. Due to this, covalent structure between hemicellulose, lignin and cellulose are disrupted due to the strong hydrogen bond acceptors of the ionic liquid (Refaat, 2012; Swatloski et al., 2002). It is reported that the most effective ionic liquids are 1-allyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium chloride (Vitz et al., 2009). As compared to dilute acid pretreatment, ionic liquid pretreatment yields less crystallinity, the large surface area of cellulose and less lignin obtained as well as an additional increase in enzymatic hydrolysis. (Li et al., 2010). Ionic liquid pretreatment advantages include zero vapor pressure, large stable temperature range and appropriate chemical stability (Ouellet et al., 2011). It has some of the disadvantages

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such as low-cost recovery technology, toxicity to microorganisms and enzymes. This process should be considered to recover lignin and hemicellulose from solutions after extraction of cellulose (Hayes, 2009; Lee et al., 2009b). The ionic pretreatment process is mention in diagram (*Fig. 7*) (Brandt et al., 2013).

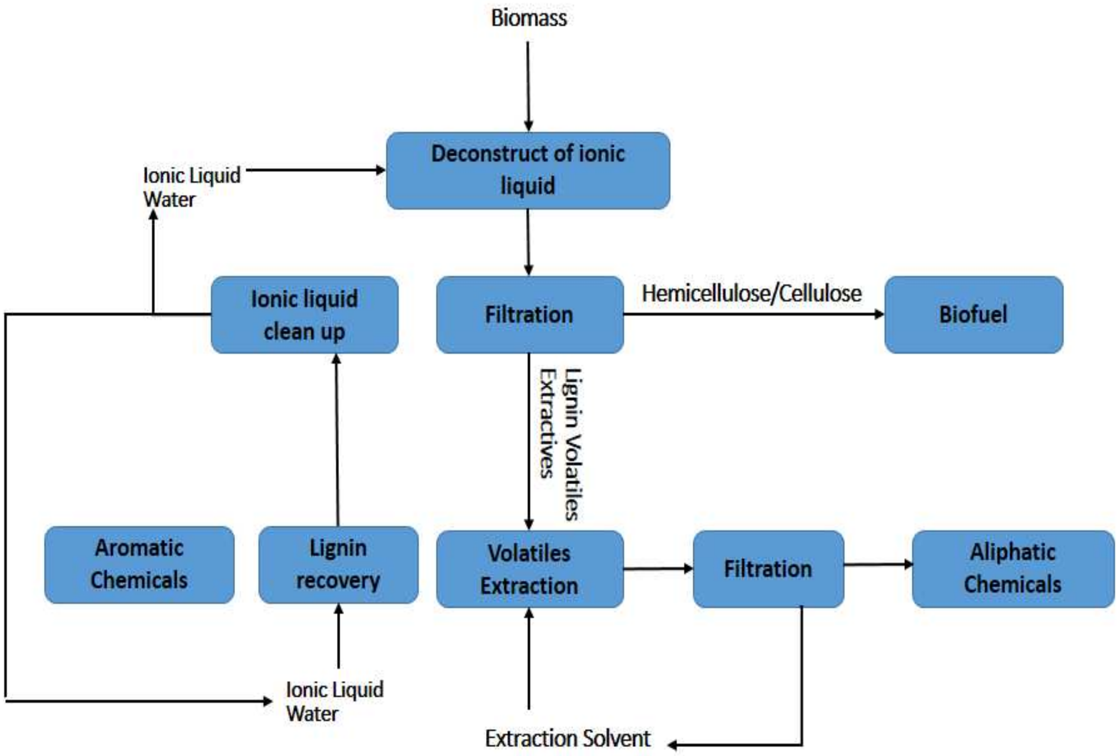

Figure 7. *Ionic Pretreatment Procedure (Brandt et al., 2013)*

Biological pretreatment

For the degradation of lignocellulose biomass, natural microorganisms possessing enzymes (bacteria, brown-white and soft rot fungi) are employed that are capable cell wall deconstruction (Talebnia et al., 2010). Biological pretreatment method does not produce any unwanted products as compared to chemical and physical pretreatment. Additionally, high pressure, acids, alkali, high temperature or any reactive species are not compulsory for this pretreatment (Zhang et al., 2011). White- and soft-rot fungi contain lignin-degrading enzymes like lignin peroxidases, manganese-dependent peroxidases, polyphenol oxidases, and laccases which are effective for the lignin degradation. Degradation by microorganisms, mode of action and features depends on targeted biomass component. For example, white and soft-rot fungi are considered most useful for degradation of lignin by using their lignin-degrading enzymes, and brown-rot fungi mainly attack cellulose (Sánchez, 2009; Sun and Cheng, 2002).

Wheat straw pretreated with low cellulose degrading fungus and high lignin degrading fungus resulted in a reduction in the concentration of inhibitors and acid loading for hydrolysis as well as increase the fermentable sugar. Ethanol yield and volumetric productivity with *Pichia stipitis* were 0.48 g/ g and 0.54 g/L h respectively (Kuhar et al., 2008). Biological pretreatment is efficient in comparison with any others

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pretreatment methods because it does not require chemicals. Furthermore, low energy and mild environmental condition are also considered as an advantage. However, the main drawback to develop biological methods is low hydrolysis rate obtained in biological materials (Sun and Cheng, 2002) (Salvachúa et al., 2011).Most lignin degrading microorganisms also degrade not only lignin but also cellulose and hemicellulose (Eggeman and Elander, 2005). Extensive research is required for the implementation of these microorganisms on a large commercial scale for biological pretreatment of lignocellulose biomass.

Conclusions

Various pretreatment methods have been developed for the delignification of lignocellulose biomass to obtain ethanol. However, we have concluded through the critical analysis, the correct choice of pretreatment should depend on the lignocellulosic biomass properties and features. Besides, a single pretreatment technique does not exist currently, which is economically practicable and environmentally friendly manner for the complete biomass delignification. Still, combined pretreatment techniques have found successful in the ethanol production, however, to achieve the total potential of the combined pretreatment method, much research required to be performed.

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AGROCLIMATIC ZONING OF SEMIARID REGION OF ZACATECAS, MEXICO

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Abstract. Agroclimatology concerns those climate processes influencing agricultural production, especially with respect to crops growing under rainfed conditions. A number of methodologies have been used to classify climate variables. The goal of this research was to obtain the agroclimatic delimitation for the Zacatecas region in Mexico using the Papadakis classification and collecting time-series data from 133 weather stations to obtain monthly thermal and monthly water parameters. The dataset is composed of information collected for the period of 1960-2015. For all weather stations data were filtered and chronologically sorted to complete any missing information using a geostatistical and Kalman filter approach. Monthly average values were interpolated using the ordinary Kriging method. Twelve maps were plotted to characterize agroclimatic conditions showing that January, February, November, and December are months that are too cool for agricultural practices. The climate conditions begin to become benevolent for agriculture from April. Three different agroclimatic zones were obtained for Zacatecas region. More than 90% of the surface is under the 2.2 climate type favoring crops such as maize, beans, and soybeans. Agroclimatic understanding is particularly important in semiarid regions because this permits proactive actions for promoting the agricultural potential of regions with scarce rainfall regimes.
Keywords: *agroclimatology, Papadakis classification, semiarid region*

Introduction

Climatology is the scientific study of regional weather patterns (Holden and Brereton, 2004). Assessment of variables such as precipitation, temperature, and evapotranspiration as well as the probability of occurrence of specific events along space and time is the most significant contribution of this branch of knowledge. In particular, agroclimatology concerns those climate processes influencing agricultural production (Holden and Brereton, 2004). Climate has been classified using methodologies such as Thornthwaite (1948), Köppen (1936), Köppen modified by García (García, 2004), Emberger (Di Castri and Hajek, 1976), and Papadakis (Campos-Aranda, 1999). Such alternatives are focused in the study of interactions temperature and humidity. Nevertheless, the Papadakis (1980) method takes into account climate aspects that influence both crops and water-soil-plant-atmosphere relations. This method allows evaluating the potential and limitations of crop growth in different climatic areas (Velasco and Pimentel, 2010).

Agroclimatic regions are characterized by interdependence between agronomy or farming systems and climate (Holden and Brereton, 2004; White et al., 2001). High climate variability in arid and semiarid lands makes farming a risky business; hence, the measuring and assessing of climate variability can be beneficial in determining the scheduling of agricultural activities for optimum production (Moeletsi and Walker,

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2013). Risks in agricultural production could be minimized, chiefly for rainfed agriculture, with an adequate geographical zoning of crops considering factors such as climatic conditions, soil characteristics, and availability of water, in order to ensure better conditions for the farming systems (Vidal and Martelo, 1993). The agroclimatic approach is used as a methodology to delimit a terrestrial space into several zones where the climate factors are favorable for certain groups of crops with similar characteristics (White et al., 2001).

Numerous studies on agroclimatic zoning have been undertaken. Falasca et al. (2012) plotted potential production areas of industrial oil and biodiesel from bean crop in arid and semiarid zones of Argentina by applying an agroclimatic model that combines precipitation, temperature and frost days. To estimate the agroclimatic potential in four places of the Bolivian Highlands, García et al. (2007) statistically analyzed more than 36 years of climatic data. Their main findings reveal that rainfed agriculture is risky due to the adverse climatic conditions, requiring the implementation of actions such as the introduction of deficit irrigation, protected intensive cropping, and cultivation of crops characterized by: (i) short growing season requirement, (ii) low water availability tolerance, and (iii) frost resistance. Previously, Geerts et al. (2006) conducted a study at the same Bolivian region to define areas with possibilities to improve quinoa production under deficit irrigation. Those authors evaluated four derived climatic indicators (reference evapotranspiration, length of rainy season, aridity index, and monthly frost risk) resulting in the agroclimatic quinoa GIS (geographic information system) map library. Morales et al. (2006) performed an evaluation and classification of edaphic and climatic resources in the Coquimbo Region of northern Chile using topoclimatic algorithms and satellite images, integrated in a GIS environment. Teran et al. (1998) executed the agroclimatic analysis of the La Mojana region in northern Colombia in order to estimate the potential for agriculture; using climate zoning, the theoretical estimate of potential crop areas and identification of consumption and excess of water in situ.

In Mexico, a number of studies report on agroclimatic patterns. Martinez et al. (2010) studied the agroclimatic conditions, chemical and nutritional characterization of kernels of *Jatropha curcas* L. (Mexican piñon), and they obtained the impact of different agroclimatic conditions in the composition of the seeds; Nuñez-Colín and Goytia-Jiménez (2009) determined the distribution and climatic patterns of current and future physic nut cultivation regions in Mexico using GIS; Diaz et al. (2000) performed agroclimatic zoning in the region of the highlands of Chiapas in southeastern Mexico, where they obtained maps of climatic suitability for cultivation of potato; Velasco and Pimentel (2010) applied the Papadakis methodology for agroclimatic delimitation to a wide region in Sinaloa in northwestern Mexico; they concluded by indicating that agroclimatic zonification can be very useful for planning purposes when different options of agricultural programs are applied; even when many crops are adapted to local conditions, it is important to know the natural climatic characteristics, so that the adaption process of crops is less hazardous if agricultural producers take into account regional climate to avoid wrong decisions as much as possible.

Zacatecas territory is depicted for corn and bean cultivars under rainfed conditions because of the local climatology. Nevertheless, yield for such crops generates low incomes for farmers whereby a part of agricultural producers use groundwater to cultivate higher income crops; furthermore, high rates of groundwater extraction originate environmental effects in Zacatecas aquifers. To contribute to the preservation of water resources, the goal of this research was to obtain the agroclimatic delimitation

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for the Zacatecas in Mexico, using the classification system of Papadakis (1980), collecting time-series data from 133 climatological stations in the region as monitored by the National Institute of Forestry, Agricultural and Livestock Research (INIFAP), and the National Water Commission (CONAGUA).

Materials and methods

Location of study

Zacatecas is located in the Northern region of Mexico, between extreme geographical coordinates 21° 01’ 45.0’’ N latitude and 100° 43’ 34.3’’ W longitude, and 25° 07’ 21.5’’ N latitude and 104° 22’ 56.4’’ W longitude (*Figure 1*). The West and Southwest of the territory is part of the Sierra Madre Occidental mountain range conformed by plateaus reaching 2,850 m above sea level. The Central portion is set in the Meseta Central Highland, with valleys at around 1,000 m above sea level. The North is part of the Sierra Madre Oriental mountain range, with the highest elevations of the State, reaching an altitude of 3,200 m above sea level (INEGI, 2017). Zacatecas climate is semiarid, with minimum and maximum mean monthly temperatures of 2.8 °C (January), and 32.6 °C (May), respectively. Average annual precipitation is approximately 500 mm, of which 80% occurs from June through September. A total of 1.7 Mha are devoted to agriculture, and 89% of this area is under rainfed conditions.

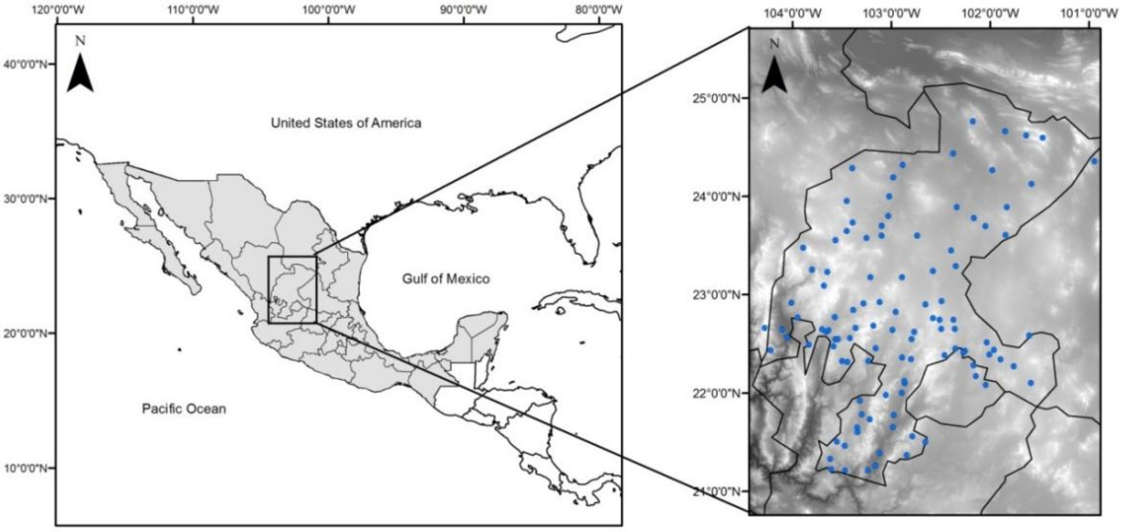

***Figure 1**. Geographical location of Zacatecas, Mexico. Blue dots represent weather stations*

Agroclimatic classification

The methodology proposed by Papadakis (1980) was used to analyze agroclimatic conditions in Zacatecas. This technique introduces the monthly climate concept to define the climate in a particular place as the sequence of 12 monthly expressions (Velasco and Pimentel, 2010). The monthly climate classification of Papadakis (1980) includes knowledge of monthly thermal and monthly water characteristics within the region under study. According to Campos-Aranda (2005), monthly thermal assessment requires obtaining average maximum temperature (T), average minimum temperature

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(t), and average extreme minimum temperature (t') of a weather dataset. Such values are expressed in Celsius degrees and their combinations result in 31 different thermal climates (*Table 1*). Monthly water climate is achieved through monthly precipitation (P) and monthly potential evapotranspiration (ET_0) as well as the amount of water previously stored in the soil (Pant). The comparison between P plus Pant and ET_0 originated 7 different water climates (*Table 2*).

***Table 1**. Monthly thermal climates for Papadakis classification Campos-Aranda (2005)*

	Climate identifier	Temperatures (°C)			Climate identifier	Temperatures (°C)			Climate identifier	Temperatures (°C)		
		t'	T	t		t'	T	t		t'	T	t
Monthly thermal climate	A	< -29	< -17.8		K	0 to 2	15 to 21		S	> 7	21 to 25	13 to 20
	B	< -29	> -17.8				> 21	< 8	T	> 7	25 to 29	< 13
	C	-29 to -10	< 0		L	2 to 7	15 to 21		U	> 7	25 to 29	13 to 20
	D	-29 to -10	0 to 5				> 21	< 8	V	> 7	29 to 33.5	< 20
	e	-29 to -10	> 5		m	-2.5 to 0	21 to 25	> 8	W	> 7	> 33.5	< 20
	E	-10 to -2.5	5 to 10		n	0 to 2	21 to 25	> 8	X	> 7	< 29	> 20
	f	-10 to -2.5	10 to 15		o	2 to 7	21 to 25		Y	> 7	29 to 33.5	> 20
	F	-10 to -2.5	> 15		M	-2.5 to 0	> 25		Z	> 7	> 33.5	> 20
	G	-2.5 to 0	10 to 15		N	0 to 2	> 25					
	H	0 to 2	10 to 15		O	2 to 7	> 25					
	I	2 to 7	10 to 15		P	> 7	< 17					
	J	-2.5 to 0	15 to 21		Q	> 7	17 to 21					
			> 21	<8	R	> 7	21 to 25	<13				

***Table 2**. Monthly water climates for Papadakis classification. Adapted from Campos-Aranda (2005)*

	Climate identifier	Description	Characteristics	Water exceeding
Monthly water climate	a	Arid	$P + P_{ant} < 0.25ET_0$	
	s	Dry	$0.25\ ET_0 < P + P_{ant} < 0.50\ ET_0$	
	i	Half-dry	$0.50\ ET_0 < P + P_{ant} < 0.75\ ET_0$	
	y	Half-moist	$0.75\ ET_0 < P + P_{ant} < ET_0$	
	p	Pre-moist	$P + P_{ant} > ET_0$	
	h	Moist	$P > ET_0$; $P + P_{ant} < 2\ ET_0$	< 100
	w	Wet	$P > ET_0$; $P + P_{ant} > 2\ ET_0$	> 100

An annual climate of the region is defined as the compendium of 12 monthly climates and the elemental components of temperature and humidity, summarizing the most frequent atmospheric behavior which is consistent with other systems of classification. However, the Papadakis (1980) methodology is targeted at the influence of climate factors during crop development (Velasco and Pimentel, 2010). Annual climate is integrated by thermal and water conditions. Thermal expression is defined as function of t' (freezing period) and a thermal number ranged between 0 (cooler month) and 9 (warmer month). On the other hand, water expression evaluates the difference between seasonal rainwater and potential evapotranspiration (exceeding seasonal rainwater) as well as the non-dry months (water number) ranging between 1 to 12.

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Climatic assessment

A network composed of 133 weather stations located within the study area was used to measure daily rainfall, and daily minimum, minimum extreme, maximum, and average air temperatures. The weather stations are monitored by the National Water Commission (CONAGUA) of the Mexican Government. The dataset is composed of information collected during the period of 1960-2015. For every weather station, data were filtered and chronologically sorted to complete missing information using a geostatistical and Kalman filter (KF) approach. To estimate potential evapotranspiration, the Hargreaves method was used due to its simplicity, reliability, minimum data requirements, ease of computation, and low impact by weather station aridity (Hargreaves and Allen, 2003; Allen et al., 1998). *Equation 1* summarizes such methodology.

$$ET_0 = 0.0023\, R_a (T - t)^{0.5} \left(\frac{T - t}{2} + 17.8\right) \quad \text{(Eq. 1)}$$

where ET_0 is reference evapotranspiration [mm·day^{-1}]; Ra is extraterrestrial radiation [MJ m^{-2} day^{-1}]; T is average maximum temperature [°C]; t is average minimum temperature [°C].

The KF is a set of mathematical equations that provide a best linear unbiased estimate (BLUE) for the state of a system containing noisy data. It also establishes a way to update these estimates when a new measurement becomes available without a need to refer to old data (Junez-Ferreira and Herrera, 2013). The KF relies upon two equations, a dynamic equation and a measurement equation. The KF that is applied using only the measurement equation is called the static Kalman filter, which calculates estimates of a given variable sequentially, starting from a prior estimate and adding new data at each step (Herrera and Pinder, 2005). The static KF was used to estimate climatic missing values of daily rainfall, daily minimum, minimum extreme, maximum, and average air temperature in the study area. Before predicting missing values, exploratory data analysis and structural analysis were realized (Diaz, 2002; Gallardo, 2006; Mendoza-Cázares and Herrera-Zamarrón, 2010; Ávila et al., 2016). The predictions of values at the unsampled sites were undertaken by using a univariate technique. The parameters of the variograms resulting from the geostatistical analyses were used to generate the a priori covariance matrices for the univariate estimates using the Kalman filter. The GSLIB and Minitab software have been used as predictive tools. The measurement equation (*Eq. 2*) for the application of the Kalman filter is

$$z_j = H_j C + v_j \quad \text{(Eq. 2)}$$

where H_j is the jth sampling matrix. The sampling matrix is a $1\ x\ N$ matrix that is non-zero only at the position corresponding to the entry of C where the jth sample is taken, and N is the dimension of the vector C. The set $\{v_j, j = 1, 2, \ldots\}$ represents the measurement error. It is a white Gaussian sequence, with zero mean and variance r_j. The measurement error sequence $\{v_j\}$ and the vector C are independent. $C = \{C_{ip}\}$ is the space-time vector, with the climatic values in the positions (x_i) and times (t_p); z_j is the measurement vector.

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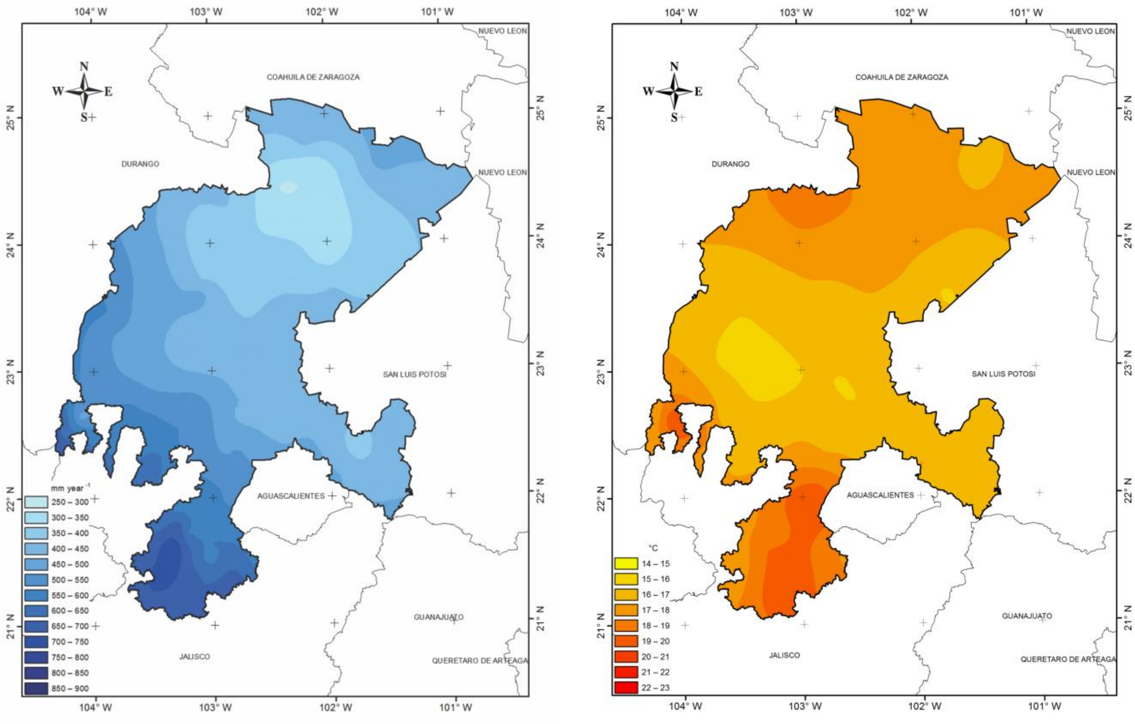
Software for data analysis and agroclimatic mapping

The database was assembled by using all the collected and processed data in a Microsoft® Excel 2016 spreadsheet. Daily rainfall, daily minimum, minimum extreme, maximum, and average air temperature data underwent quality checking to identify spurious values, and the daily values were transformed into monthly average series. Monthly average values were interpolated using the ordinary Kriging method that embraces a set of methods for local estimation, including simple and ordinary kriging, co-kriging, universal kriging and disjunctive kriging. Ordinary kriging was used in this research. The kriging weights were determined using the variogram and the configuration of the data. It is an optimal interpolator in the sense that estimates are unbiased and have known minimum variances (Oliver and Webster, 1990). Several investigations recommend the use of kriging as interpolator for hydrological variables (O'Conell and Todini, 1996; Holdaway, 1996; Borga and Vizzaccaro, 1997; Bargaoui and Chebbi, 2009). Kriging is a standard method of optimal interpolation included in ArcMap for ArcGIS®.

Results and discussion

Mean annual values of precipitation and temperature for the period of 1960 to 2015 are shown in *Figure 2*.



(a) (b)

***Figure 2**. Mean annual values of precipitation (a) and temperature (b) for the period of 1960 to 2015 of Zacatecas, Mexico*

For the period under study, the spatial distribution of the mean precipitation ranges between less than 300 mm year^{-1} in the northeast region and 750 mm year^{-1} in the south region of Zacatecas state. Notwithstanding, critical years due to both scarcity and excess

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pluvial precipitation were recorded during analysis of the time series. The recent cases occurred in 2011 and 2013 with precipitation rates of 286 mm year^{-1} and 678 mm year^{-1}, respectively. Regarding temperature variations throughout the year, minimum and maximum annual averages were around 14 °C and 23 °C; nevertheless, minimum mean temperature of 2.8 °C and maximum mean temperature of 32.6 °C were reached in January and May, respectively.

Monthly thermal and monthly water climates from January to December for the Zacatecas region following the Papadakis (1980) methodology are plotted in *Figure 3*.

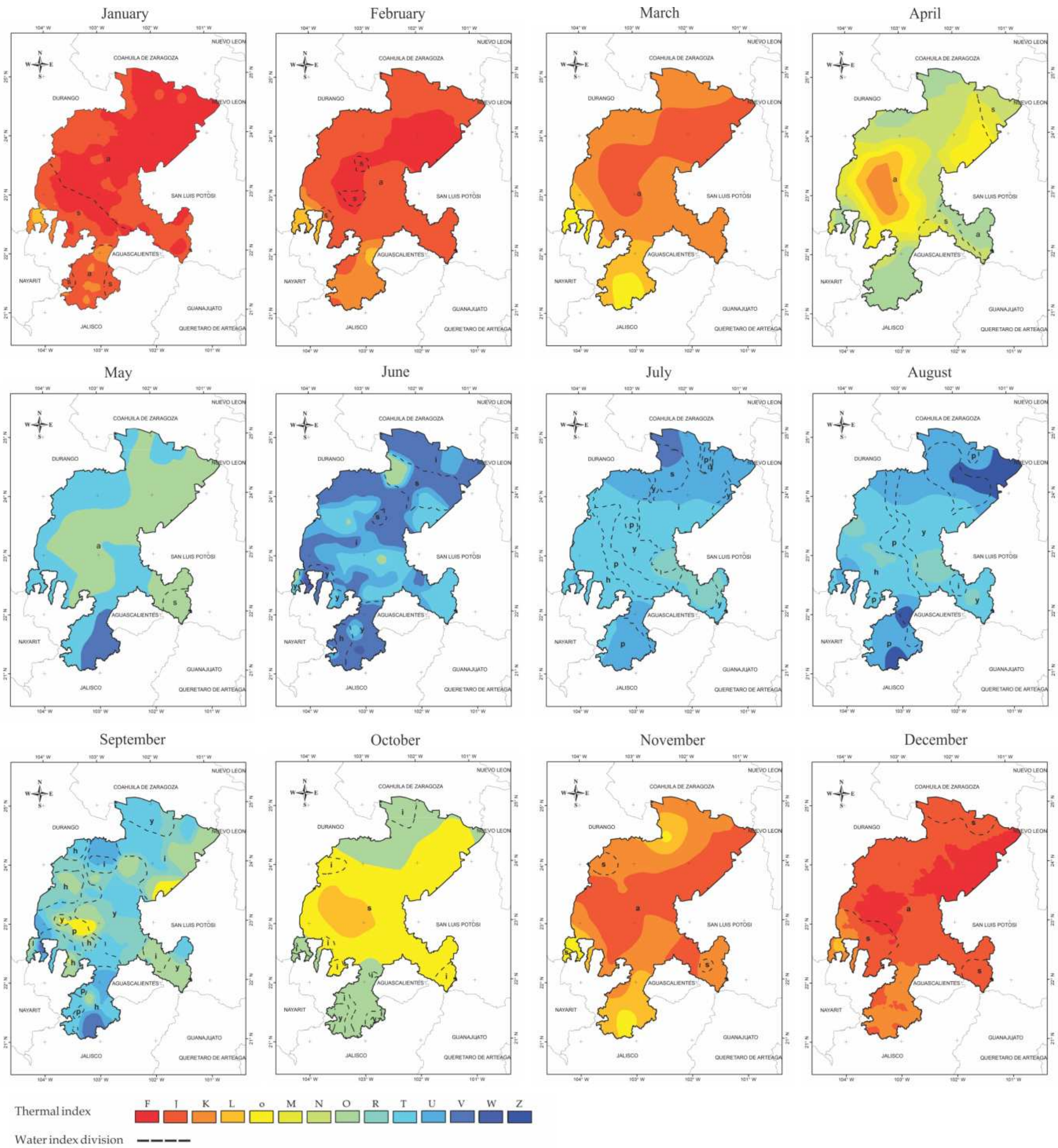

***Figure 3**. Thermal and water indexes from January to December for Zacatecas, Mexico*

Maps show that January, February, November, and December are months that are too cool (more than 85% of the area is under F or J thermal indexes) to cultivate citrus but grass (*Festuca*) grows with respect to water availability. Actually, orange and lemon

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trees are a way of undertaking subsistence farming in a small portion of the South of the region where the thermal index is between J and K in January, the cooler month of year. Coupled with unfavorable thermal indexes for agricultural activities, these months do not reach enough rainfall to satisfy crop water requirements (water indexes between a and s in the whole region); nevertheless, the Zacatecas region is provided with groundwater resources that make it possible to establish perennial crops such as alfalfa (*Medicago sativa* L.) and grass (*Festuca*).

Temperature begins to become benevolent for agriculture from the month of April. This month is usually adopted by farmers who irrigate to sow and take advantage of the market (high prices for early producers). Farmers who do not irrigate wait for the best opportunity that precipitation can provide, choosing the sowing date empirically after the first significant rainfall (Bautista-Capetillo et al., 2016). For rainfed crops, the sowing period usually oscillates between April and July, depending on the type of crop established and once precipitation starts (Luna, 2014; Medina et al., 2003). These facts are in agreement the with Papadakis (1980) classification. The thermal (O and R), and water (i, y, and p) monthly indexes for such a period are in optimal climate conditions for crops as maize and beans, two of the main rainfed crops established in Zacatecas. *Table 3* shows a description of monthly thermal index and monthly water index is included as well as their influence on growing crops.

***Table 3**. Description of thermal and water indexes for Zacatecas territory*

Thermal index	Water index	Month	Zacatecas territory		Climatic conditions for growing crops
			(Km²)	%	
F	a	January	39,750	53.00	Winter too cold and rainfall shortage. Climatic conditions not suitable for rainfed growing crops
		February	9,750	13.00	
		December	18,750	25.00	
	s	January	9,000	12.00	Winter too cold. Rainfall remains deficient for rainfed crops
		February	6,000	8.00	
		December	1,500	2.00	
J	a	January	13,500	18.00	Frosts are common and rainfall shortage. Winter cereals grow because of vernalization but they need irrigation water
		February	46,500	62.00	
		March	27,750	37.00	
		April	9,000	12.00	
		November	32,250	43.00	
		December	36,000	48.00	
	s	January	5,250	7.00	Frosts are common. Rainfall remains deficient for rainfed crops but winter cereals grow under irrigation
		February	2,250	3.00	
		December	9,750	13.00	
K	a	January	3,750	5.00	Minimum risk of frost and rainfall shortage. Corn, alfalfa, grass, and cryophilic trees grow under irrigation
		February	6,750	9.00	
		March	31,500	42.00	
		April	8,250	11.00	
		November	24,000	32.00	
		December	5,250	7.00	
	s	January	2,250	3.00	Minimum risk of frost. Rainfall remains deficient for rainfed crops but corn, alfalfa, grass, and cryophilic trees grow under irrigation
		February	750	1.00	
		November	3,750	5.00	

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		December	3,750	5.00	
L	a	February	3,000	4.00	Frosts are too rare but cold is appropriate for some cryophilic trees. Corn, beans, cereals, alfalfa, and grass need irrigation water for optimal growth
		March	10,500	14.00	
		April	7,500	10.00	
		November	7,500	10.00	
	s	January	1,500	2.00	Frosts are too rare but cold is appropriate for some cryophilic trees. Even though rainfall is not enough, corn, beans, cereals, alfalfa, and grass grow under irrigation
		October	12,000	16.00	
		November	3,000	4.00	
o	a	March	5,250	7.00	No frosts and cold is not appropriate for vernalization. Rainfall shortage, then legumes and vegetables need irrigation water for growth
		April	10,500	14.00	
		November	3,750	5.00	
	s	April	1,500	2.00	No frosts and cold is not appropriate for vernalization. Rainfed beans grow with considerable risk
		October	28,500	38.00	
		November	750	1.00	
	i	September	2,250	3.00	No frosts and cold is not appropriate for vernalization. Bean crop and corn crop grow under rainfed conditions
		October	6,000	8.00	
	y	September	750	1.00	
	p	September	2,250	3.00	
M	a	April	8,250	11.00	Temperature is adequate for initial season development stage of legume and vegetables grow but need irrigation water
	s	April	2,250	3.00	
N	a	April	12,750	17.00	
	s	April	3,750	5.00	
O	a	April	9,750	13.00	
		May	39,750	53.00	
	s	April	1,500	2.00	
		May	3,750	5.00	
		June	4,500	6.00	
		October	14,250	19.00	Temperature is adequate for garlic, onion, and carrot grow but need irrigation water
	i	October	8,250	11.00	Temperature is adequate for garlic, onion, and carrot grow but rainfall has to be complemented using irrigation water
	y	October	6,000	8.00	
R	i	July	5,250	7.00	Temperature and rainfall are quite adequate for bean crop and corn crop at midseason development stage. Vegetables as pepper grow using irrigation water
		September	4,500	6.00	
	y	July	750	1.00	
		August	5,250	7.00	
		September	6,750	9.00	
	p	September	3,750	5.00	
	h	August	4,500	6.00	
		September	6,000	8.00	
T	a	May	26,250	35.00	Temperature is adequate for initial season development stage of legume and vegetables grow but need irrigation water
	s	June	3,750	5.00	
	i	June	10,500	14.00	Temperature and rainfall are quite adequate for bean crop and corn crop at midseason development stage as well as for Mexican wheat crop. Vegetables as pepper grow using irrigation water
		July	11,250	15.00	
		September	6,750	9.00	
		August	3,750	5.00	
T	y	June	3,000	4.00	Temperature and rainfall are quite adequate for bean crop and corn crop at midseason development stage as well as for Mexican wheat crop. Vegetables as pepper grow using irrigation water
		July	12,750	17.00	
		August	6,750	9.00	
		September	9,750	13.00	

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	p	July	12,000	16.00	
		September	4,500	6.00	
		August	6,000	8.00	
	h	July	7,500	10.00	
		August	14,250	19.00	
		September	6,750	9.00	
U	s	June	9,750	13.00	Temperature is adequate for bean crop but rainfall is not enough to satisfy crop water requirements
		July	3,750	5.00	
	i	June	9,000	12.00	Temperature and rainfall are quite adequate for bean crop and corn crop at midseason development stage as well as for Mexican wheat crop. Vegetables as pepper grow using irrigation water
		July	11,250	15.00	
		August	7,500	10.00	
		September	9,000	12.00	
	y	June	4,500	6.00	
		July	3,000	4.00	
		August	5,250	7.00	
		September	750	1.00	
	p	July	2,250	3.00	
		August	8,250	11.00	
		September	2,250	3.00	
	h	July	750	1.00	
		August	3,750	5.00	
		September	4,500	6.00	
V	a	May	5,250	7.00	High temperatures during day but refreshing nights which harms some cultivars. Rainfall shortage
	s	June	6,750	9.00	
		July	2,250	3.00	
	i	June	12,000	16.00	High temperatures during day but refreshing nights which harms some cultivars. Rainfall is suitable for bean crop and corn crop
		July	2,250	3.00	
	y	June	6,750	9.00	
	h	June	3,750	5.00	
		September	4,500	6.00	
W	y	June	750	1.00	
Z	y	August	3,750	5.00	
	i	August	3,000	4.00	
	h	August	3,000	4.00	

The annual agroclimatic classification (Papadakis, 1980) for the Zacatecas region is mapped in *Figure 4*. Three different agroclimatic zones were obtained (2.1, Semitropical cold zone; 2.2, Low cold zone; and 2.3, Middle cold zone). More than 90% of the surface is under the 2.2 climate type favoring crops such as maize, beans, and soybeans but dryland farmers have to wait for the best opportunity that precipitation can provide, choosing the sowing date empirically after the first significant rainfall. The sowing period oscillates between July 1 and August 1. In winter, the temperature permits the growing of wheat but since this season of the year is generally dry, it should be irrigated. The annual water regime varies from semiarid to humid; rainfed crops depend on the availability of rainfall occurring in May or June. About 9% and 1% of Zacatecas are under 2.3 and 2.1 climate types, respectively. The same crops as the 2.2 climate type can be established as well as forage crops such as maize, oats, sorghum, grasses and alfalfa. Additionally, rainfed bean crop is sown in spring and summer

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seasons. It should be noted that the classification of Köppen modified by Garcia places the Zacatecas region in a subtropical environment with some days having temperatures below 0 °C. Almost 90% of the region is mainly covered by semiarid and arid environments affected by droughts of different intensities (Bautista-Capetillo et al., 2016; Mojarro et al., 2013; Medina et al., 1998).

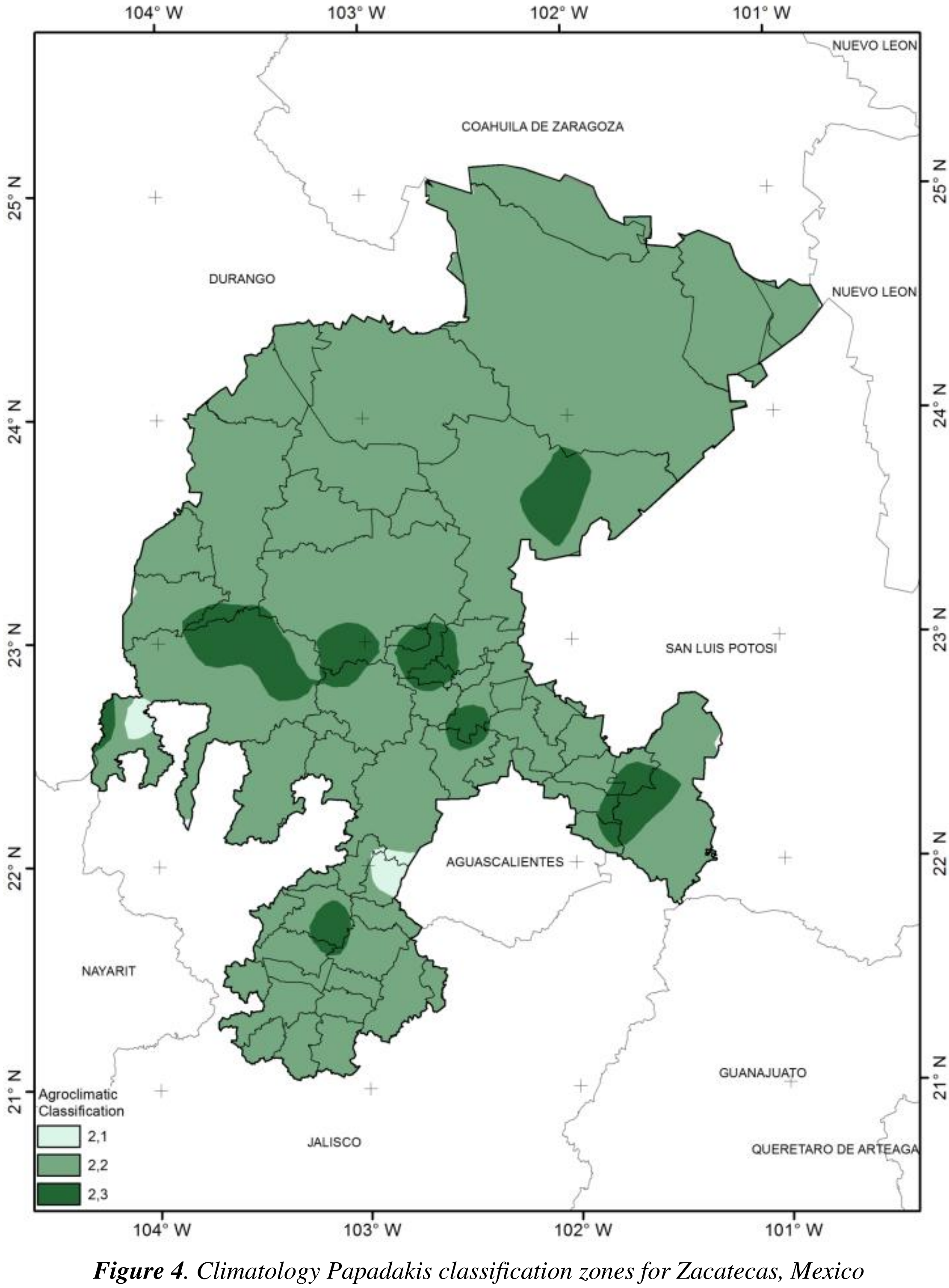

***Figure 4**. Climatology Papadakis classification zones for Zacatecas, Mexico*

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According to the agroclimatic classification of Papadakis (1980), the Zacatecas region is suitable for maize and bean crops. This region of Mexico is the major producer of bean crop in the country (Flores and Bautista-Capetillo, 2015). Around 400,000 ha of bean crop are harvested annually (95% is dryland and 5% is irrigated land); meanwhile, maize crop harvests reach 235,000 ha per year (85% is dryland and 15% is irrigated land). The importance for Mexico of beans cultivated in Zacatecas is contextualized as follows: between 20 and 23 Mha are annually farmed in the country (CONAGUA, 2016). Dry beans are established in 12% of the total agricultural land. Most of this area is rainfed (1.6 Mha), while only 0.3 Mha is irrigated. The current average yields are 0.53 Mg ha^{-1} and 1.53 Mg ha^{-1} for rainfed and irrigation conditions, respectively (SIAP, 2013). Factors such as crop genetics, soil physics, fertilizer use, and water stress affect crop production. Dry beans are grown all over the country during the Spring-Summer season but the main producers are the states of Zacatecas, Durango, and San Luis Potosi with 0.75 Mha cultivated. In the Fall-Winter season only 0.27 Mha are cultivated, the main producers are the states of Chiapas, Nayarit, Veracruz and Sinaloa.

Maize and bean crop yield in rainfed agriculture generates low incomes for farmers because economic productivity of these products is barely 200 USD Mg^{-1} and 150 USD Mg^{-1}, respectively. These conditions entail socio-economical aspects that should be considered. The agroclimatic context of the Zacatecas region is useful for such crops but the economic profit margin is lower than farmer expectations. For this reason, agricultural producers have taken advantage of natural resources such as soil and groundwater which allow them to cultivate vegetables (chilli, pepper, garlic, onion, tomato, carrot, lettuce) and perennial crops that are mainly alfalfa and grass. In fact, chilli (both fresh and dry) produced in the Zacatecas region places this region as the second producer in the Mexican market; the value of production is around 3,400.00 USD Mg^{-1} (SAGARPA, 2017) yielding 1.1 MMg per year approximately. Nevertheless, some environmental effects occur in Zacatecas aquifers because of high water extractions. CONAGUA (2016) estimates that 1,397.3 Hm^3 of groundwater is used annually for agriculture in Zacatecas. As a consequence of the intensive exploitation of aquifers, undesirable effects have been documented in the body of scientific literature, such as: the accelerated extraction rates of water tables (Carrillo-Rivera et al., 2008; Maderey-Rascón and Carrillo-Rivera, 2005), the degradation of water quality in wells (Kresic, 2007; Freeze and Cherry, 1979), subsidence (Fetter, 1994; Bear, 1979), as well as alterations to ecosystems (Carabias et al., 2005).

Conclusions

Mexico frequently faces adverse climatic conditions. In southeastern Mexico, the country has undergone large floods, while central and northern Mexico experienced critical droughts. Agroclimatic understanding is particularly important in semiarid regions because this permits proactive action in promoting the agricultural potential of regions with scarce rainfall regimes. Bautista-Capetillo et al. (2016) note that 73% of the Zacatecas region (75,284 km^2) is characterized by dry climate (rainfall is lower than potential evapotranspiration) with significant limitations for agriculture (INEGI, 2016). The agroclimatic maps presented in the present paper confirm such asseverations. The climate variables analyzed for the period of 1960-2015 show that large areas of Zacatecas are not dedicated to rainfed agriculture during months of January, February, November, and December but such a condition starts to change from April. In that

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month, the average temperature increases by up to 24 °C; nevertheless, precipitation still is not enough to satisfy crop water requirements of a low consumption of this resource. According to Papadakis (1980) agroclimatic classification, at least 90% of Zacatecas is adequate to establish maize and bean crops under rainfed conditions but these types of crops generate low incomes for farmers. For this reason, they opted for vegetables and taking advantage of natural resources such as soil and groundwater with environment consequences such as depletion of aquifers. The agroclimatic analysis carried out for Zacatecas state can be useful to design policies concerning the preservation of natural resources, such as water. Because of this, our contribution could support the management of regional rainfed agriculture, providing fresh information about incidence of temperature and rainfall conditions for Zacatecas territory through maps that show thermal and water indexes of the Papadakis agroclimatology classification.

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MANAGEMENT OF IRON DEFICIENCY STRESS IN KIWIFRUIT TREES (*ACTINIADIA DELICIOSA*) BY SOIL INJECTION

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Abstract. A factorial experiment using the randomized complete block design was conducted in 2013-2014 to study the effects of methods of controlling iron deficiency stress, and the chlorosis resulting from it, on the qualitative and quantitative characters of kiwi fruits. The factors included three levels of iron sulfate, sulfuric acid and organic matter in four replications. Results showed that the treatments had a significant effect on SPAD readings, maximum leaf iron content, the largest proline content, vitamin C and crop yield in both years. The treatments did not significantly influence the TSS/TA index in the two years. In all, results indicate that injection of two kilograms of iron sulfate together with half a liter of sulfuric acid and four kilograms of organic matter can be a suitable replacement for iron chelates in correcting iron chlorosis, and in increasing the qualitative and quantitative yield of kiwi trees for a period of at least two years.
Keywords: *chelate, chlorosis, ferrous sulfate, sulfuric acid, organic matter*

Introduction

Kiwi (*Actinida deliciosa*) enjoys relative advantages due to its high crop yield per hectare, its nutritional and medicinal values of its fruits and desirable postharvest storage life. Kiwi trees need suitable weather conditions and fertile soils. High soil lime content is one of the factors that threaten kiwi orchards. The high sensitivity of kiwi trees to high pH values, and to iron deficiency, causes chlorosis, loss of vigor, and reduced crop yield and fruit quality in kiwi orchards, especially in those with heavy- textured soils. Iron (Fe) is an important micronutrient that plays crucial roles in plant growth, development, and reproduction (Vigani et al., 2013). Iron chelates such as Fe-EDDHA are used to quickly correct iron chlorosis in plants, but they are expensive and, besides that, must be applied every year (Pestana et al., 2003). Local placement of mineral iron salts together with organic matter (deep placement) is an effective method of controlling iron chlorosis, and improves fruit quality too, but it is labor-intensive and costly. Iron chlorosis symptoms in pear orchards can be corrected by adding acids to a limited volume of soil for neutralizing calcium carbonate (Kalbasi, 1986). Several experiments have shown the effectiveness of the method of injecting fertilizers into the soil as either solutions or suspensions. In one of

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them, iron (II) phosphate fertilizer was injected into the soil around mature kiwi trees in four places at the depth of 25-30 centimeters, into the soil of potted kiwi trees, and into the soil around one-year-old kiwi saplings. Results indicated that injection of the iron fertilizer Vivianite could be a suitable replacement for iron chelates in correcting chlorosis and in improving kiwi fruit quality (Rombola et al., 2003). Reducing the pH of the injected fertilizer can also be effective in increasing iron solubility. Solubility of iron containing minerals increases 1000- fold for every one-unit decrease in pH (Samar et al., 2011). Adding acidic materials to mineral fertilizers will result in a longer duration of iron solubility, and will thus increase iron absorption by roots (Havlin et al., 2005). Some species, such as Arabidopsis thaliana, produce phenolic compounds (Schmidt et al., 2014) while other species, including cucumber and melon, produce flavin compounds (Rodríguez-Celma et al., 2013). Although the function of flavin compounds in plant Fe deficiency is not well defined, they may function in reduction or complexation of extracellular Fe to facilitate Fe acquisition (Sisó-Terraza et al., 2016). In our experiment, the effectiveness of injecting iron sulfate fertilizer into the soil with a Biolift machine, and the probability of increasing its efficiency by adding acids and organic matter, in correcting chlorosis and improving the qualitative and quantitative characters of kiwi fruit were studied.

Materials and Methods

Mazandaran Province is considered one of the suitable places for growing kiwi trees and for producing kiwi fruit in Iran. This experiment was conducted in the central part of the Mazandaran with the latitude of 36°28′ north, longitude of 52°53′ east, altitude of 51.2 meters, and average annual temperature and rainfall of 16.7 °C and 725 millimeters, respectively, in 2013-2014. Physical and chemical characteristics of soil described in *Table 1*. The experiment was conducted in the factorial form using the randomized complete block design (28 treatments in four replications on 112 trees). The treatments included three levels of iron sulfate application (0, 1, and 2 kg per tree represented by Fe_1, Fe_2 and Fe_3, respectively), three levels of sulfuric acid (0, 0.5, and 1 liter per tree represented by AC_1, AC_2 and AC_3, respectively), and three levels of organic matter (0, 2, and 4 kg of organic matter per tree represented by OM_1, OM_2 and OM_3). In an additional treatment (other than those usually performed), the iron chelate treatment was employed as an effective fertilizer for correcting iron deficiency and for evaluating the effectiveness of the above-mentioned treatments. This treatment received 100 grams of the iron chelate Sequestrine (FeEDDHA) per tree in two stages. The first stage was in the first month of spring, and the second in the second month of spring and, in each stage, 50 grams of this iron chelate were dissolved in 20 liters of water and applied as fertigation at the base of each tree trunk in both years that the experiment was carried out. This treatment was considered as the sham treatment, and the treatment that did not receive any fertilizer as the control treatment. We mixed the materials needed in each treatment with 20 liters of water, turned the mixture into a suspension, and then injected the suspension into the soil with a Biolift machine at four points in the area shaded by each tree at the depth of 40-50 centimeters. These four points were at a distance of 75 centimeters from the tree trunk and the injections were carried out in the early part of the first month of spring in 2013. The force and pressure applied by the

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machine at each injection point spread the fertilizer combination in an area of about 0.12 square meters. With 20 liters of the suspension injected at four points around each tree (five liters per point), the volume of soil around each tree that was impregnated with the suspended materials was 0.2 cubic meter out of the total of 0.7 cubic meter. In other words, close to 30 percent of the volume of the soil around each tree was affected by the applied materials. The powdery type of iron sulfate was used, the sulfuric acid was of the commercial type with a concentration of 98 percent, and the organic matter was decomposed cow manure. To prepare the suspensions, the quantity of the organic matter specified by the levels used in the experiment was first mixed with 20 liters of water and the acid was then added. Twenty-four hours later, the calculated amount of iron sulfate for each treatment was poured into the mixture. We passed the prepared suspension through a filter and injected the filtrate into the soil using a fertilizer injection machine. This machine, with the trade name of MTM, had a compressor with a gasoline motor to produce pressurized air at the rate of 1260 liters per minute at the pressure of 9-11 bars, and had a nozzle with the maximum diameter of three millimeters for injection. At entrance into the soil, the compressed air created a network of small and large air channels that reduced soil compaction and, hence, improved soil aeration and infiltrability (for easier injection of the suspensions). After carrying out the treatments, other maintenance operations such as irrigation and pest and weed control were performed as are customary in the region.

Table 1. *Physical and chemical characteristics of soil*

Soil texture	Zn	Mn	Mg	Fe	P	K	Total N (%)	T.N.V (%)	pH	EC (d.S/m)	dept (cm)
p.p.m											
L	1.82	5.44	612	9	56	347	0.11	8.75	7.7	0.42	0-30
L	-	-	-	-	52	307	0.1	10.5	7.8	0.4	30-60

The studied characters were as follows: SPAD reading with a SPAD-502 chlorophyll meter, leaf iron content with an atomic absorption machine, proline content by drawing the standard curve at the wavelength of 520 nanometers (the method introduced by Bates, 1973) , and the proline concentration measured in milligrams per gram of fresh leaf. The ratio of sugar to acid or TSS/TA was calculated (the TSS was measured using a refractometer and TA, or titratable acidity, by titration with 0.1N sodium hydroxide), and vitamin C content was measured by using the 2,6-dichloroindophenol titrimetric method. We used MSTATC to analyze the data and Duncan's test to compare the means.

Results and Discussion

Chlorophyll assay (SPAD reading)

The individual effects of iron sulfate, sulfuric acid, and organic matter on the chlorophyll index that were measured in the early part of the last month of summer in both years were statistically significant at the 1% probability level. The effects of the interactions between the treatments of applying iron sulfate, sulfuric acid, and organic

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matter on the chlorophyll content of the leaves were significant at the 1% probability level, as compared to effects of iron chelate application (*Table 2*). Leaf chlorophyll content in the $Fe_3AC_3OM_3$ treatment increased by 39.9% compared to the chelate treatment, and that of the chelate treatment by 18.8% compared to the control ($Fe_1AC_1OM_1$) (*Table 5*). Conducted studies on the sensitivity of kiwi varieties to iron deficiency have shown that iron deficiency reduces leaf chlorophyll content in all kiwi varieties (Rombola et al., 2002). Injection of a solution containing Fe-EDDHA and organic matter into the soil in a peach orchard significantly increased leaf chlorophyll index (SPAD reading) (Tsipouridis et al., 2006). Injection of iron (II) phosphate into olive trees improved yield and leaf color stability index for more than two years (Rosado et al., 2002). Iron deficiency was followed by leaf yellowing, by reduced vegetative growth and leaf surface area, by diminished flowering and fruit set and, hence, by lowered crop yield and quality (Alvarez-Fernandez et al., 2003). These results conform to ours because we found that there were significant differences between leaf chlorophyll concentrations in the control, iron chelate, and iron sulfate + sulfuric acid+ organic matter treatments. Since one of the most important roles played by iron is in enzymatic activities, and because iron forms a part of the structure of enzymes (and activates them), it has an essential role in the metabolism of nucleic acids and proteins because nucleic acids contain considerable amounts of iron and other heavy metals. Accumulation of nitrates, amino acids, and amides, and reduction of proteins, under conditions of iron deficiency are signs of the effects of iron in protein synthesis; and these conditions return to normal when required iron is provided. Among various proteins, chlorophyll is strongly affected by iron deficiency leading to lack of development, and reduced quantities, of leaf chlorophyll, less photosynthesis, and diminished vegetative growth. Increased availability of iron results in more chlorophyll production and in less yellowing and chlorosis. Following that, photosynthesis increases, more raw xylem sap is used, more water and minerals are absorbed from the soil, more phloem sap is produced and more nutrients will be available, leading to increased vegetative growth, especially longitudinal growth of branches and greater leaf surface area (Havlin et al., 2005).

Table 2. *Analysis of variance of SPAD reading, leaf Fe concentration, proline, yield, fruit TSS/TA and vitamin c in 2013 year*

Mean square						DF	Source
Vitamin c	**TSS/TA**	**Yield**	**Proline**	**Leaf Fe**	**SPAD reading**		
510.16*	7.631*	334.02*	0.0001*	1524.75*	94.3*	3	Replications
138.12^{ns}	2.693^{ns}	775.88**	0.001**	1711.29**	206.2**	27	Treatment
94.19	2.341	142.99	0.0001	671.93	29.03	81	error
16.58	14.93	40.6	23.74	13.28	14.18		**(%)** CV

ns and *, **, respectively, according to F-test non-significant and significant at 5 and 1 percent

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Leaf iron concentration

Individual effects of applying iron sulfate and sulfuric acid on iron leaf concentration were statistically significant in both years at the 5% probability level. The interaction of the effects of applying a combination of iron sulfate, sulfuric acid, and organic matter were also significant at the 1% probability level (*Tables 2, 3*). Comparison of the iron chelate and the iron sulfate + sulfuric acid+ organic matter treatments also indicated significant differences in leaf iron concentrations at the 1% probability level (*Tables 2* and *3*). In 2013, the treatment $Fe_2AC_2OM_1$ yielded the highest leaf iron concentration that was 19.9 percent more than the iron chelate treatment and 28.5 percent more than the control, while leaf iron concentration in the iron chelate treatment was 10.7 percent higher compared to the control. In 2014, the $Fe_2AC_3OM_3$ treatment showed the highest leaf iron concentration that was 4.9 percent more than the superior treatment of the first year and 23.5 and 34.4 percent higher compared to those of the iron chelate and the control treatments, respectively (*Table 4*). Comparisons indicated that leaf chlorosis of untreated trees (the control) worsened in the second year. Crane et al. (2007) showed that leaf iron content in sulfuric acid treatments (or in treatments of citric acid plus iron sulfate) resulted in the highest leaf iron content compared to the iron sulfate, acid, or iron chelate treatments. Presence of organic matter in soil is effective in the reduction reaction of iron because decomposing organic matter transfer electrons to trivalent iron and reduce it and, thus, increase iron concentration in soil solution. Organic molecules form organometallic complexes with iron or with some of the other cations and these complexes increase the capability of absorbing elements. Moreover, the beneficial effects of adding organic matter together with mineral iron compounds sustain the capability of absorbing the iron present in mineral compounds (i. e., prevent or delay precipitation) and thus make it possible for plants to absorb more of this element (Samar et al., 2011).

Table 3. *Analysis of variance of SPAD reading, leaf Fe concentration, proline, yield, fruit TSS/TA and vitamin c in year 2014*

Mean square						DF	Source
Vitamin C	**TSS/TA**	**Yield**	**Proline**	**Leaf Fe**	**SPAD reading**		
158.10*	1.11*	261.06^{ns}	0.0001^{ns}	617.94^{ns}	65.6^{ns}	3	Replications
88.50*	1.80^{ns}	1177.98**	0.001**	2114.87**	265.4**	27	Treatment
47.35	2.44	113.25	0.0001	399.90	27.1	81	error
11.36	14.42	33.44	24.77	9.89	13.95		**(%)** CV

ns and *, **, respectively, according to F-test non-significant and significant at 5 and 1 percent

Proline

The individual effects of iron sulfate and organic matter on leaf proline content were statistically significant at the 5% and 1% probability levels, respectively. Effects of the

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interactions of applying iron + acid + organic matter on leaf proline content were very significant at the 1% probability level (*Table 2*). In 2013, the highest leaf proline content (0.104 milligram per gram of fresh leaf) was that of the $Fe_3AC_3OM_3$ treatment , which was 76.9 and 35.8 percent more than the control and the iron chelate treatments, respectively. In 2014, the $Fe_2AC_3OM_3$ treatment had the maximum leaf proline content, which was 61.8 and 25 percent higher compared to the control and the iron chelate treatments, respectively (*Table 4*).

Table 4. *Effect of application of ferrous sulfate, sulfuric acid and organic matter on SPAD reading, iron leaf concentration, leaf proline content during 2013-2014*

Proline (mg/gr)		**Leaf Iron (micgr/gr)**		**(SPAD reading)**		**Treatment**		
2014	**2013**	**2014**	**2013**	**2014**	**2013**	**OM**	**Asid**	**Fe**
0.029 k	0.024c	162 k	168 e-f	20.3m	24.4 L	OM_1	AS_1	Fe_1
0.03 k	0.029 bc	180 i-k	181 d-f	22.7 l-m	26.6 k-l	OM_2	AS_1	Fe_1
0.065 a-f	0.065 a-c	173 j-k	175 e-f	25.8 k-m	30.8 g-l	OM_3	AS_1	Fe_1
0.059 a-g	0.06 a-c	190 f-k	186 b-f	30.5 h-l	30.3 h-l	OM_1	AS_2	Fe_1
0.068 a-d	0.072 a-c	199 e-k	197 a-f	28.9 i-l	29.1 i-l	OM_2	AS_2	Fe_1
0.054c-i	0.065 a-c	180 h-k	177 d-f	38.2 c-h	39.4 b-g	OM_3	AS_2	Fe_1
0.057 b-h	0.052 a-c	188 f-k	198 a-f	26.7 j-m	27.3 j-l	OM_1	AS_3	Fe_1
0.042 h-k	0.046 bc	191 f-k	185 b-f	34.5 e-j	35.5e-k	OM_2	AS_3	Fe_1
0.049 f-j	0.054 a-c	206 c-j	209 a-e	36.2 d-i	35.4e-k	OM_3	AS_3	Fe_1
0.053 d-j	0.058 a-c	228 a-e	224 a-c	35.4 d-i	35.5e-k	OM_1	AS_1	Fe_2
0.048 g-j	0.052 a-c	177 i-k	157 f	34.1 f-k	36.9 d-i	OM_2	AS_1	Fe_2
0.067 a-e	0.073 a-c	184 g-k	174 e-f	31.6 g-k	33.8f-k	OM_3	AS_1	Fe_2
0.037 i-k	0.04 bc	243 ab	235 a	42.5 a-f	40.7a-f	OM_1	AS_2	Fe_2
0.048 f-j	0.049 bc	235 a-d	219 a-d	45.6 a-c	43.9a-e	OM_2	AS_2	Fe_2
0.051 e-j	0.051 a-c	196 e-j	184 c-f	49.1 a	46.7a-c	OM_3	AS_2	Fe_2
0.037 Jk	0.035 bc	172 j-k	158 f	43.1 a-e	43.2a-e	OM_1	AS_3	Fe_2
0.056 b-h	0.055 a-c	220 a-f	202 a-e	45.9 a-c	45.4 a-d	OM_2	AS_3	Fe_2
0.076 a	0.08 ab	247 a	234 a	43.3 a-d	44.1 a-e	OM_3	AS_3	Fe_2
0.058 b-h	0.059 a-c	e-j198	190 b-f	36 d-i	36 e-j	OM_1	AS_1	Fe_3
0.071 ab	0.081 ab	210 b-i	202 a-e	35.1 d-j	35.2 e-k	OM_2	AS_1	Fe_3
0.056 b-i	0.059 a-c	216 a-g	206 b-f	39.7 b-g	38.4 c-h	OM_3	AS_1	Fe_3
0.070 a-c	0.07 a-c	213 b-h	197 a-f	43 a-e	43.4 a-e	OM_1	AS_2	Fe_3
0.069 a-d	0.073 a-c	221 a-f	202 a-e	42.9 a-e	47.1 a-c	OM_2	AS_2	Fe_3
0.055 b-i	0.06 a-c	190 f-k	187 b-f	48 a-b	47.8 ab	OM_3	AS_2	Fe_3
0.037 Jk	0.038 b-f	204 d-j	199 a-f	37.8 c-h	39.8 b-g	OM_1	AS_3	Fe_3
0.027 K	0.028 c	212 b-h	193 a-f	46.2 a-c	47.1 a-c	OM_2	AS_3	Fe_3
0.068 a-d	0.0104a	239 a-c	228 ab	51.1 a	49.2 a	OM_3	AS_3	Fe_3
0.057 b-h	0.067 d-e	189 f-k	188 b-g	31.6 g-k	30 h-l	(Fe	Chel	ate)

* values followed by same letters do not differ significantly at 0.05% significant level
Fe_1, Fe_2 and Fe_3, respectively, 0, 1 and 2 kg of ferrous sulfate, AC_1, AC_2 and AC_3, respectively, 0, 5/0, and 1 L of sulfuric acid and OM_1, $OM_2$and OM_3, respectively, 0, 2 and 4 kg organic matter per tree

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In general, proline accumulation declined in all treatments except for the control in the second year, after the treatments were performed. In a research on two species of the monocot plant Triglochin, the effects of providing food nutrients and salinity on growth, on quantities of absorbed elements and on proline content were studied. It was found that increases in the soil content of heavy metals such as iron, manganese, zinc, and copper, from the optimum to the usual content (which in the case of this experiment was excessive), raised the proline content of leaves and roots. This shows that plants respond to the stress of excessive content of heavy elements including iron (Karlsons et al., 2011). Biochemical studies of the effects of iron deficiency on leaves of macadamia trees indicated that non-protein amino acids such as arginine and lysine, organic acids, and citric acid accumulated in chlorotic leaves (Gilfillan, 1967).

In an experimented that was conducted on safflower, it was concluded that, when plants were not under water stress, applying iron fertilizer to the soil resulted in the highest proline accumulation compared to foliar application (Fathi Amirkhiz et al., 2011). Research on tomatoes also showed that deficiency stress of micronutrients such as iron, zinc, copper, and manganese had significant effects on the contents of amino acids and amides, and that iron deficiency stress increased the contents of amides more than it did those of amino acids such as proline (Possingham, 1956). In a study on the effects of phosphorous fertilizer and foliar spray of iron chelates on protein and dissolved carbohydrate contents of wheat, it was found that, although proline content increased by applying phosphorous fertilizer at flowering, iron fertilizer application at stem elongation and flowering stages had no significant effects on proline content (Akbari et al., 2013).

Wong (2009) conducted a study on wheat and concluded that the effects of iron deficiency stress on leaf proline content were not significant, but that the effects of iron fertilizer application, especially when plants faced salinity and UV stress, somewhat increased wheat leaf proline content. Various reports indicate that plant protein content declines when iron is deficient, and since no relationship has been established between proline content and iron deficiency, proline cannot protect plants under conditions of iron deficiency (Wong, 2009). Based on the findings of various researchers, it has been proved that nutritional stresses, such as potassium, phosphorous, magnesium, chlorine, and copper deficiencies, are accompanied by proline accumulation, but that iron deficiency causes accumulation of amino acids like arginine, asparagines, histidine, lysine, and serine in plants (Rabe, 1999). Under environmental stresses, organic solutions with low molecular weights such as amino acids and sugars accumulate in plants. Increases in the concentrations of dissolved free sugars and proline (which are among the most important potential participants in osmotic regulation) under stress conditions have been reported repeatedly. In general, there are two major pathways of proline synthesis: the glutamate pathway in which cytoplasmic enzymes are involved, and the ornithine pathway in which mitochondrial enzymes take part. The glutamate pathway is of greater importance in plants, and it seems the main enzymes of this pathway respond positively to zinc and iron sprays (Delaney et al., 1993). In our experiment, iron deficiency stress did not significantly increase proline accumulation either, but application of iron fertilizer and organic matter increased proline synthesis in kiwi trees.

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Fruit yield

Individual and mutual effects of treatments significantly influenced fruit yield at one percent probability level in both years the experimented was conducted (*Tables 2* and *3*). Injection of two kilograms of iron sulfate, or 0.5 liter of sulfuric acid, or four kilograms of organic matter, increased fruit yield by 48 and 60.7 percent, by 30.8 and 33 percent, and by 49.3 and 49.1 percent compared to the control treatment in the first and second years, respectively. In comparison, as for the interactions of the effects of iron sulfate + sulfuric acid + organic matter in 2013, fruit yield was largest in the $Fe_3AC_2OM_3$ treatment, 73 percent more than the iron chelate treatment or sham treatment and 80.1 percent compared to the control, and that of the iron chelate treatment 24.7 percent more compared to the control. In the second year of the experiment, although no new fertilizer treatments were considered for the studied trees, the treatments that had received suitable amounts of iron fertilizer in the previous year exhibited desirable fruit growth and development. The maximum fruit yield, like the previous year, belonged to the treatment of applying one kilogram of iron sulfate, 0.5 liter of sulfuric acid, and four kilograms of organic matter.

This was the $Fe_3AC_2OM_3$ treatment with fruit yield of 79.9 percent higher than the iron chelate (or sham treatment) and 84.4 percent more than the control, while the fruit yield of the iron chelate treatment was 22.2 percent more than the control (*Table 5*). In research conducted by Iglesias et al. (2003), injection of iron (II) sulfate into calcareous soil effectively prevented iron chlorosis in pear trees. Chlorophyll levels in treated trees significantly differed from those of the control, but did not show significant differences with trees treated with iron chelate. Studies in the following years indicated that the extents of flowering and fruit set increased significantly compared to the control treatment, but were lower than were those of the chelate treatments. The mean four-year yield in trees treated with iron (II) sulfate did not significantly differ from that of trees treated with iron chelate but differed very significantly from that of the control. Rombola et al. (2003) conducted an experiment in which they injected iron (II) phosphate fertilizer into the soil at the depths of 25-30 centimeters at four points around mature kiwi trees and compared the effects with those of Fe-EDDHA solution. They found that injection of this fertilizer into the soil could be an effective replacement for iron chelate fertilizers in improving quantitative indices of kiwi. Faust (1989) stated that iron chlorosis mainly occurred in young leaves due to the immobility of iron in plant leaves, but that lower leaves were also affected by chlorosis and intangible reduction of chlorophyll levels happened in lower leaves too. This condition led to a reduction in the extent of photosynthesis and in the efficiency of leaf photosynthesis in all parts of the plants including old parts that did not exhibit chlorosis and, hence, efficiency, carbohydrate accumulation, flower induction and fertilization, and plant yield decreased due to the general decline in plant photosynthesis. Since cytochromes b and c (that contain iron) play an important role in the electron transfer of the respiration process, iron deficiency can disturb plant respiration and, as a result, disturb other plant physiological mechanisms leading to reduced growth, development, and yield. Considering the role iron plays in the reduction of nitrates in plants, and given that nitrates and ammonium are important sources of mineral nitrogen absorbed by higher plants, nitrates must be reduced to ammonium to enter into the structure of organic compounds. This process happens under the influence of various enzymes, and iron plays

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the major role in the activities of these enzymes (Rombola and Tagliavini, 2006). Since iron plays a role in the metabolism of organic acids and other biomolecules, its deficiency leads to a reduction in sugar content, especially a reduction in the content of reducing sugars, cofactors such as riboflavin, other vitamins, flavins, and mononucleotides. Any of these disturbances can cause disorders in the natural plant growth and development system and, hence, can reduce growth and development, fruit size, weight, and quality, and, eventually, can reduce yield.

Table 5. *Effect of application of ferrous sulfate, sulfuric acid and organic matter on yield, fruit TSS/TA and vitamin C during 2013-2014*

Vitamin C (mg/100gr)		Fruit TSS/TA (%)		Yield (kg)		Treatment		
2014	**2013**	**2014**	**2013**	**2014**	**2013**	**OM**	**Asid**	**Fe**
52.6 de	54.13 a-c	10.90 ab	11.32 ab	11.2 i-k	12.7 ij	OM_1	AS_1	Fe_1
60 a-e	59.3 a-c	10.01 ab	8.27 c	10.5 Jk	11.7 ij	OM_2	AS_1	Fe_1
53.9 c-e	53.83 a-c	10.57 ab	9.63 bc	16.1 g-k	19 h-j	OM_3	AS_1	Fe_1
54 c-e	51.65 a-c	10.90 ab	9.42bc	6.48 K	7.5 j	OM_1	AS_2	Fe_1
49.3 e	48.8 bc	9.99 ab	10.61 a-c	17.5 g-k	18.8 h-j	OM_2	AS_2	Fe_1
61.9 a-d	65.15 ab	11.52 ab	10.68 a-c	31.2 e-h	36.5 c-g	OM_3	AS_2	Fe_1
64.2 a-d	62.85 ab	9.35 b	10.97 ab	16.9 g-k	17 h-j	OM_1	AS_3	Fe_1
58.6 a-e	56.08 a-c	11.88 ab	9.55 bc	17 g-k	18.8 h-j	OM_2	AS_3	Fe_1
59.8 a-e	58.83 a-c	10.32 ab	9.80 bc	29 e-i	35.1 c-g	OM_3	AS_3	Fe_1
62.7 a-d	64.73 ab	10.47 ab	9.26 bc	25.4 e-j	22.9 g-i	OM_1	AS_1	Fe_2
55.5 b-e	49.18 bc	11.15 ab	9.83 a-c	21.1g-k	17.7 h-j	OM_2	AS_1	Fe_2
61 a-d	59.25 a-c	10.17ab	11.38 ab	26.4 e-j	24.7 e-i	OM_3	AS_1	Fe_2
62.5 a-d	60.98 a-c	10.63 ab	11.12 ab	25.2 e-j	21.2 h-j	OM_1	AS_2	Fe_2
60.8 a-e	60.43 a-c	11.11 ab	9.67 bc	48.5 b-d	43 b-d	OM_2	AS_2	Fe_2
65.5 a-c	68.48 a	12.16 a	10.14 a-c	48.8 b-d	40.8 b-d	OM_3	AS_2	Fe_2
63.1 a-d	55.68 a-c	11.57 ab	9.95 a-c	28.5 e-j	29.7 d-h	OM_1	AS_3	Fe_2
65.8 a-c	52.03 a-c	10.37 ab	10.49 a-c	42.7 c-e	38.3 b-e	OM_2	AS_3	Fe_2
62.9 a-d	66.93 a	11.52 ab	10.75 a-c	58 ac	50.9 b	OM_3	AS_3	Fe_2
60.7 a-e	57.03 a-c	10.19ab	10.31 a-c	33 d-g	29.3 d-h	OM_1	AS_1	Fe_3
64.5 a-d	55.23 a-c	11.64 ab	10.42 a-c	39.3 d-f	35 c-g	OM_2	AS_1	Fe_3
58.6 a-e	56.58 a-c	10.25 ab	10.57 a-c	39.8 d-f	37.1 c-f	OM_3	AS_1	Fe_3
62.3 a-d	58.43 a-c	11.05 ab	9.45 bc	27 ej	23.9 f-i	OM_1	AS_2	Fe_3
61.6 a-d	64.05 ab	11.27ab	10.35 a-c	57ac	48.1 bc	OM_2	AS_2	Fe_3
63.4 a-d	63.5 ab	10.77ab	9.85 a-c	71.8 a	64.3a	OM_3	AS_2	Fe_3
61.9 a-d	45.63 c	11.51 ab	12.45 a	22.3 f-k	17.8 g-i	OM_1	AS_3	Fe_3
68.5 a	65.4 ab	10.65 ab	10.95 ab	42.1 c-e	34.8 cg	OM_2	AS_3	Fe_3
67.5 ab	64.15 ab	11.33 ab	10.14 a-c	64.1 ab	50.6 b	OM_3	AS_3	Fe_3
52.9 de	61.03 a-c	10.16 ab	9.69 bc	14.4 h-k	16.9 g-i	(Fe	Chel	ate)

* values followed by same letters do not differ significantly at 0.05% significant level
Fe_1, Fe_2 and Fe_3, respectively, 0, 1 and 2 kg of ferrous sulfate, AC_1, AC_2 and AC_3, respectively, 0, 5/0, and 1 L of sulfuric acid and OM_1, OM_2 and OM_3, respectively, 0, 2 and 4 kg organic matter per tree.

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Fruit sugar/acid ratio

The treatments did not significantly affect fruit sugar/acid ratio in the two years the experiment was conducted (*Tables 2, 3*). However, the highest fruit sugar/ acid ratio in the first year was that of the treatment $Fe_3AC_3OM_1$ and that of $Fe_2AC_2OM_3$ in the second year, which were 9.07 and 10.4 percent higher compared to the control treatment. The noteworthy point in the effects of the treatments on fruit sugar/acid ratios was the inverse effects of organic matter application in the first year that reduced fruit sugar content and increased fruit acid content in most treatments in which organic matter was applied.

Such results were obtained in a research that was conducted on kiwi and apple trees in which organic methods were compared with customary ones, and the reason for obtaining these results can be the increased access to nitrogen that increases fruit acid and reduces fruit sugar (Pecket et al., 2006). The sugar/acid ratio in the treatment of applying iron chelate decreased compared to the control and the $Fe_3AC_3OM_1$ treatment (*Table 5*). Different levels of iron sulfate together with sulfuric acid application did not very significantly affect the sugar/acid ratio, but increased ratio of sugar/ acid ration was observed in our experiment too because the treatments corrected iron deficiency and chlorosis in kiwi trees. This increase in the sugar/ acid ratio is one of the most important qualitative factors in marketing the crop.

Vitamin C

Kiwi fruit is naturally rich in vitamin C. This raises the health benefits (because vitamin C is an antioxidant) and nutritional value of this fruit. Kiwi fruits usually contain 25-155 milligrams of vitamin C per 100 grams of fresh fruit (White et al., 2005).Various factors such as kiwi variety, local environmental conditions, and especially tree nutrition, affect nutritious compounds, including vitamin C, found in kiwi fruit. Only the individual effects of organic matter on fruit vitamin C content was significant at the 5% probability level in our experiment. The interactions of the treatments did not have any significant effects in the first year, but in the second year significant differences were observed at the 5% probability level after the treatments were performed. The highest fruit vitamin C content in the first year was that of the $Fe_2AC_2OM_3$ treatment and in the second year that of $Fe_3AC_3OM_2$, which were 20.95 and 23.2 percent higher than fruit vitamin C in the control treatment, respectively (*Table 5*). Application of iron chelate did not show any significant differences in vitamin C content compared to the other treatments in the first year, but in the second year these differences became significant so that in the best treatment ($Fe_3AC_3OM_2$) the vitamin C content was 22.8 higher than of the iron chelate treatment (*Table 5*). Kiwi fruit has greater antioxidant activity and higher vitamin C, polyphenol, and mineral matter contents in the organic method compared to the customary method, in which chemicals are used (Amodio et al., 2007). Comparison of the nutritional value and antioxidant contents of kiwi fruit in the organic agriculture method (in which manure replaces chemical fertilizers) and the integrated and the customary methods indicated that the vitamin C content in the organic method was significantly higher (Ashoorinezhad et al., 2011). Use of organic methods in tangerine fruit production also increased vitamin C, mineral matter, and carotenoids contents compared to chemical treatments (Beltran et al., 2008). Application of organic matter together with iron fertilizer and acid in our experiment prepared suitable

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conditions for kiwi trees to overcome chlorosis and, hence, increased photosynthetic capability of the trees and the raised the level of photosynthetates production including sugars, which increased vitamin C synthesis.

General Conclusions

In our experiment, we tackled the problem of chlorosis in kiwi orchards in a region in the central part of Mazandaran Province by injecting a combination of iron sulfate, sulfuric acid, and organic matter with a Biolift machine into the soil around kiwi trees. Iron sulfate provided the required iron element, sulfuric acid reduced pH locally and increased iron absorption, and organic matter raised the efficiency and the ability to absorb iron sulfate. This method is an effective and useful strategy and can replace that of deep placement of iron chelates which entails high costs, has short- time effectiveness, and is accompanied by environmental problems too. Based on obtained results of our experiment, injecting one kilogram of iron sulfate, half a liter of sulfuric acid, and four kilograms of organic matter increased iron availability for kiwi trees and, hence, increased leaf chlorophyll concentration, solved the problem of iron chlorosis, and increased photosynthetic capability and efficiency. As a result, many of the qualitative and quantitative characters of kiwi trees (including yield, vitamin C content, and sugar/acid ratio) were affected for a period of at least two tears.

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AN ESTIMATION OF THE ROTATION AGE USING AUTOREGRESSIVE PRICE MODEL AND TRUNK ANALYSIS DATA: RESULTS FOR *PINUS BRUTIA* TEN

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Abstract. The present study was conducted in southwestern Iran and it aimed to determine the optimal rotation age of Turkish pine (*Pinus brutia* Ten) at which the marginal revenue equaled to the marginal cost. The volume growth, stumpage price, setup cost and interest rate were considered as essential factors for calculating the optimal rotation age. The data of volume growth were extracted through Trunk Analysis Method and stumpage price of *Pinus brutia* was estimated through autoregressive model. The results showed that the optimal harvest age of *Pinus brutia* occured in the age range of 18 to 23 years when the land's expected value maximizes. It is noteworthy that cultivation of the stated species was found to be economically justifiable in this area.
Keywods: *Pinus brutia, Turkish pine, stumpage price, optimal rotation age, Faustmann model*

Introduction

Forest rotation refers to the period of time between stand establishment and harvest which is applied for forests with even-aged management. On the other hand, rotation age refers to the age when stand must be harvested or clear-cut. The calculation of this period is necessary to achieve the economic and sustainability goals of the harvester (Posavec et al., 2011). The most commonly used criteria to maximize productivity of forest stands are: (1) maximum single-rotation physical yield (2) maximum single-rotation annual yield (3) maximum internal rate of return (4) maximum annual net revenues (5) maximum discounted net revenues from an infinite series of rotation (Newman, 1988). The idea of optimal economic age was first introduced by Faustmann (1849) and is extensively used in evaluating the financial maturity of a forest stand. Regarding this criterion, foresters and natural resources managers have traditionally assumed that forestlands will be under timber production for ever, under the condition that the harvested stands are rapidly replaced by other ones. Therefore, Faustmann criterion is called discounted benefits to infinity (DBI) or land's expected value (LEV). In this model, optimal solution occurs when the land's expected value is in its maximum level and marginal revenue of production equals marginal cost of input (Chang, 1984).

Since 1957 optimal rotation age has been studied by various researchers using the models with different levels of complexity (Gaffney, 1957; Näslund, 1969; Anderson, 1976; Reed, 1986; Engindeniz, 2003; Petit and Montagnini, 2004; Posavec et al., 2011; Pourmajidian et al., 2013; Brazee and Dwivedi, 2015). Some of these studies have used Faustmann criterion (Zhang, 2001; Brazee and Dwivedi, 2015) and other studies have applied other criteria for calculating the optimal rotation age (Engindeniz, 2003; Petit

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and Montagnini, 2004). Chang (1984) investigated the relationship among the Land's Expected Value model, Net Present Value model, Forest Renting model and Traditional Biologic model and showed that the three latter models are special cases of the Land's Expected Value model. Land's Expected Value model is the most accurate model for calculating the optimal rotation age by taking into accounts the opportunity cost of stand as well as opportunity cost of land (Newman, 1988; Pearse, 1967). To determine the optimal economical rotation in even-aged forests, a combination of information including forest growth, stumpage price, cost and interest rate is required (Mohammadi Limaei et al., 2013) and it is usually assumed that: 1) The forest growth and stumpage price are constant. 2) Utilization costs are not undertaken by the owner and trees are sold as stand stock. 3) The risk of pests and tree uprooting is very low and it is ignored. 4) Tax is not paid to government. 5) Thinning is not performed (Amacher et al., 2009).

Since Iran is not rich in terms of native coniferous species, the government has to import paper pulp and long-fibers of coniferous species for the pulp and paper industries demand. These imports remarkably increase the currency outflow. Hence, it must be attempted to develop plantations of fast-growing species or wood farming in various parts of the country (Nouri, 1999; Arian et al., 2017). In the present study, *Pinus brutia* was selected because of its good physical and mechanical properties in commercial pulp production (Üner et al., 2011; Fakhryan Roghani, 2015). The aim of this research is to determine whether this species is economically justifiable or not, and to calculate the maximum net present value in an infinite series or estimate the optimal rotation age.

Materials and Methods

This study was carried out in a 33-year old area under *Pinus brutia* plantation in western Iran. The study area is located at 48º 20ˊ E and 33º 30ˊ N and 1270 m above sea level. The area is characterized by Mediterranean climate with mean annual temperature and mean annual rainfall of 17º C and 552 mm respectively. This plantation was established in 1978 with a planting distance of 2×3 m and is managed by Khorramabad Park and Green Space Organization. As mentioned above, growth value, stumpage price, interest rate and setup costs are essential factors to calculate the optimal age by Faustmann model (Mohammadi Limaei et al., 2013). These parameters are calculated as follows:

Growth of Pinus brutia

Stand measurement method and trunk analysis method are two prevailing approaches to measure the growth characteristics of a tree. In the second method, which is more accurate to estimate growth parameters, trees are cut down and growth component is calculated according to trunk analysis and annual rings count (Zobeiry, 1994).

In this study, the data of growth was extracted from a previous study conducted by Ostakh (2013) to evaluate the effect of climatic factors on the growth rings of *Pinus brutia*. They selected 31 trees using selective method and cut them down. Then five disks were prepared from various heights of trunk. After preparation of disks, the width of annual rings was measured with an accuracy of 0.001 mm for various years. Finally they have estimated the growth components including current annual increment (CAI), mean increment (MI) and volume stock in different ages of considered stand which will be used in the present study (*Table 1*). *Figs. 1.a* and *1.b* show the amount of volume stock (m^3/ha) in various ages and the relationship between CAI and volume stock (m^3/ha).

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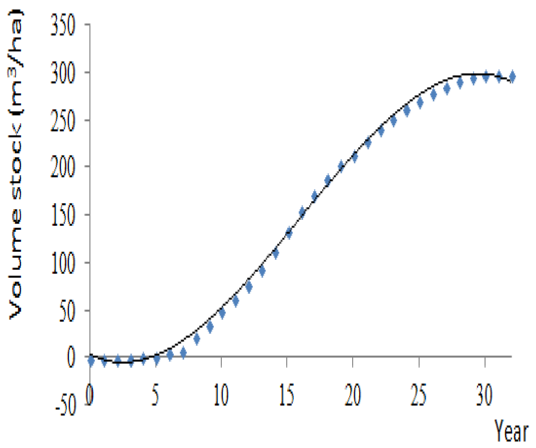
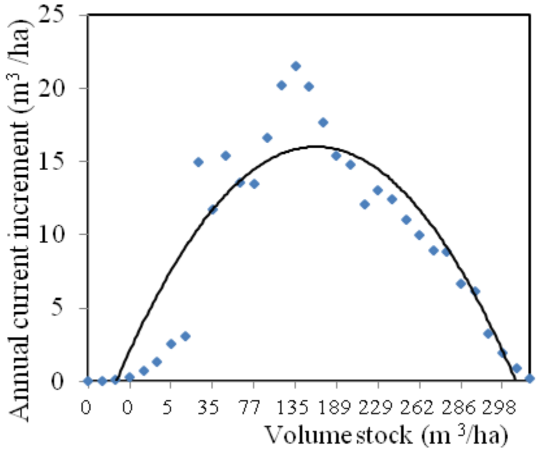


(a)



(b)

***Figure 1.** a: Volume stock per ha in various ages of the Pinus brutia stand, b: Relationship between CAI and volume stock per ha of the Pinus brutia stand*

***Table 1.** Annual increment, mean increment, volume*

Age	Current Annual Increment (m^3/ha)	Mean Increment (m^3/ha)	Volume (m^3/ha)
1	0.0119	0.0119	0.0119
2	0.0357	0.0238	0.0477
3	0.0903	0.0460	0.1380
4	0.2544	0.0981	0.3925
5	0.7468	0.2278	1.1394
6	1.3592	0.4164	2.4986
7	2.5155	0.7163	5.0141
8	3.0516	1.0067	8.0537
9	14.922	2.5542	22.987
10	11.750	3.4737	34.737
11	15.355	4.5539	50.093
12	13.557	5.3042	63.651
13	13.508	5.9353	77.159
14	16.586	6.6961	93.746
15	20.214	7.5972	113.95
16	21.531	8.4681	135.49
17	20.066	9.1504	155.55
18	17.658	9.6230	173.21
19	15.435	9.9289	188.65
20	14.813	10.173	203.46
21	12.088	10.264	215.55
22	12.996	10.388	228.54
23	12.396	10.475	240.94
24	11.068	10.500	252.01
25	9.9699	10.479	261.94
26	8.9584	10.420	270.94
27	8.8442	10.362	279.78
28	6.7028	10.231	286.48
29	6.0968	10.089	292.58
30	3.2545	9.8613	295.84
31	1.9349	9.6056	297.77
32	0.8587	9.3323	298.63
33	0.1744	9.0548	298.80

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Estimation of the stumpage price equation of Pinus brutia

There are two approaches to estimate the wood price among economists. According to the first approach, the price follows a stationary autoregressive model, that is to say, the changes which occur in a period will have no significant effect on the price of the next period and the best method for forecasting the price is to calculate the average of the previous prices. This way, prices can be estimated using the following formula: $P_{t+1} = \alpha + \beta P_t$, where $0 < \beta < 1$.

In the second approach, the price is non-stationary and does not follow the aforesaid stationary condition. In this situation, the price in the next year or period completely depends on the price in previous year or period. The price in this case, can be evaluated as $P_{t+1} = \beta P_t$, β=1 (Lindahl and Plantinga, 1997; Dickey and Fuller, 1981; Mohammadi Limaei, 2006).

After testing the price data, it was found that due to the stationary type of data, stationary autoregressive model was used for estimating the parameters. It should be noted that the data of the period 1993-2012 was collected from the Natural Resources Organization and interview carpenters. Then it was adjusted to Consumer Price Index (CPI) of Iran for the base year of 2011 (*Fig. 2*) (Central Bank of Iran, 2014).

$$P_r = \frac{P_t}{y_t} \times 100 \quad \text{(Eq.1)}$$

Where Pr is the real price, Pt is the price in year t and yt is the price index in year t and 100 is the value of price index in basic year (Branson, 1989).

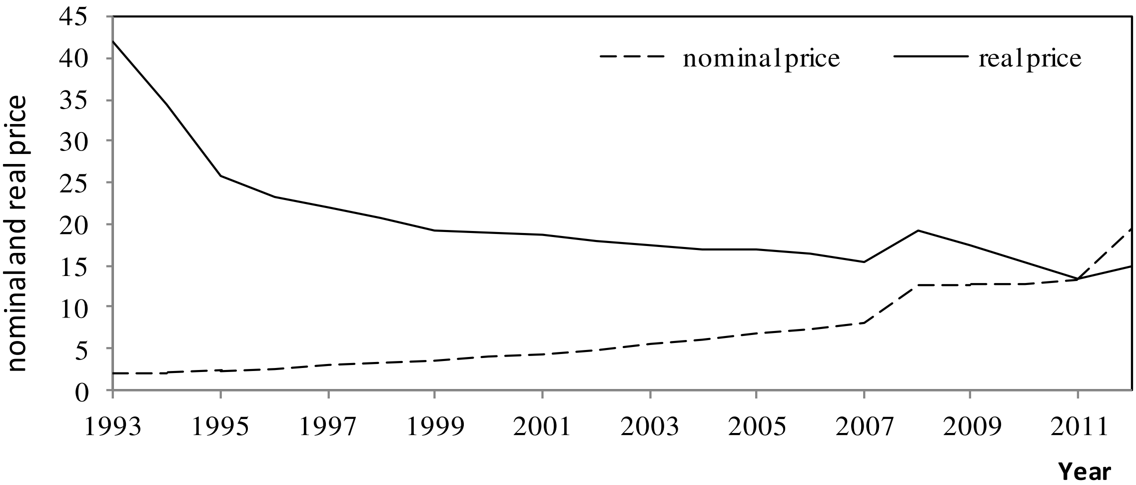

Figure 2. *Nominal (non-adjusted) and real (adjusted) prices of Pinus brutia in period of 1993-2012*

Then, a regression model was used for estimating the price of each m^3 of the *Pinus brutia Ten* (Lindahl and Plantinga, 1997; Mohammadi limaei and Lohmander, 2007).

$$p_{t+1} = \alpha + \beta p_t + \varepsilon_t \quad \text{(Eq.2)}$$

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Here, it is assumed that ε_t is a sequence of errors with normal distribution, mean of zero and zero autocorrelation. P_{t+1} is the price in the year t+1 and P_t is the price in year t. Expected mean stumpage price is calculated as follows:

$$P_{eq} = \frac{\alpha}{1-\beta} \quad \text{(Eq.3)}$$

Faustmann expected value

Since Faustmann formula includes economic parameters in estimation of rotation age, it was chosen to be applied in this study. In this method it is attempted to select the length of rotation so that the land's expected value is maximized.

Details of Faustmann model, which assumes that a land will be under forest cultivation forever, is explained as follows: net present value resulting from harvesting after the first rotation is shown in Eq. 4, where T is the decision variable or rotation period, p is the price per m^3 of stumpage, f (T) is the standing volume stock in time T, r is the interest rate, c is set up cost in time 0, and e is Nepper number. Here, e^{-rT} is referred to as discount rate which is continuous time approximation of $\frac{1}{(1+r)^T}$.

$$pf(T)e^{-rT} - c \quad \text{(Eq.4)}$$

Net present value of an infinite series with similar rotations is stated as follows:

$$pf(T)e^{-rT} - c + [pf(T)e^{-rT} - c]e^{-rT} + [pf(T)e^{-rT} - c]e^{-2rT} + [pf(T)e^{-rT} - c]e^{-3rT} + \dots \quad \text{(Eq.5)}$$

By factoring $(pf(T)e^{-rT} - c)$, an infinite converging series can be obtained:

$$(1 + e^{-rT} + e^{-2rT} + e^{-3rT} + \cdots)(pf(T)e^{-rT} - c) \quad \text{(Eq.6)}$$

By summing the converging function to infinite which is obtained by multiplying the first term by $\frac{1}{1-q}$ (q is common ratio of progression as e^{-nr}) and by substituting the sum of converging function in the series shown in number 6:

$$v = pf(T)e^{-rT} - c(1 - e^{-rt})^{-1} \quad \text{(Eq.7)}$$

Here, numerator of the fraction shows the present value of a rotation and denominator indicates the discount factor of future rotations to infinite. This equation is called Faustmann equation, land's expected value (LEV), or soil's expected value (SEV). To obtain the age at which net present value of forest is maximized, the derivation of the above term is set equal to zero.

$$v_T = pf'(T) - rpf(T) - rv = 0 \quad \text{(Eq.8)}$$

And finally, optimality condition of the Faustmann model is as follows:

$$pf'(T) = rpf(T) + rv \quad \text{(Eq.9)}$$

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According to the optimality condition of the Faustmann model, age is increased to a point that marginal revenue equals marginal cost. Here, f'(T) is the current increment of the year T.

Results

Growth of Pinus brutia

According to *Table 1*, maximum annual current increment of the species is 21.531 m^3 per ha and volume stock in age 33 is 298.80 m^3 per ha. Current Annual Increment (CAI) and Mean Annual Increment (MAI) will be equal at the age of 24 which is called biologically optimal rotation age.

Stumpage price model for Pinus brutia

As explained in *Fig. 2*, the change in trend of nominal price is in line with real price, however, real price increases with a higher slope in comparison to nominal price. It means that a major portion of increase in the price of *Pinus brutia* in the study period has occured as a result of inflation. Assessing the details of the nature and reasons of inflation in various years which may be due to increase in demand, structural inflation, inflation expectations and inflation as a result of expenses pressure is out of the limits of this work.

After adjusting the data for the base year (*Fig. 2*) autoregressive analysis was used to estimate the expected prices. According to the results and the t-value with a confidence level of 95%, it was found that there was a significant correlation between parameters P_{t+1} and P_t. Moreover, the results of this analysis revealed that β varied between 0 and 1, and stationary condition was observed (*Table 2*).

Table 2. *estimated parameters using autoregressive analysis*

	α	β	R	R^2	**Standard deviation of** ε
Parameter value	186379.95	0.655	0.957	0.916	46598.47804
Standard deviation	34132.095	0.048			
T-statistics	5.461	13.644			

Price predicting equation for *Pinus brutia* was calculated through the estimated parameters obtained from the regression analysis, and substituting the value of parameters α and β in Eq. 2, as follows:

$$P_{t+1}=186379.95+0.655P_t \qquad \text{(Eq.10)}$$

And using Eq. 3, expected mean stumpage price of *Pinus brutia* was obtained to be 540229 RLs (16$) per 1 m^3.

$$P_{eq}=\frac{186379.95}{1-0.655}=540229 \qquad \text{(Eq.11)}$$

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Economic rotation age of Pinus brutia

As mentioned above, four main components are needed for calculating the LEV. The Growth data was extracted from the trunk analysis table provided by Ostakh (2013), and the expected price mean was predicted by autoregressive model. Interest rate and set up cost were considered 2%-8% and 500000-1000000 RLS (15.26 – 30.52 $), respectively. After substituting the mentioned components in Faustmann formula, optimal rotation age was calculated, where land's expected value was maximized or marginal revenue of production equaled marginal cost of input. As seen in *Table 3* optimal rotation age fluctuates from 18 to 23 years owing to different interest rates and various setup costs. It can be said by increasing interest rate, optimal rotation age will decrease and by reducing the setup cost, optimal rotation age will increase.

Table 3. *The Optimal rotation age considering various interest rates and setup costs*

Interest rate setup cost	2%	3%	4%	5%	6%	7%	8%
500000	22	20	20	19	19	18	18
600000	22	20	20	19	19	18	18
700000	23	20	20	20	19	18	18
800000	23	20	20	20	19	18	18
900000	23	20	20	20	19	18	18
100000	23	22	20	20	19	18	18

Discussion and Conclusion

One of the most important goals of planting the coniferous species in Iran is rehabilitation and restoration of degraded forests as pioneer species during succession stages (Sardabi, 1998). Although most of *Pinus brutia* plantations are planted for aesthetic and protective purposes, research has revealed that they are suitable in terms of growth efficiency for wood production (Fattahi, 1994; Radaei, 2002).

As mentioned above, in this study, the growth data were extracted from the trunk analysis table which was calculated by Ostakh (2013), Since the trunk analysis method has higher accuracy in estimating the stand volume in comparison to stand measurement method (Zobeiry, 1994), in the present study, the required growth data for calculating the optimal rotation age was extracted through trunk analysis method for the first time. While in most of the previous studies focusing on the rotation age, stand measurement method was used for gathering the growth data (Mohamadi Limaie et al., 2013; Ranjbar et al., 2009).

As shown in *Table 2*, studied plantation with CAI, MAI and stock volume equal 20.066 (m^3/ha), 9.1504 (m^3/ha) and 155.55 (m^3/ha), respectively at the age of 17, and it is of relatively proper efficiency for wood production. Therefore, the studied region can be considered as a good site for plantation according to the provided product table by Usta (1991).

It should be noted that this stand has been affected by drought in the middle of its age (Ostakh, 2013) which has affected the process of growth stand and has reduced volume growth in the region under study in comparison to other regions of Iran with similar ecological conditions (Yousefi et al., 2013; Sadegh Zadeh Hallaj and Rostaghi, 2011).

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CAI and MAI became equal after 24 years and it can be inferred that MAI reaches its peak at this point (*Table 2*). This peak point varies owing to different site conditions (Engindeniz, 2003). The optimal rotation age which was calculated through the stated method relies entirely on biological information.

Faustman model is the best method for determining the optimal rotation age due to considering the opportunity cost of land as well as opportunity cost of stand (Newman, 1988; Pearse, 1967). It should be noted that the optimal rotation age, calculated by Faustmann model, is shorter than the optimal biological rotation age because of considering economic parameter. Various factors contribute to the optimal rotation age including site quality (Marutani, 2010), carbon subsidiary and tax (Kooten et al., 1995), fire risk and discount rate (Pasalodos, 2010), establishment costs and interest rate (Mohamadi Limaei et al., 2011; Alvarez and Koskela, 2003), non-monetary benefits of forest (Hartman, 1976) and these factors change extensively in various conditions.

According to Petit and Montagnini (2004), determination of optimal rotation age and estimation of the amount of wood produced at that age is necessary for forest sustainability and management; therefore, this issue was taken into consideration. In recent years, farmers have used poplar species around their farms and most of the research in this area has been conducted to determine the optimal rotation age of this species. Coniferous species are planted and utilized by farmers to a lower degree, while a large volume of paper pulp of these species is imported from other countries (Adeli et al., 2012).

According to the studies carried out in Iran, *Pinus brutia* is a suitable species for foresting and restoring of forests in Iran owing to its ecologic conditions which are similar to *Pinus brutia*'s native country; therefore, this paper investigated this species in terms of economic conditions. According to the results, the age of economic rotation in Khorramabad was found to be between 18-23 years.

It must be noted that the studied region is classified as a good site because this species has a relatively suitable growth rate and short optimal rotation age for plantation in this area. It is proposed that other parts of Iran especially those with suitable conditions be investigated in order to avoid a huge currency outflow which occurs due to importing the paper pulp of coniferous species.

Owing to the limitations in wood extraction from Iran's forests, it seems necessary to plant fast-growing species in order to solve the problem of wood shortage.

In this study, factors contributing to determination of the optimal age using Faustmann model such as price and increment were considered constant. As a future line of research, it is recommended to compute the optimal age by considering stochastic states.

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TELESTES SOUFFIA (RISSO, 1827) SPECIES CONSERVATION AT THE EASTERN LIMIT OF RANGE – VIŞEU RIVER BASIN, ROMANIA

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Abstract. The aim of this study implemented in 2011-2016 was to assess the status of *Telestes souffia* (Risso, 1827) and its habitats in its extreme eastern limit, a small island-like area in the upper Tisa Basin including the Vişeu Basin, which was under a long term significant human impact. The survey was based on electrofishing at 370 sampling stations for fish sampling and mapping. There where *T. souffia* was found, the habitats quality were assessed, and the causes of the identified situation were described. The studied lotic habitats are large enough, but their quality is mostly mediocre and occasionally reduced due to: poaching, riverbed morphodynamic changes, disruption of water and sediment flow, destruction of riparian vegetation, habitat fragmentation, organic pollution, and flood washing of fish. The main finding is that the effects of the identified human impact induced a moderate decrease of *T. souffia* distribution. Specific management elements were suggested in order to offer amelioration of the decreasing trend of the studied species, and for its long term survival in this sensitive zoogeographical isolated area. Integrated future monitoring and management of the basin are the main corner stones needed for this extreme population of *T. souffia* to survive and thrive.
Keywords: *Vairone, fish ecology, riparian ecosystem, isolation, conservation, management*

Introduction

The *Telestes souffia* (Risso, 1827) (Actinopterygii, Cypriniformes, Cyprinidae) (Code Natura 2000: 1131/6147) geographical distribution includes an extreme eastern small island-like area in the upper Tisa River at the Romanian-Ukrainian border (Bănărescu, 1964; Ardelean and Beres, 2000; Bănărescu and Bănăduc, 2007) including the Vişeu River basin, which was under a constant and significant human impact in the last half of the 20th century (Staicu et al., 1998; Curtean-Bănăduc, 2008; Curtean-Bănăduc et al., 2014). The local ichthyofauna status including the survival of the *T. souffia* in this isolated part of its distribution area depends in general on human activities (Bănărescu, 2005), and the different categories of human impact which have significant effects on this species should be identified and inventoried. This paper proposes to make this work and also to offer some management elements for the studied basin, with special regards to the *T. souffia*.

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A rather great variety of habitats and their species of conservation interest in the Vişeu Basin, including fish, are indubitable valuable (Bănărescu, 1964; Staicu et al., 1998; Ardelean and Beres, 2000; Curtean-Bănăduc et al., 2008). Around 50% of the fish species sampled in the Vişeu River basin are of important conservation interest: *Eudontomyzon danfordi* (Regan, 1911), *Thymallus thymallus* (Linnaeus, 1758), *T. souffia* (Risso, 1827), *Romanogobio uranoscopus* (Agassiz, 1828), *Barbus meridionalis* (Risso, 1827), *Sabanejewia aurata* (De Filippi, 1863), *Cottus gobio* (Linnaeus, 1758), and *Hucho hucho* (Linnaeus, 1758).

T. souffia is under the protection of the Bern Convention (at which Romania joined through the Law 13 of 1993), the European Directive 92/43/EEC Annex II, O.U.G. 57/2007/Law 49/2011 of the Romanian Government concerning the protected areas, habitats conservation, and of wild fauna and flora. This species is also listed in the Red Book of Ukraine as vulnerable (Akimov, 2009) and it is a critically endangered species in Hungary (Guti et al., 2014).

The studied species inhabits middle reaches of streams and rivers in the grayling ichthyologic zone, in general in the sectors with clear and moderate-fast flowing water, on gravel bottom. The required spawning habitats are rocky fast flowing sectors and its food consists of insect larvae, rheophilic crustaceans, algae and diatoms. It can reach 20 cm in length. The abundance of this species decreased in the second half of the 20 century in the studied area, the human impact has an important role (Bănărescu, 1964; Kottelat and Freyhof, 1972).

In the context in which the Vişeu Basin faced significant human pressures as a whole, with negative impacts on different fish species (Staicu et al., 1998), the central needed objective of this study was to assess the distribution and ecological status of the locally threatened *T. souffia* in one of its small isolated areas of distribution in the Eastern Carpathians (Romania), and the particular results to propose specific *in situ* management elements for the studied populations.

The identification and mapping of human pressures form the frame for *in situ* management of *T. souffia* studied populations.

Material and methods

The study on mapping and assessment of the *T. souffia* conservation status, or the identification of anthropogenic elements that can disturb this fish species in the Vişeu River basin, was carried out in 2011-2016 at 370 sampling stations (*Fig. 1, Table 1*). The following elements were evaluated in all 370 sampling stations: human pressures on habitats, habitat state, and presence of *T. souffia* populations.

To assess the spatial distribution and ecological condition of the Vişeu River basin *T. souffia* population, in the context of this species conservation value, biological samples were taken in the studied area from around two to three kilometres between two successive sampling stations along the Vişeu River and its tributaries. The sampled lotic sectors of the studied basin were selected for their appropriate/possible habitats for the fish species of interest. Additional sampling stations were done upstream from the last appropriate habitat for this fish species in order to acquire a good coverage of the studied basin. This setting of the sampling stations, based on the authors field experience during a quarter of a century in this basin of interest, guarantees the appropriateness of the obtained data and recognition of the modifications in the target fish species population under the human induced habitat changes.

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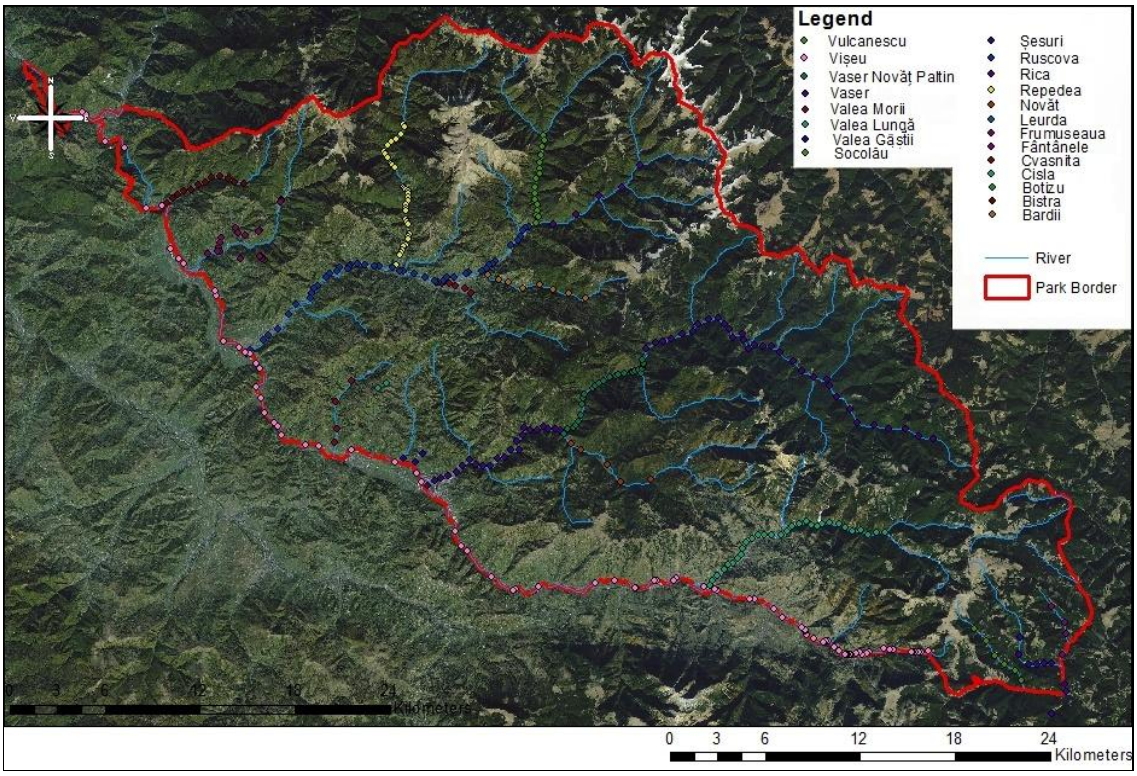

Figure 1. *The location of the 370 sampling stations in the studied area*

Table 1. *Telestes souffia sampling sites in the Vişeu River basin*

No. crt.	River	Station code	Lat. (N')	Long. (E')	Catch index no. ind./time unit (3 h in Vişeu River and 2 h in Vişeu tributaries)	Characteristic habitat state
1.	Vişeu	60	47 45 01.0	24 17 20.7	1	Reduced
2.	Vişeu	66	47 47 01.2	24 16 15.4	1	Reduced
3.	Vişeu	70	47 49 04.9	24 14 45.1	1	Average
4.	Vişeu	77	47 54 00.7	24 09 06.6	6	Average
5.	Vişeu	78	47 54 53.1	24 08 07.9	2	Average
6.	Vişeu	79	47 54 58.2	24 07 55.3	3	Average
7.	Ruscova	9	47 49 53.9	24 28 29.3	1	Reduced
8.	Ruscova	11	47 49 44.7	24 27 34.0	1	Reduced
9.	Ruscova	20	47 49 42.0	24 24 48.1	3	Average
10.	Ruscova	27	47 49 59.8	24 21 58.4	9	Average
11.	Ruscova	31	47 49 52.0	24 21 01.0	2	Average
12.	Ruscova	35	47 49 16.9	24 19 68.1	1	Average
13.	Vaser	43	47 43 36.4	24 29 26.9	1	Average
14.	Vaser	47	47 43 19.6	24 27 59.3	4	Average
15.	Vaser	49	47 43 19.9	24 27 17.8	1	Average
16.	Novăț	31	47 47 15.4	24 36 35.0	1	Average

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The quantitative fish sampling was carried out by catch per unit effort (CPUE) of electrofishing. Five longitudinal lotic sectors of one km in length for three hours on the Vişeu River and for two hours in the tributaries, were researched. All specimens of *T. souffia* (green spots in *Fig. 2*) were released on their habitat.

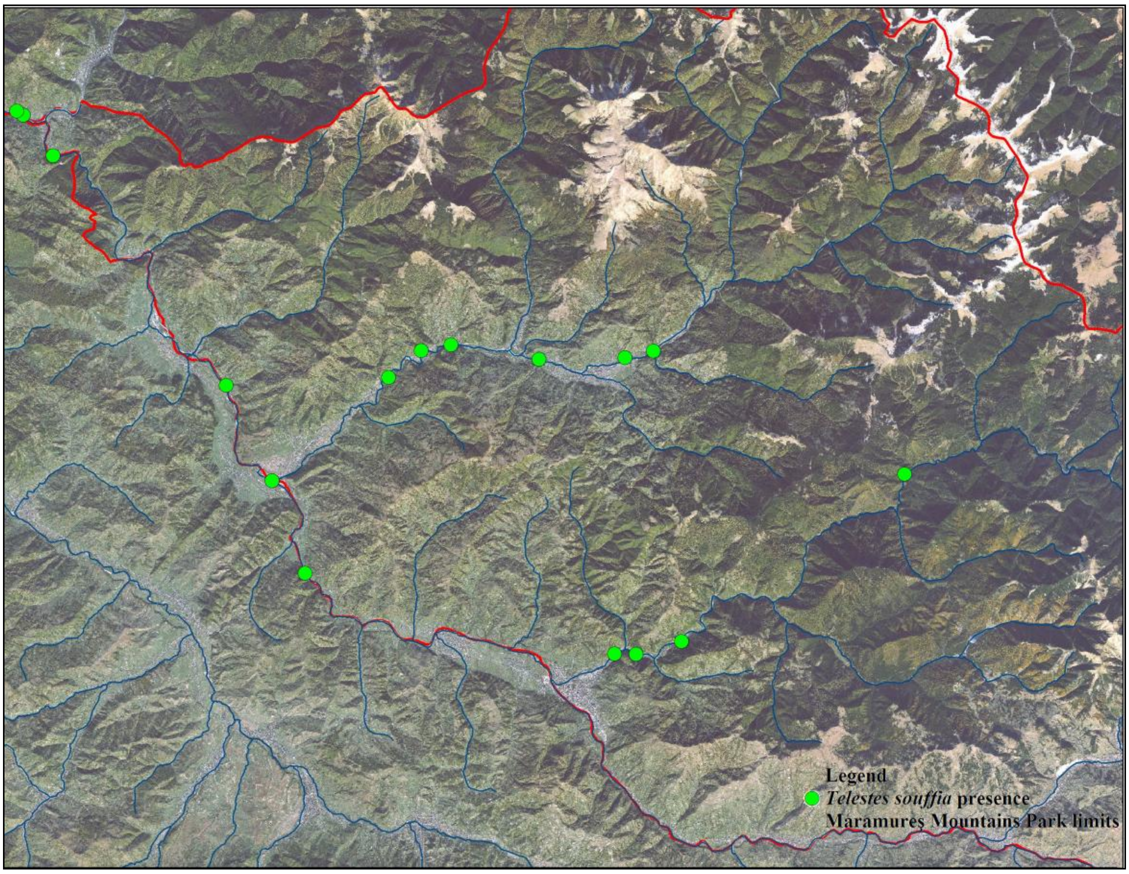

Figure 2. *The sampling stations, where Telestes souffia were observed*

The CPUE can be transformed through the correspondence in different categories: (C) – common species, (R) – rare, or (V) – very rare according to the Natura 2000 standard data form guidelines, "In mammals, amphibians, reptiles and fishes, no numeric information can be indicative and then the size/density of the population is evaluated as (C) – common, (R) – rare, or (V) – very rare".

The criteria used to assess *T. souffia* population status are the following: 1) equilibrated allocation of fish by age categories, 2) the size of population, 3) the size of distribution area and 4) the relative percentage of individuals of the fish species of interest in the fish communities.

According to the Natura 2000 guidelines, the criteria "The conservation degree of specific habitats" in the standard data form incorporate two subcriteria: i) degree of conservation of habitat features, which are important for the species; ii) recovery possibilities.

The criteria i) need an all-encompassing assessment of habitat characteristic concerning the necessities of the species of concern. "The best expertise" way of dealing with the issue was used to rank this criterion in the following elements: I in excellent condition, II well preserved, III in moderately/partially degraded.

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When the subclass I is accepted "I elements in excellent condition" or "II well preserved elements," the criteria B (b) should be classified completely as "A: excellent conservation" or "B: good conservation", indifferent of the other sub-criterion classification. The criteria ii) which can be use only if the items are degraded moderately or partially, an assessment of the viability of the studied population is needed. The acquired ranking system is: I easy recovery; II possible restoration with moderate effort; III restoration is or difficult or impossible.

The synthesis applied for classification is based on two sub-criteria: A-excellent conservation = excellent condition; B-good conservation = well preserved elements; C-average or reduced conservation = all other combinations.

Result and discussion

The running lotic sections where this fish species (*Fig. 3*) was sampled during this study are presented in *Table 1;* the catch index values were detailed for each sampling section (fish individuals number per time and unit effort).

Figure 3. *Sampled Telestes souffia*

Within the studied area, *T. souffia* form permanent populations in the Vișeu River and some of the tributaries such as Ruscova, Vaser, and Novăţ. The studied habitats of this fish species are large enough, but their quality is mostly mediocre and occasionally reduced (*Table 1, Figures 4* and *5*), with good perspective for long term survival of this species in the studied area.

The identified types of human pressures in the studied area have a long term medium cumulative result, affecting somewhat the long-term ecological status of the *T. souffia*.

Based on this field data, some risk elements related to the biological and ecological requirements of *T. souffia* were identified as pressures and threats: poaching, minor changes in riverbed morphodynamics due to river engineering; disruption of water flow and natural sediment transport; destruction of riparian tree and shrub vegetation; habitat fragmentation/isolation of fish populations; organic pollution; flood washing of fish (*Figs. 4* and *5*).

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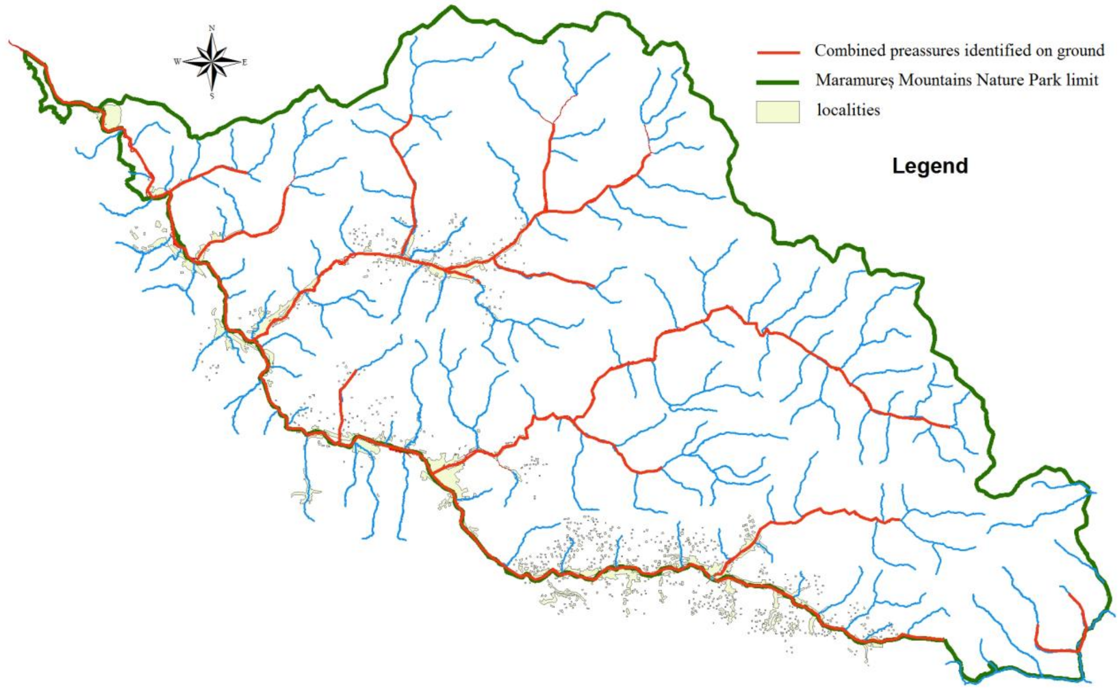

Figure 4. *Identified (in red) combined pressures (poaching, minor changes in riverbed morphodynamics due to river engineering, disruption of water flow and natural sediment transport, destruction of riparian tree and shrub vegetation, habitat fragmentation/isolation of fish populations, flood washing of fish) on Telestes souffia*

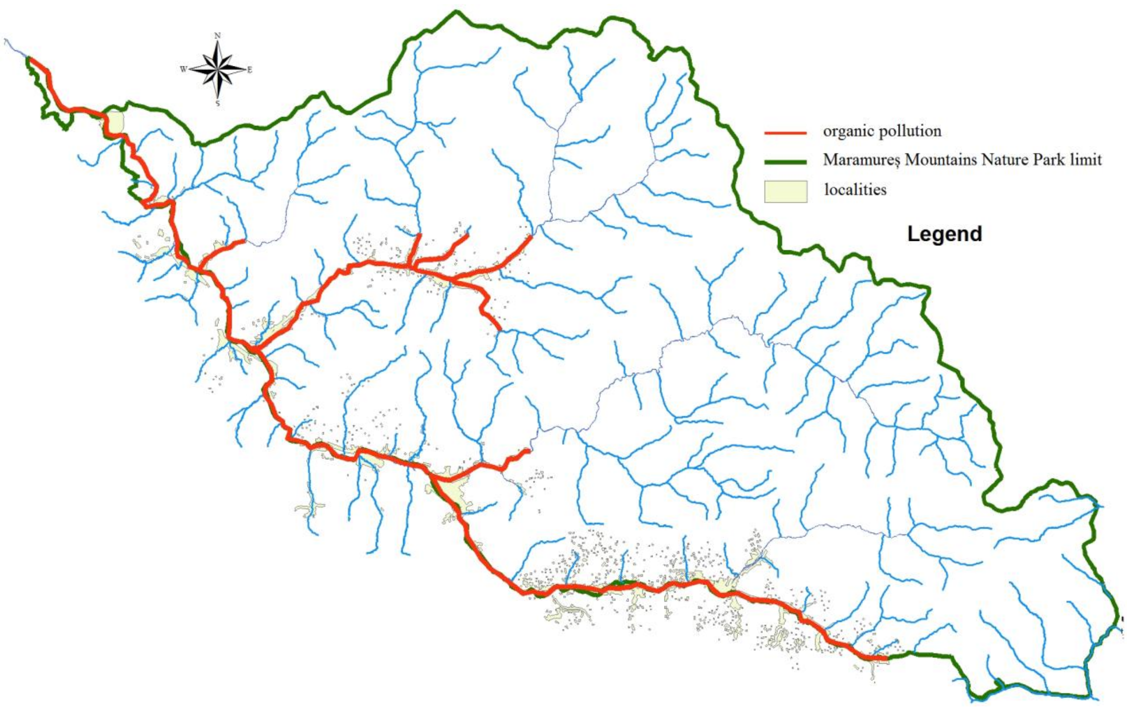

Figure 5. *Identified organic pollution (in red) sectors as pressure on Telestes souffia*

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Poaching

During the study, poaching activities (in over 20% of our field sampling activities) with the use of electrofishing devices were noted. Sometimes, poachers were observed to use diverse substances for fishing. By interviewing 431 local people in the Vişeu River basin, it is obvious that poaching is an usual activity here, and these illegal actions induce a significant decrease in the *T. souffia* population. *T. souffia* is not a targeted species for poachers due to its small size, but non-selective poaching activities endanger this species too. *T. souffia* also occurs in poacher's catches done by a variety of illegal fishing techniques in the neighbouring Transcarpathian region of Ukraine, where poaching is also quite a common activity (Didenko et al., 2011; 2014).

Minor changes in riverbed morphodynamics due to river engineering

T. souffia has relatively diverse habitat requirements, including different natural processes of riverbed morphology. Dams, dykes, sills, roads in riverbeds, sediment transport modification, gravel excavation (*Fig. 5*), etc., have significant impacts on natural morphodynamics of the riverbed and therefore the *T. souffia* key habitats are damaged, a fact that induces a decrease in the population size. On the other hand, this species avoids places with very fast currents and can be often found in pools near barrages, small dams, hydrotechnical facilities, etc. (Danko, 1956) and therefore their construction can create favourable conditions for *T. souffia.*

Disruption of water flow and natural sediment transport

The unnatural water flow and sediment transport create unfavourable circumstances for forming of the needed key habitats for different fish species in the studied basin, and these processes may induce fish population decline (Bănăduc, 2008). The boost of the turbidity of water due to negligent forestry activities is only one case of the elements resulting in the modification of the natural sediment transport in the rivers.

Destruction of riparian tree and shrub vegetation

Destruction of riparian tree and shrub vegetation and therefore reduction of the riverine vegetation (*Fig. 6*), by fractional/total destruction, both in the context of depreciating the microclimate protection and that of trophic resources, can lead to a reduction in abundance of fish fauna, including *T. souffia* populations. The shrubby and woody riparian vegetation should be as intact as possible for a minimum of 5-10 m width in the upper part of the studied rivers and of 10-25 m width in their lower part (Curtean-Bănăduc et al., 2014).

Habitat fragmentation/isolation of fish populations

Habitat fragmentation/isolation of fish populations often creates the situations of fish genetic isolation, reduction of genetic diversity resulting in inbreeding and local extinction (Popa et al., 2016). Interruption of streams and rivers by dams restricts longitudinal migration of *T. souffia* and its access it's to key habitats, which can induce a decrease in the abundance and finally to the disappearance of this fish species.

Organic pollution

Organic pollution from municipal sewage systems, aquaculture and riverine agriculture is a persistent problem in the most part of the Vișeu River basin, mainly along the Vișeu River (Staicu et al., 1998), which affect the *T. souffia* populations. Comprehensive development of the sewage systems and the wastewater treatment are urgent tasks everywhere in the Vișeu Basin.

Figure 6. *Anthropogenic reduction of the riverine vegetation on Vaser River*

Flood washing of fish

During flood periods, downstream drift of fish is significantly more intense along the channelized and straightened river stretches, as it was noted in all the field campaigns in spring.

Suggested protection measures

The authors developed specific suggestions for the *T. souffia* protection measures in the researched basin to compensate for the identified threats.

We underlined the need for the support from the local Maramureş Mountains Nature Park administration based on expanding field activities of the park personnel in order to control and diminish the poaching. The insufficient financial resources for these activities can be somewhat counteracted through cooperative actions with forestry, gendarmerie, and police. It is indisputable that few centralised national/regional institutions, an excellent example in this context being the Romanian National Agency for Fishing and Aquaculture, are not capable of stopping these activities. The establishment of a volunteer corps can also help in controlling this phenomenon, only if

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the involved personnel have the proper specific knowledge in identifying all the fish species and in understanding fish biology and ecology in natural habitats. A simple legitimation cannot replace the needed professional background for this work.

Water engineering interventions and development of low control facilities (dams, bottom sills, dykes, micro hydropower plants, water extractions, riverbed mineral over-exploitations, etc.) should not be admitted by Maramureș Mountains Nature Park Scientific Council and Administration without professional environmental impact assessment concerning the effect on the target species and/or their habitats.

The natural-like water flow and sediment transport can be maintained in a state similar to the unregulated conditions, if forestry activities and/or river gravel mining will not significantly alter the self-sustaining natural processes in the river ecosystem. It can be carried out by adapting human activities to the appropriate seasons, when the local natural conditions are relatively similar to those to be generated (i.e. significant turbidity). Seemingly inoffensive, many present (*Fig. 7*) or potential works on the investigated lotic systems must be of specific management concern. Examples like dams, embankments, fords, bank modifications (*Fig. 8*), water extractions, alterations of thalwegs, etc., should not be admitted by the local park administrator without the agreement of specialists studying the species of interest, based on the specific/local stressors and the biological and ecological requirements of the species of concern. In this specific case, for example, no ford should be higher than 10-15 cm in the shallow sectors and dry season. We also propose the all year round control of the forestry activities, like the interdiction of dragging and storage of lumber through/in creeks, streams and rivers. We also propose the control of the development of works for lumber depository and exploitation platforms (*Fig. 9*) and the obligation of reforestation. In this general context, the planed rotation of forestry activities in the sub-basins of the studied Vișeu Basin is needed.

Figure 7. *Works in the investigated lotic systems*

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Figure 8. *Vişeu River banks anthropogenic modifications*

Figure 9. *Lumber depository and exploitation platform on Ruscova River*

The restoration and maintenance of longitudinal connectivity, including all the sub-drainage basins of the Vișeu Basin, is an important element for conservation of natural habitats.

We propose studying the potential future constructions situated on or near the water courses very cautiously, in the condition in which they could interrupt the longitudinal connectivity along the lotic systems, by different crosswise barriers in the riverbed, and/or by diminishing the water volumes.

The water courses should be not straightened and channelized but kept as much as possible in their natural/semi-natural hydro-geomorphologic state, which provides refuge habitats for fish during floods and reduces the downstream drifting of the aquatic organisms. In the lotic sectors, which are relatively uniform due to river engineering, steps of maximum 15 cm high should be created, which can provide shelter areas during flood periods.

Combinations of various human pressures affect numerous lotic sectors in the Vişeu Basin (*Figs. 4* and *5*), and the assessment score for the *T. souffia* was determined as average.

Some management elements should be enforced in the studied area: poaching control, creation of buffer/refuge sectors on lotic systems; proper management of water use according to seasonal habitat requirements of fish, treatment of sewage and polluted surface waters; recalibration of hydro-energetic utilisation of streams and rivers; integrated management of the Vişeu Basin water resource; creation of lotic systems ecological networks; restoration of longitudinal connectivity in the river system.

T. souffia occurs in other rivers of the Tisa River basin, however, its populations are not abundant like in the past, at least in the Romanian rivers. Its occurrence in the Hungarian section of the Tisa was observed close to the border between Hungary and Ukraine (Harka and Sallai, 2004). On the neighbouring Ukrainian territory, very little information is available on *T. souffia*, because no large-scale ichthyologic surveys were carried out recently and no routine monitoring is performed due to the ban on the use of electrofishing devices for fish sampling in Ukraine. Therefore, the current data on this fish in Ukraine are very fragmentary. This species is listed in the Red Book of Ukraine as vulnerable (Akimov, 2009). *T. souffia* was mentioned to inhabit middle and upper half of lower reaches of the rivers flowing in the eastern part of the modern Transcarpathian region of Ukraine and was listed as absent in the western part (Vladykov, 1926; 1931) In the middle of the twentieth century, *T. souffia* was listed as a common species and reported for the upper and middle reaches of the Tisza, Teresva, Tereblia, Rika, and Borzhava rivers, however, it was not detected in Uzh and Latorica rivers (Vlasova, 1956). Nevertheless, this species was found in the Uzh and Latorica rivers as well as in the Teresva, Tereblia, Rika, Borzhava, and Tisza rivers and by Danko (1956), who indicated that this fish was quite abundant in some rivers composing up to 20-30% of the total catches in abundance. Harka and Bănărescu (1999) recorded this fish in the Tisza River near Vynogradiv in 1995. Later, *T. souffia* was reported to be sampled in the Tisza, Teresva, and Tereblia rivers but was not detected in the other eight investigated rivers including Rika, Latorica, and Uzh (Koščo et al., 2004). According to Movchan (2011), *T. souffia* occurs in the Uzh, Latorica, Borzhava, Rika, Tereblia, Teresva, Shopurka, and Tisza rivers as well as in the Tereblia reservoir.

Due to the fact that *T. souffia* populations of the Vişeu and Tisa rivers are interconnected and connections with populations from other Tisa tributaries on the Ukrainian side can be active as well (nevertheless, these connections should be studied

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based on genetic and other alternative methods), special measures to protect *T. souffia* and its habitats should be put into action by the Romanians and Ukrainian authorities, based on transboundary partnership programmes.

Conclusions

The state of characteristic *T. souffia* habitats in the Vişeu Basin, and consequently this species populations status are in majority average with only few reduced sectors, and no bad and good sectors from this point of view.

The principle found human impact types, which induced in the degradation of *T. souffia* habitat state in the Vişeu Basin were: poaching, minor changes in riverbed morphodynamics due to river engineering, disruption of water flow and natural sediment transport, destruction of riparian tree and shrub vegetation, habitat fragmentation/isolation of fish populations, organic pollution, and flood washing of fish.

All of these human impact types synergic effects induced a moderate decrease of *T. souffia* distribution and abundance in the last century in the Vişeu Basin.

The survival/thrive of *T. souffia* species populations in this sensible zoogeographical isolated area of its range will depend on an integrated basin long term monitoring and management programmes which should include as a base this study results and proposals.

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POTENTIAL DISTRIBUTION OF PERSIAN GAZELLE *(GAZELLA SUBGUTTUROSA SUBGUTTUROSA)* IN BAMOO NATIONAL PARK, SHIRAZ, IRAN: A PRESENCE-ONLY MODEL APPROACH

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Abstract. Using 30 *Gazella subgutturosa subgutturosa* presence-only data related to maximum entropy (Maxent) and ecological niche factor analysis (ENFA) models, but gained from field surveys, the present study was an attempt to assess how the Persian gazelle (*subgutturosa)* was distributed in Bamoo National Park located in Shiraz, Iran. While Maxent is thought to be predictive of satisfaction of environmental requirements, ENFA is considered as a tool for investigating the niche and habitat preferences of the species. All of the analyses by the models demonstrated the species distribution and the *subgutturosa* habitat. According to Maxent, vegetation, trough, and predator were witnessed to be most influential in the distribution, whereas ENFA revealed that elevation and vegetation were of highest value in terms of *Gazella subgutturosa subgutturosa* distribution. Interestingly enough, the predator variable was found to be highly contributing by Maxent but avoidable by ENFA. Also, the prediction level of the models turned out to be higher than chance occurrence under curve (AUC) = 0.5.
Keywords: *ENFA, Gazella subgutturosa subgutturosa, habitat distribution, habitat preferences, Maxent, presence-only data*

Introduction

To date, the issue of species distribution has gained much importance. According to Rebelo and Jones (2010), to gain an accurate piece of knowledge about how a given species is distributed is an area of investigation which is of high interest as far as conservation management is concerned. A careful review of the related literature reveals that a multitude of techniques has been developed to predict species distribution (e.g. Hirzel et al., 2002; Phillips et al., 2006). These techniques are, as Rebelo and Jones (2010) asserted, based upon the description of data related to the species in the form of presence or absence data in a set of sampled locations. It is the presence/absence data that facilitates the process by which the distribution of the species is predicted through the techniques or models (e.g. Osborne and Tigar, 1992; Brito et al., 1999; Carroll et al., 1999). Presence data, as Hortal et al. (2005) argues, usually correspond to the true

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presence of the species, but absences could be due to an insufficient sampling effort. Hence, false absences, according to Palmer et al. (2003), are much more common than false presences, and therefore there is a need for removing such inaccurate data from distributional maps (Palmer et al., 2003) and to assure the reliability of absences (Anderson, 2003). Zaniewski et al. (2002) argued that modeling based on presence–absence data is more likely to reflect the present natural distribution of a species (i.e. the realized niche), whereas presence-only methods are more likely to predict potential distributions, more closely resembling the fundamental niche.

The most frequently used and common techniques for assessing species distribution on the basis of occurrence-only records include kernel smoothing techniques, the Ecological-Niche Factor Analysis (ENFA) approach developed by Hirzel and Guisan (2002), and the Maximum entropy method (Maxent) introduced by Phillips et al. (2006). Interestingly enough, it has never been proven that any of these techniques outperforms its competitors. Zaniewski et al. (2002) comparatively evaluated the performance of General Additive Models and ENFA models and concluded that ENFA was a better candidate for detecting the potential distribution hot spots, especially if occurrence-only data was used. Jiménez-Valverde et al. (2008) argues that making a comparison between SDM models including potential and realized distribution ones could be a controversial issue.

According to a multitude of research studies, Maxent outperforms ENFA and GARP models (e.g. Phillips et al., 2006; Sérgio et al., 2007). Phillips et al. (2006) stated that the Maxent model yields better results even when it comes to small-size samples. Maxent, according to Phillips and Dudik (2008), is optimal for predicting within the realized niche, even though it should be used with caution when it is used to predict outside the realized distribution.

Rebelo and Jones (2010) assert that the results gained from their study support the use of presence-only modeling as an indispensable tool within any survey design as shown by the discovery of B. barbastellus populations outside of the previously known range, and ENFA seems to be more suited to determining a species' potential distribution. In contrast, Maxent is better suited to determining a species' realized distribution. It is more successful in predicting occurrence in previously unsurveyed areas and can be recommended as a technique for determining the conservative distribution for a species. Maxent modeling can aid biodiversity conservation, especially when it is obliged to develop survey plans or first assessments of a species' distribution.

In this study, we compared two presence-only modeling techniques, namely ecological niche factor analysis (ENFA) and maximum entropy (Maxent), in order to predict the Persian gazelle species' distribution. These techniques differ in their modeling approaches; Maxent is a complex technique for establishing a flexible relationship between the dependent and independent variables, and therefore it is theoretically more suited to predict the realized distribution of a species (the locations and range of environmental conditions in which a species actually lives). ENFA, on the other hand, is a presence-only method, but it reduces the shape of a species' response to an Eco-geographical Variable (EGV) to a normal distribution. The predictions it makes are generally closer to the potential distribution of a species (for a detailed discussion see Jiménez-Valverde et al., 2008).

The Persian gazelle (*G. subgutturosa subgutturosa*) is a sub-species of the goitered gazelle (*G. subgutturosa*) in the genus gazelle, family Bovidae and order Artiodactyla. This subspecies is distributed from eastern Turkey to Iran, Pakistan, Turkmenistan, and

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Central Asia (Kingswood and Blank, 1996). It is mow categorized as Vulnerable (VU) and if conservation efforts are not implemented for this species in the near future, this could change into the Extinction (EX) category (IUCN Red List, 2013).

Hence, the main objectives of this study were to: (i) investigate both modeling techniques in terms of their predictions and discrepancies; (ii) determine which environment factors are relevant for each model; and (iii) validate these modeling techniques by comparing predicted distributions with the results obtained from ground sampling.

Materials and methods

Study area

Bamoo National Park (BNP) covers an area of 46913 hectares and is located in the northeastern part of Shiraz, Iran (52°29' to 52°56' E, 29°39' to 29°50' N) (see *Fig. 1*). This park has been under protection since 1962. This park is covered by *Astragalus spp*, annual grass, and *Artemisia spp*. It is also characterized by mountainous areas and plains. Its maximum elevation equal 2700 m.

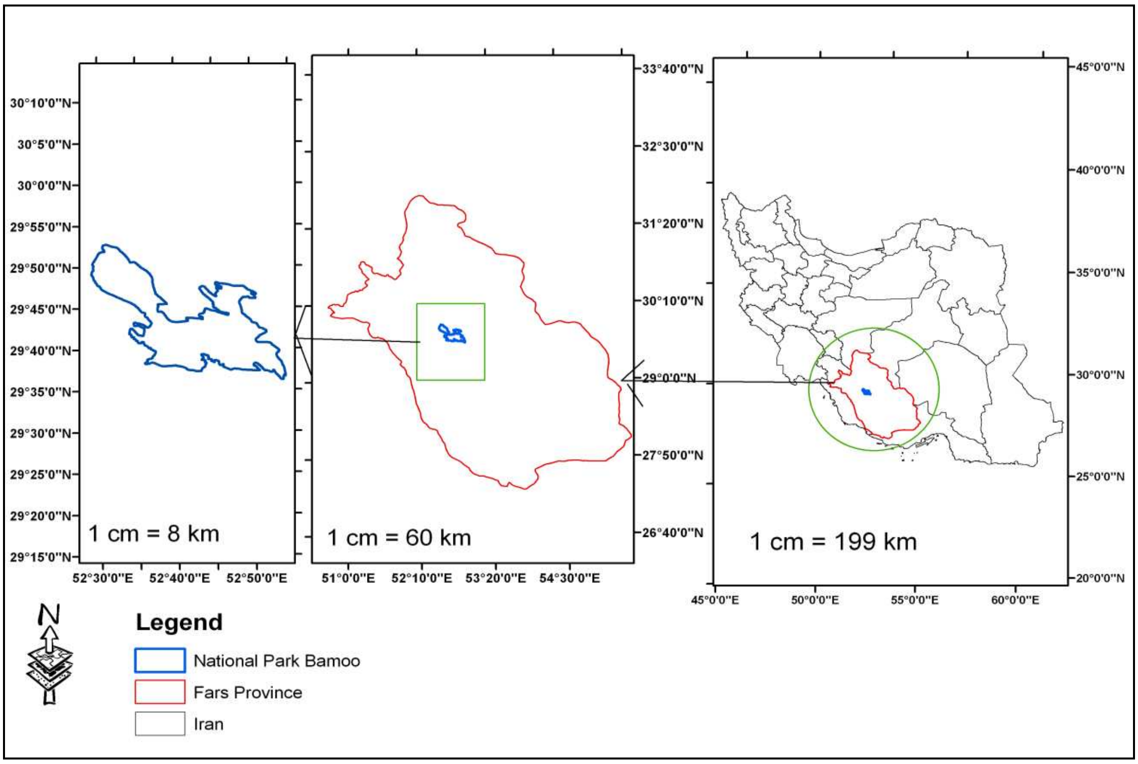

Figure 1. *BNP geographical map*

Target species and occurrence data

Thirty occurrence records of *Gazella subgutturosa* species collected in random systematic transect conducted in plain area of national park. Park ranger drive on line transect for 500 (m) and stop using binocular in 360 around to find out about the presence of Gazella. These data representing the total distribution of the species that are under severe threatening circumstances due to eco-geographical factors were obtained.

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Environmental variables

This study was based on such environmental variables as clouding, vegetation, trough, predator, soil structure, elevation, slope and road. As a potential predictor of the *Gazella subgutturosa* habitat distribution, a map of work layer (see *Fig. 2*) was also utilized.

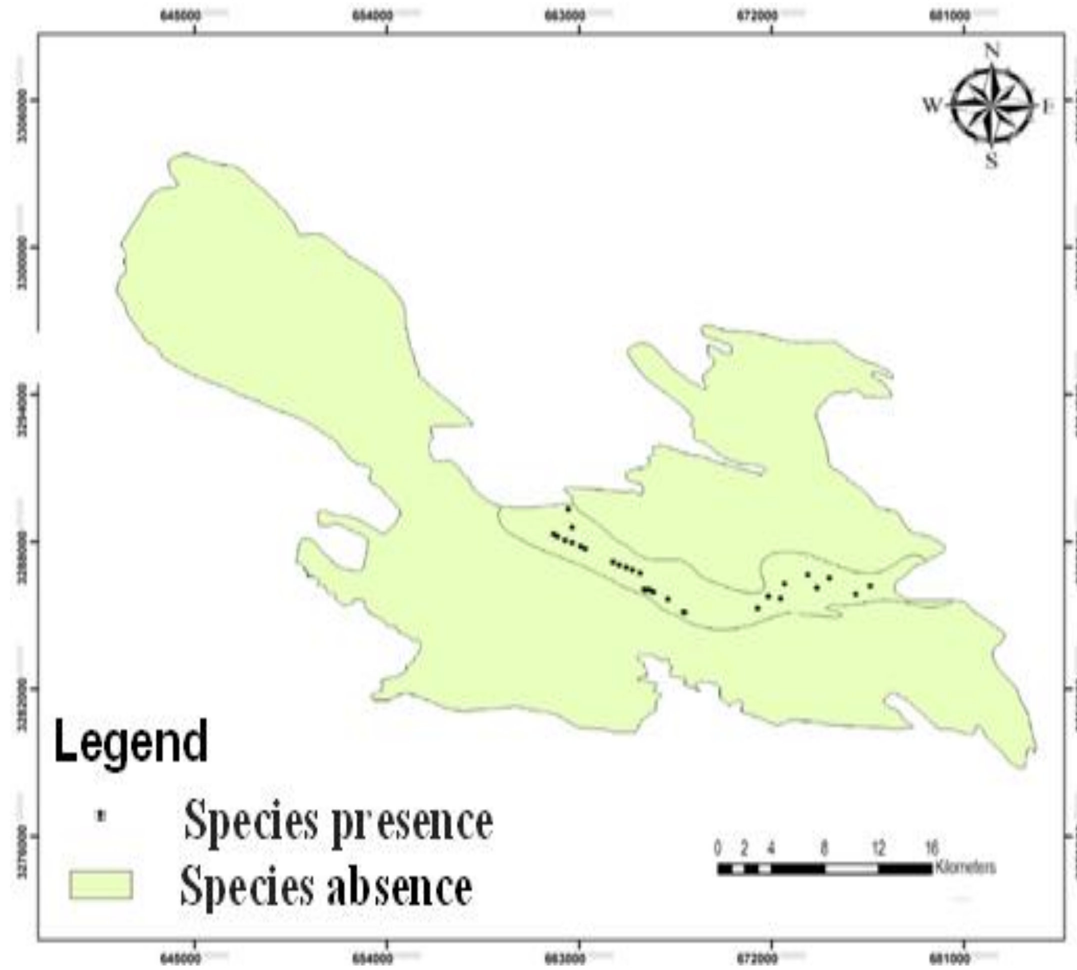

Figure 2. *Map points (GPS) of the gazelle distribution in Bamoo National Park*

These variables were chosen based on their biological relevance to animal species distributions and other habitat modeling studies (for example, Hu and Jiang, 2010; Bagherirad et al., 2010). Elevation (Digital Elevation Model; DEM) data were also obtained from the National Cartographic (IRNCC25K, 1 km spatial resolution). The DEM data were used to generate slope using Environmental Systems Research Institute's ARC GIS version 9.3. All environmental variables were resampled to 1 km spatial resolution. All the variables were tested for multicollinearity by examining the cross-correlations based on 30 localities species occurrence records as well as 30 randomly generated samples from the area. Only one variable from a set of highly cross-correlated variables (r > 0.75) was included in the model based on the potential biological relevance to the distribution of the species and the ease of interpretation.

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Modeling procedure

Maxent

Maxent has been found to perform best among many different modeling methods (Elith et al., 2006; Ortega-Huerta and Peterson, 2008). It is based on a machine-learning program that estimates the probability distribution for a species' occurrence based on environmental constraints (Phillips et al., 2006). It requires only-presence data (not absence) and environmental variable (continuous or categorical) layers for the study area. We used the freely available Maxent software (version 3.1 at http://www.cs.princeton.edu/~schapire/maxent/) which generates an estimate of presence probability associated with the species. The 30 occurrence records and 7 environmental predictors were utilized to model the potential habitat distribution for *Gazella subgutturosa*. As previously mentioned, 30 independent data points were used to test the reliability level of the model using a binomial test of omission to evaluate the statistical significance of the prediction.

Also, the importance of each environmental variable was evaluated through a heuristic estimation during the model training as well as a jackknife test that was conducted by initially excluding one variable in each run, running the model with only one variable, and finally including all of the variables in the model (Fielding and Bell, 1997). Afterwards, the performance of each model was assessed, and the response curves were created for each environmental variable. This showed how each variable influenced the logistical prediction by keeping the remaining variables at their average value. It should be emphasized that to make a change in one of the variables included in the model had a marginal effect.

The model was evaluated in light of the threshold-independent receiver operating characteristic (ROC) approach by calculating the area under the ROC curve (AUC) as the measure of the prediction success (Hanley and McNeil, 1982) . The ROC curve is obtained by plotting all true positive values (sensitivity fraction) against their equivalent false positive values (1-specificity fraction, Phillips et al., 2006). Analysis was performed 10 times to generate 95% confidence intervals.

ENFA

ENFA makes a comparison between the total area combinations of habitat variables available to the species and those of habitat variables at the locations where the species is found by means of two terms including marginality and specialization. Marginality is a measure of the separation between the optimal habitat combinations (those of the actual presence sites) and the average available environmental conditions within the study area (Liu et al., 2005).

Specialization contrasts the global distribution variance with the species habitat variance. It measures how restricted the species niche is in comparison with the habitat combinations available (Hirzel et al., 2002; Basille et al., 2008). ENFA is implemented in the Adehabitat package (Calenge, 2006) of R software (R Development Core Team, 2010). The analysis performs a principal component analysis by calculating the factors that have a biological meaning. Of the factors, the first one explains marginality (m), but the remaining ones explain the specialization (s). Larger values of marginality indicate that the species is not equally distributed in the environment and that the habitats utilized strongly differ from the average conditions in the study area (Hirzel et al., 2002; Basille et al., 2008). Alternatively, small values of specialization represent less restricted niches on some particular environmental variable (Basille et al., 2008) and high values of specialization mean that the species do not

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tolerate variation in that dimension. Adehabitat package also provides global marginality (M) and global tolerance (T) of the species to the habitat evaluated: the greater the marginality, the more the niche deviates from the available conditions; the smaller the tolerance, the more restricted the niche is, i.e. more specialized is the species. These global estimations, however, must be used cautiously as they only apply to the specific area covered in a specific study and assume that the environmental variables do not change over time (Hirzel et al., 2002; Basille, per. comm). Marginality, the specialization axes and tolerance were evaluated with Monte-Carlo tests to assess their significance after 999 replicates (Basille et al., 2008; 2009; Calenge and Basille, 2008). As in Maxent, the analysis was also evaluated by ROC approach. In order to perform this validation, ENFA was applied to a set of 200 random absence points created by Dismo package (Hijmans et al., 2010) of R software, avoiding land areas and areas nearby to presence points to minimize 'false' absences in these points. Then, the predicted suitability of both training and test data was contrasted with the ENFA results performed by the absence points by means of ROCR package (Sing et al., 2009) of R software.

Results

Given the results gained from the heuristic estimations of the Maxent model, the variables that make the most meaningful contribution to the model include vegetation (56%), trough (18.4%), and predator (12.1%) (*Table 1*). The jackknife test presented by *Figure 3* manifests that the variables mentioned above lead to the greatest results when considered in isolation, so they can be claimed to be more relevant for the *Gazella subgutturosa* distribution.

Table 1. *Relative contributions of the environmental variables to Maxent and ENFA models*

Variable	MAXENT percent contribution	ENFA				
		Marginality (100%) 71.18% S1	S2 (14.3%)	S3 (9.18%)	S4 (4.34%)	S5 (1%)
Vegetation	56.6	0.476	0.345	-0.123	0.043	0.067
Trough	18.4	0.637	0.354	0.316	0.105	0.185
Predator	12.1	-0.372	-0.248	0.031	0.041	0.059
Soil structure	2	0.326	-0.223	-0.128	0.063	0.196
Elevation	10.7	0.598	0.367	0.249	0.024	-0.108
Slope	0.2	0.241	0.162	0.026	-0.045	0.276
Road	0	0.243	0.126	-0.141	0.043	-0.063

On the other hand, the variables that cause the highest amount of gain when they are omitted include the road, slope, elevation and soil structure. The Maxent model indicates that the predicted suitability of the *Gazella subgutturosa* increases in zones where the vegetation range is 9, 10 and 12 with such dominant species as *Scariola orientalis, Astragalus gossypimus, Ebenus stellata, Achillea eriophora, Centaurea intricate, Ebernus stellata, Astragalus gossypimus, Astragalus rhodosemius, Stipa hohenackeriana, Helichrysum leucocephalum, Scariola orientalis, Astragalus fasciculifolius, Astragalus curviflorus, Astragalus cephalanthus and Astragalus susianus, Astragalus rhodosemius, Astragalus gossypinus, Astragalus cephalanthus, Astragalus fasciculifolius, Artemisia aucheri* as well as the distance to trough, and

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distance to predator. Also, the elevation range associated with the classes is 0-<5000 m, 5000-15000 m and 1600-1800 m, respectively (*Fig. 4*).

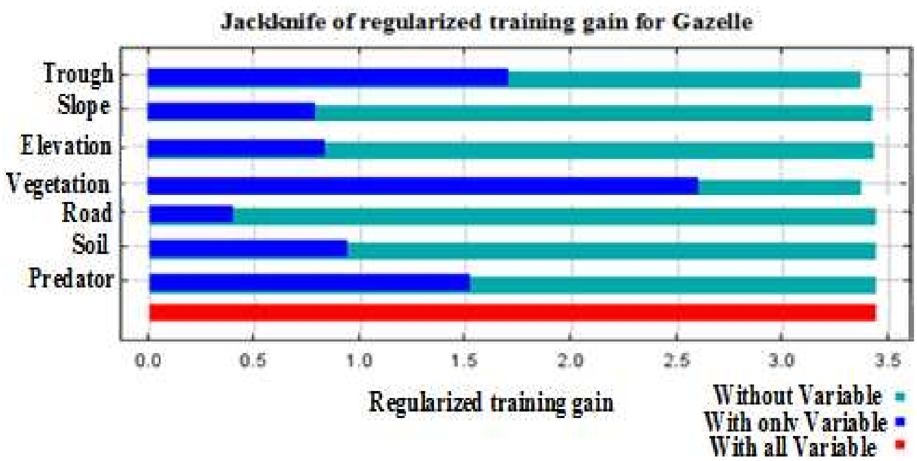

***Figure 3.** Results of the jackknife test of variable importance in the Maxent*

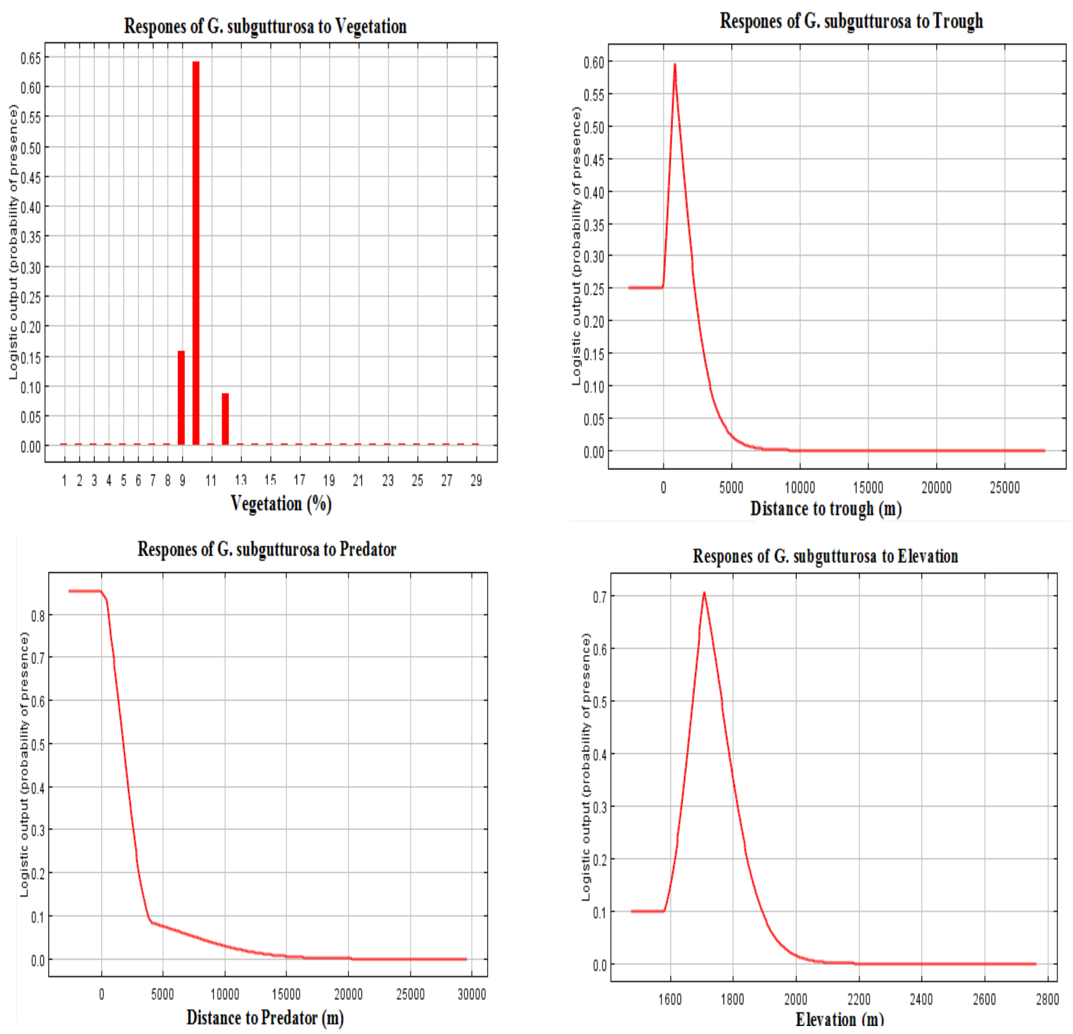

***Figure 4.** Response curves related to the most important variables of the Maxent model*

According to the model, the area under the curve (AUC) for the training data and the test data was 0.904 and 0.929, respectively (*Fig. 5*). This means that the model prediction is higher than the chance (AUC = 0.5). The map produced by the Maxent models is indicative of the areas having the best conditions for *G. subgutturosa* occurrence (*Fig. 6*), and it is congruent with the known distribution of *G. subgutturosa* (*Fig. 2*). In addition, several areas with higher probability of presence were identified in the marginal parts that are not yet under the influence of human activities.

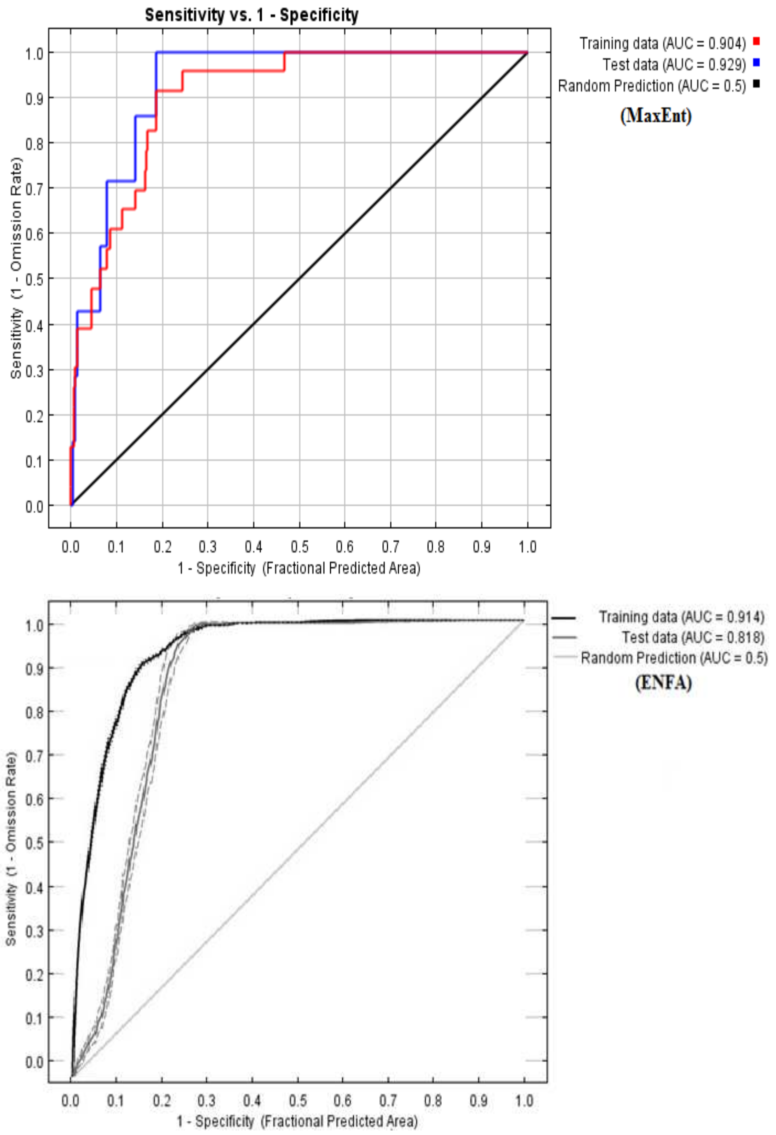

Figure 5. *Receiver operating characteristic curve using Maxent and ENFA models*

The global marginality estimated by ENFA was 1.35 (P = 0.001). It indicates that the area used by *Gazella subgutturosa* differs from the average conditions in the Bamoo National Park (*Table 1*) niche centroid shown in (*Fig. 7*) and that it requires a specific habitat niche. As for the importance of the variables, the analysis indicates that trough, vegetation and elevation are the main variables that contribute to marginality (*Table 1* and *Fig. 7*).

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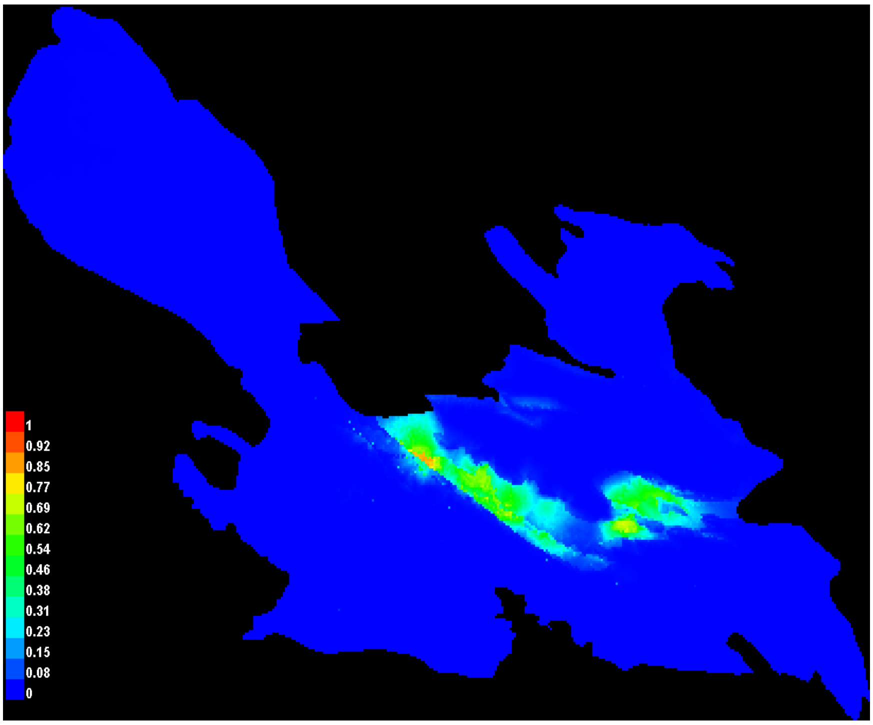

Figure 6. *Distribution map by Maxent model*

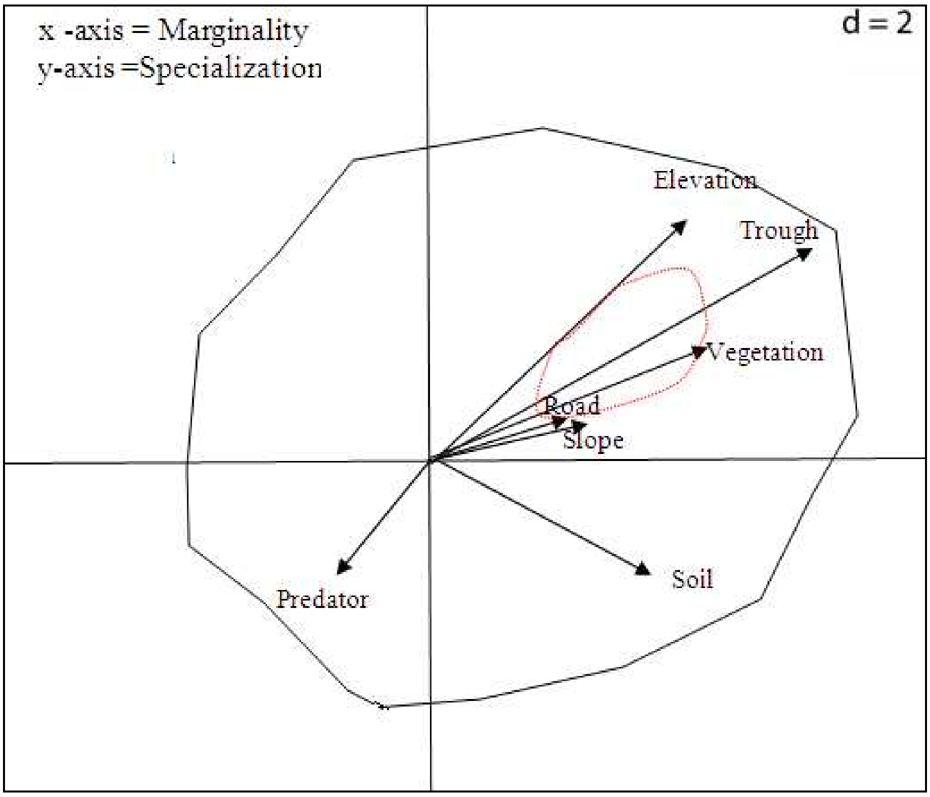

Figure 7. *Ecological niche display of G. subgutturosa*

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That trough, vegetation and elevation have large and positive values mean that *Gazella subgutturosa* prefers areas where these variables have an average greater than that of the environment (*Fig. 7*). Global specialization estimated by ENFA was 1.86 (P = 0.001) indicating that *Gazella subgutturosa* is a specialized species which has a narrow field of ecology.

Trough and elevation are the variables that have higher specialization coefficients, thus they are most critical in terms of habitat selection (*Fig. 7*). The AUC related to the training and test data was 0.914 and 0.818, respectively (*Fig. 5*) meaning that the prediction of the model is also better than the randomness (AUC = 0.5), so the ability of the Maxent model to predict suitability areas is inferior to that of the ENFA model.

Habitat suitability maps obtained by ENFA were classified into classes, namely suitable habitat, intermediate habitat and unsuitable habitat (*Fig. 8*). It shows the areas with the best-predicted conditions for *G. subgutturosa* occurrence. Map obtained by the ecological niche factor analysis showed that the gazelles were observed mostly in areas with vegetation types include annual grasses, *Astragalus spp*, *Artemisia sieberi*, and other pasture species that have provided suitable habitats for this species. The unsuitable habitats are located in the southern area of the BNK. This was incompatible with the known distribution of *G. subgutturosa* (*Fig. 2*).

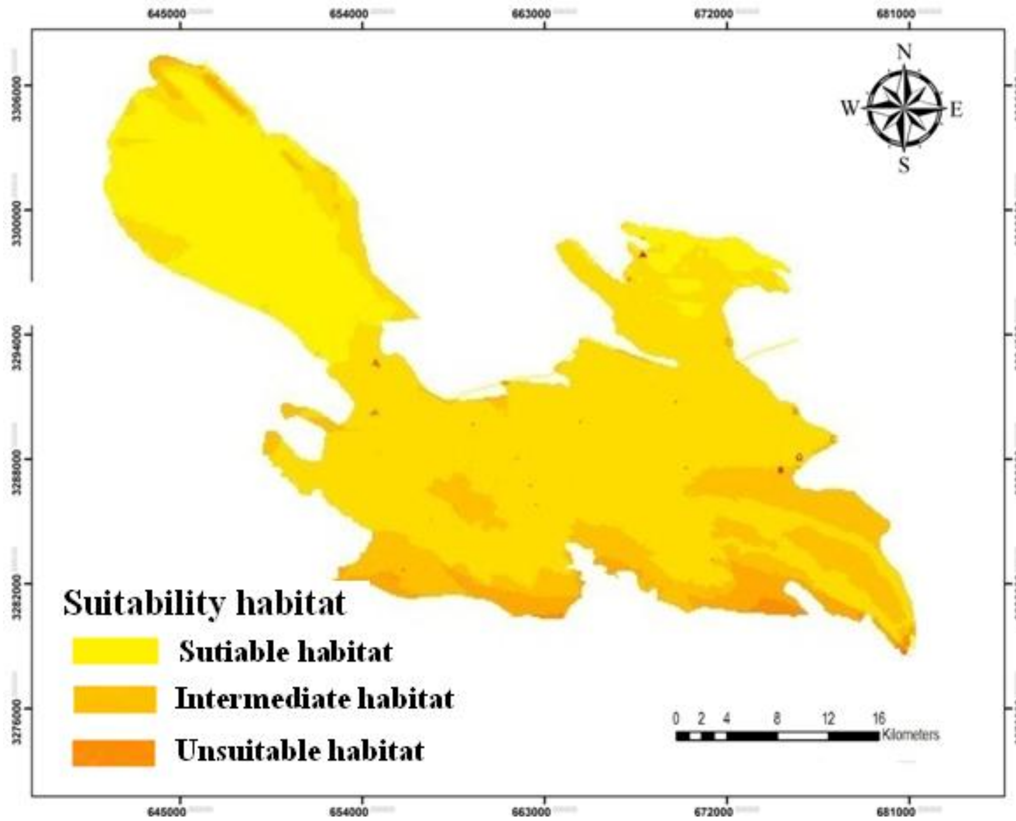

Figure 8. *Habitat suitability map by ENFA model*

As indicated by the figure above, the great polygons represent total available areas, but the small polygons represent used area (the niche centroid is located in the center of the small polygons). The arrows represent the projections of the environmental variables on the marginality and specialization axes.

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Discussion

Integrating multiple environmental variables and presence-only data, this study was an attempt to shed light on *G. subgutturosa* habitat preferences and distribution in the Bamoo National Park situated in Iran using such techniques as Maxent modeling and ENFA analysis. To date, some studies have been focused upon the local abundance of *G. subgutturosa* in light of presence-only data and ENFA model in relation to some environmental variables like elevation, slope, aspect, distance to road, distance to villages, distance to watercourse, distance to livestock herds, Normalized Difference Vegetation Index (NDVI), and predator (Bagherirad et al., 2014).

Maxent has achieved a robust performance showing a good accuracy with low sample sizes and an excellent predictive ability (Hernandez et al., 2006; Pearson et al., 2007; Wisz et al., 2008). The results gained from this study are in agreement with these studies, because, in terms of analysis comparison, ENFA model is more robust than Maxent as a predictive tool: both training and test AUC plots confirmed its high performance (*Fig. 5*). The test AUC of the Maxent had a lower value, although test data included the entire area that was incorporated in the training data. In contrast, ENFA had a lower fitness because the absence model was built using random points that covered most of the total available habitat, thus the model could not perform adequately. In addition, Maxent did not predict broad areas of distribution for the *G. subgutturosa*. Maxent proved to be robust at this scale which allowed the discovery of new populations and the extension of known distribution. In fact, Maxent and ENFA had different levels of success in predicting the occurrence of new populations.

Zaniewski et al., (2002) showed that ENFA produced accurate results although it had a tendency to overestimate the spatial extent of distributions, especially on the periphery of ranges (Brotons et al., 2004). Our results showed a similar pattern. A broad and accurate area of high-suitability was identified in the regions of BNK. ENFA was shown to be inaccurate in its predictions outside the geographical range of the training data.

It is important to understand why these two techniques yielded such different results in the discovery of new populations. Tsoar et al. (2007) concluded that more complex techniques (i.e. Maxent) are better predictors than the simple ones as they establish more flexible relationships between the dependent and independent variables. In fact, models that have no predefined shape of response curves can build models closer to the training data such as those based on smoothing techniques like Maxent (Randin et al., 2006). On the other hand, parametric methods like ENFA are limited by the normal distribution making them more sensitive to bias or extrapolations (Elith et al., 2006; Randin et al., 2006). Overall, Maxent seems prone to over fitting presence data (Peterson et al., 2007), so it is more likely to develop omission errors or false absences (the species exists in low suitability areas) while ENFA seemed to have greatest problems in reducing the commission error rate or false positives; it predicts occurrence where the species does not exist in areas outside the range of the training data. Both analyses agreed that trough, vegetation and elevation are variables relevant to *G. subgutturosa* spatial distribution and habitat preferences (*Figs. 3* and *6*), although they showed a small discrepancy in terms of the remaining variables. For instance, vegetation, trough, predator and elevation contribute most to the Maxent model (97.8%), while the road and slope make a 0.2% contribution (*Fig. 2*). Gazelles are known to eat a variety of grasses, forbs, and shrubs during the different seasons (Olson et al., 2010; Xu et al., 2012). Persian gazelles feed mostly on Chenopodiacea, Gramineae, and forbs which are comprised of 38.8% to 85.1% (Karami et al., 2002; Wenxuan et al., 2008). In previous studies by Vallentine (2000) and

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Bagherirad et al. (2010), a positive relationship between these plant families and gazelles was observed. Although the Persian gazelles used all vegetation types, Types I and II were the most suitable habitats because of the higher density of annual forbs and grasses such as *Astragalus spp, Bromus tectorum, Trigonella arcuata*, and *Eremopyrum bonaepartis* and shrub species such as *Artemisia herba-alba, Anabasis aphylla, Salsola rigida,* and *Aellenia sp*. Vegetation type I in the low steppe area of the habitat was located at less than 1100 m, but type II at 1100-1200 m elevation (Bagherirad et al., 2010). Using the Maxent model, Hu and Jiang (2010) showed that slope made a small contribution to the model development in terms of predicting the potential distribution of the endangered Przewalski's gazelle. Predator, on the other hand, made a significant contribution; However, it does seem to be a variable relevant (in terms of marginality and specialization) for the *G. subgutturosa*. These are reflections of the differences between both analyses. Although the ENFA model deals with spatial distribution, the Maxent counterpart focuses on habitat preferences. Therefore, in the former, a measurement of the distance to the predator is essential to the spatial distribution of the species, because the low distance of the predators is in direct relationship with high-risk areas of the *G. subgutturosa.* On the other hand, the predator is influential in its habitat preferences, which make sense because *G. subgutturosa* species should not select its habitat according to predator values. The results gained from this study are not suggestive of a major effect of the road on the *G. subgutturosa* distribution. In fact, the road was discarded during preliminary evaluations due to its low contribution to the analyses (Bagherirad et al., 2014). The ENFA analyses indicated a high marginality (1.40) and low tolerance (0.46) scores, suggesting a strong tendency for the species to live in a particular habitat throughout Golestan National Park as the context of the study. The interpretation of the factors in terms of its EGVs turned out to be very consistent with the experience of field specialists. In particular, the EGVs that correlated with the marginality factor were precisely those that were most often particularly relevant for the ecology of the gazelles.

The narrow distribution of the *G. subgutturosa* in the study area suggests that the species prefers a small range of environmental factors for their habitat confirmed by the high marginality and specialization factors. Recent studies about the interaction between human activities and the presence of the gazelle have shown that a negative relationship exists between the presence of the species and most types of human interference (Wangdwei and Fox, 2008; Hu et al., 2010; Ying et al., 2009; Ying et al., 2010; Bagherirad et al., 2014) whereas in our study the reverse was true. The road variable of the BNP with low marginality and specialization factors encompassed the main human activities and it was positively related to the occurrence of the *G. subgutturosa.* Previous studies have shown the effect of vegetation on *G. subgutturosa* habitat preferences, gazelles preferred an intermediate range of vegetation productivity, presumably facing quality-quantity trade-offs where areas with low NDVI are limited by low ingestion rates, and areas with high NDVI are limited by the low digestibility of mature forage (Mueller and Fagan, 2008).

Conclusion

In this study, we attempted to investigate the distribution of the species in the Bamoo National Park in order to develop a robust model based on environmental variables which are capable of predicting its distribution. Maxent and ENFA models helped identify habitat preferences and spatial distribution patterns of *G. subgutturosa*. Main

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environmental variables related to them are congruent with other life species in previous studies. To Maxent, vegetation, trough and predator are the variables most relevant to its spatial distribution; whereas to ENFA, trough, elevation and vegetation are the key variables in terms of habitat preferences. Also predator is a variable from which *G. subgutturosa* runs away. Maxent can successfully predict *G. subgutturosa* spatial distribution with the capability to evaluate which variables are more important to its distribution. This could be very useful in understanding the distributional patterns and geography of the species. In the future, this model can be applied in a wider geographical area to locate other habitable areas for *G. subgutturosa,* to add information about more or less suitable areas for the species and help elucidate the possible presence of the *G. subgutturosa* in regions where the actual identity of the species is uncertain.

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THE ROLE OF *HALOXYLON* PLANTATIONS IN IMPROVING CARBON SEQUESTRATION POTENTIAL OF SAND DUNES OF IRAN

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Abstract. Rehabilitation of desertified land in semi-arid and arid regions through *Haloxylon* plantations has a great potential to increase carbon sequestration. In this study, carbon distribution and sequestration were examined in different parts of *Haloxylon spp.* and depths of soil surface. Afterward, the economic value of carbon sequestration in the *Haloxylon* plantation was estimated. In order to investigate vegetation variables, a systematic random method with 10 nested plots was applied. Plant properties including diameter at breast height, tree height, height to crown, and the small and large diameters of the crown were measured. Tree and soil sampling was conducted in 10 × 10 m^2 and 5 × 5 m^2 plots, respectively. Soil was sampled at 0-15 and 15-30 cm depths of *Haloxylon* plantation and control area. Litter were harvested at1 × 1 m^2 plots. Algometric equations and Walkley-Black method were used to determine plant biomass and soil organic carbon sequestration. The results showed that planting *Haloxylon* increased carbon sequestration by up to 24.46 ton/ha compared to the control area. Economic value of carbon sequestration in the *Haloxylon* plantation was estimated at $3.74 million. Carbon was mostly sequestrated in the branches and roots. Carbon sequestration in different parts of the plant was calculated as 16.6 ton/ha (54% of total sequestration). Soil organic carbon sequestration was computed as 13.9 ton/ha (46% of total sequestration). *Haloxylon* species has a high potential for carbon sequestration. Nevertheless, the species used in rehabilitation of desertified lands need to be capable of maintaining other resources, especially water resources.
Keywords: *carbon sequestration, economic value, Haloxylon plantation, drylands, sand dunes, Ala region*

Introduction

Throughout the history of the Earth, the planet's surface has transformed dramatically due to various organic and inorganic phenomena (Mahdavi et al., 2011). Climate change and global warming are among the serious challenges to sustainable development and pose negative impacts on aquatic and terrestrial ecosystems (UNDP, 2000). A number of scientists have identified increased atmospheric concentrations of greenhouse gases (GHGs) to be responsible for climate change and global warming

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(Brooks, 1998). GHGs may have natural and human-caused sources. On the other hand, their levels can be reduced through chemical changes in the atmosphere or absorption of the gases by sinks. Each GHG has a particular lifetime and percentage contribution to the greenhouse effect.

Carbon dioxide (CO_2) has the greatest global warming potential (GWP) among all GHGs and is thus generally considered as the main determinant of the effects of GHGs on global warming (Hashemi and Karvani, 2010). Atmospheric GHGs, particularly CO_2, methane (CH_4), and nitrous oxide (N_2O), emitted from both natural and human-caused sources, absorb infrared radiation, generate heat, and consequently increase the temperature of the troposphere (the lowest portion of the Earth's atmosphere). While CO_2, accounting for 81% of GHG emissions, is produced during the combustion of petroleum products, natural gas, coal, and all other fossil fuels, CH_4 (making 10% of emissions) comes from landfills, coal mines, oil and natural gas operations, and agriculture. On the other hand, N_2O (responsible for 5% of emissions) is the result of using nitrogen fertilizers, burning fossil fuels, and industrial and waste management processes (Shuman, 2011). Two types of strategies, i.e. emission reduction and GHG capture, have been proposed to counter the greenhouse effect. Typical examples of emission reduction are improving energy saving technology and renewable energy development. GHG capture methods mainly involve carbon capture and storage and afforestation.

Numerous researchers have suggested afforestation and forest management as effective countermeasures against global warming (Srivastava et al., 1993; Silver et al., 2000; Kumar et al., 2001; Niles et al., 2002). According to the United Nations Framework Convention on Climate Change (UNFCCC, 2006), afforestation is effective for carbon mitigation. The emission reductions that are attributable to an afforestation or reforestation project activity (called "net anthropogenic greenhouse gas removals by sinks") can be calculated as the "actual net GHG removals by sinks" minus the "baseline net GHG removals by sinks" minus "leakage" in five carbon pools (aboveground and below-ground biomass, litter, dead wood, and soil organic carbon pools). Of these five carbon pools, the aboveground and belowground biomass will change rapidly after afforestation. Based on the Clean Development Mechanism and Joint Implementation (CDM/JI) guidelines set by the UNFCCC, Suganuma et al. (2012) evaluated the sequestered carbon amount (as carbon credit) following the afforestation of a 45×50 km^2 arid area. They found that carbon mitigation amount by the applied arid land afforestation was equivalent to 0.88% of CO_2 emission in Japan in 2008 (Suganuma et al., 2012).

Carbon sequestration is the process through which atmospheric carbon dioxide is absorbed, stored, and deposited as carbohydrates in plant tissues (Abdi et al., 2008). In addition to synthesis of carbon compounds by plants, the viability and stability of carbon in plant tissues are also critical. In fact, as the decomposition rate of carbon compounds decreases, carbon sequestration in the ecosystem increases. Therefore, since the lowest rate of decomposition is observed in arid areas where there is too little moisture, such areas are important in terms of carbon sequestration (Gao et al., 2007; Ardo and Olsson, 2003). Drylands, many of which may have degraded soils, cover about 40% of the global land area (FAO, 2000). Although dryland soils are commonly low in carbon content, the above-mentioned facts may suggest their high potential for carbon sequestration (Scurlock and Hall, 1998; Rosenberg et al., 1999).

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Desertification is defined as land degradation in arid, semi-arid, and dry sub-humid areas due to various reasons such as climate change and human activities (UNEP, 1990). Land desertification can damage soil structure and reduce soil aggregates. This will affect the global carbon cycle through reducing the total soil carbon pools and increasing the emission of CO_2 from the soil and vegetation into the atmosphere (Lal, 2001; Jabro et al., 2008; Yu et al., 2007). Su et al. (2010) compared soil carbon sequestration at 0-30 cm depths of rehabilitated desert lands and a control area in northwest China. They reported that the values reached 1.8-9.4 and 7.5-17.3 Mg/ha over 7- and 32-year rehabilitation periods, respectively. They hence concluded that desertification control could be beneficial to soil carbon sequestration and soil quality improvement (Su et al., 2010).

Amani and Maddah Arefi (2003) estimated the amount of carbon sequestrated in aerial biomass of 1.5×10^6 ha of Iran's desert lands planted with *Haloxylon* at about 5.7 million tons. They believed that considering equal amount of carbon sequestration in the underground organs and soil, total carbon sequestration would reach about 15 million tons.

Research has indicated significantly higher carbon concentration in samples from natural forests than in those from plantations (Elias and Potvin, 2003). Haghdoost et al. (2012) compared destroyed natural *Quercus castaneifolia* forests with *Acer velutinum* and *Alnus subcordata* plantations in Chamestan region (Mazandaran Province, Iran). They found soil carbon sequestration to have increased by up to 33.61 ton/ha in *Acer velutinum* plantations and to have decreased by up to 20.55 ton/ha in *Alnus subcordata* plantations. All previous measurements have confirmed the difference in carbon concentrations among various species, organs, treatment types, drying temperatures, provenances (natural/plantation), and climates. In addition, studies have assessed carbon concentrations of tree components by age not only in China (Zhang et al., 2009) but also in other parts of the world. Fu et al. (2013) ranked carbon concentration of aboveground tree organs as living branch > bark > foliage > dead branch > stem. In the ranking of belowground tree organs, large and small roots had the highest and lowest carbon concentrations, respectively (large roots > stumps > thick roots > medium roots > small roots). They concluded that despite the significant differences in carbon concentrations of various tree organs, trees of unalike ages were not significantly different in this regard. Forozeh et al. (2008) compared carbon sequestration potential of three shrub species, namely *Helianthemum Fire Dragon, Dendrostellera lessertii*, and *Artemisia sieberi* in the arid rangelands of Iran. According to their findings, *Artemisia sieberi* had the greatest carbon sequestration potential. Moreover, among the four tree parts (leaves, branches, stems, and roots), stems and leaves had the highest and lowest potential, respectively.

The rate of carbon sequestration is much lower in young areas than in old areas. Moreover, organic carbon concentrations in sand dunes elevate as the age of *Haloxylon* plantations increases. For instance, high carbon sequestration (15 ton/ha) was observed in a 41-year-old *Haloxylon* plantation (Sarparast et al., 2013). On the other hand, conifers have greater carbon sequestration potential compared to broad-leaved plants (Abbas Nejad and Khajodin, 2012).

Biomass is the basis for estimating the cost of carbon sequestration (McDicken, 1997). Masses of *Acacia* and *Fraxinus excelsior* (covering 207 and 90 ha in Cheetgar Park of Tehran, Iran) increased carbon sequestration by 99877.5 and 12600 ton, respectively. Since the average cost of these masses is $200 per ton, the economic value

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of carbon sequestration is about 20 and 2.5 million dollars in these pilot areas, respectively (Varamesh et al., 2011).

The present study aimed to investigate carbon sequestration potential of different parts of *Haloxylon spp*. and various soil depths of *Haloxylon* plantations. It also sought to estimate the cost of carbon sequestration through rehabilitation of sandy desertified lands in Ala region of Semnan Province, Iran.

Methods

Introduction to Haloxylon species (saxaul)

Haloxylon ammodendron (saxaul) belongs to the Amaranthaceae (Salsoloideae subfamily) family placed in the Caryophyllales order. The genus exists in shrub or small tree forms with very small leaves that join in the base. It resembles conifers due to its needle-like leaf appearance. Two major species of *Haloxylon* are *Haloxylon aphyllum* (black saxaul) and *Haloxylon persicum* (yellow or white saxaul). Studies on natural and artificial saxaul lands have shown that the plant can live only as long as 15-25 years depending on species and germination conditions. In fact, older saxaul plantations require more germination (Jafari et al., 2009). *H. aphyllum* and *H. persicum* are dominant plants in the deserts of the Irano-Turanian region (Pyankov et al., 1999). Stabilization of Iran's sand dunes with vegetation was initiated in October 1959. *Haloxylon* plantations are currently covering an area of about 2 million ha (Baghestani Meybodi et al., 2006). In Ala region of Semnan Province, planting *Haloxylon* and rehabilitation of sandy desertified land started in 1974 and germination was performed in 1990 (*Fig. 1*).

Figure 1. *Haloxylon plantation, gaps, and underground water level changes (increase and decrease)*

Site description

The study area (35°32′0″N 53°30′5″E/35°33′50″N 53°30′49″E) covered 766 ha of Semnan Plain. It was located on a playa and had a geomorphological structure containing clay plains and sand ripples (*Fig. 2*). The mean temperature and mean absolute minimum and maximum temperatures during 1965-2005 were 18.4°C, -0.4°C

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(in January), and 37.7°C (in July), respectively. The mean precipitation was 140.8 mm in the same period.

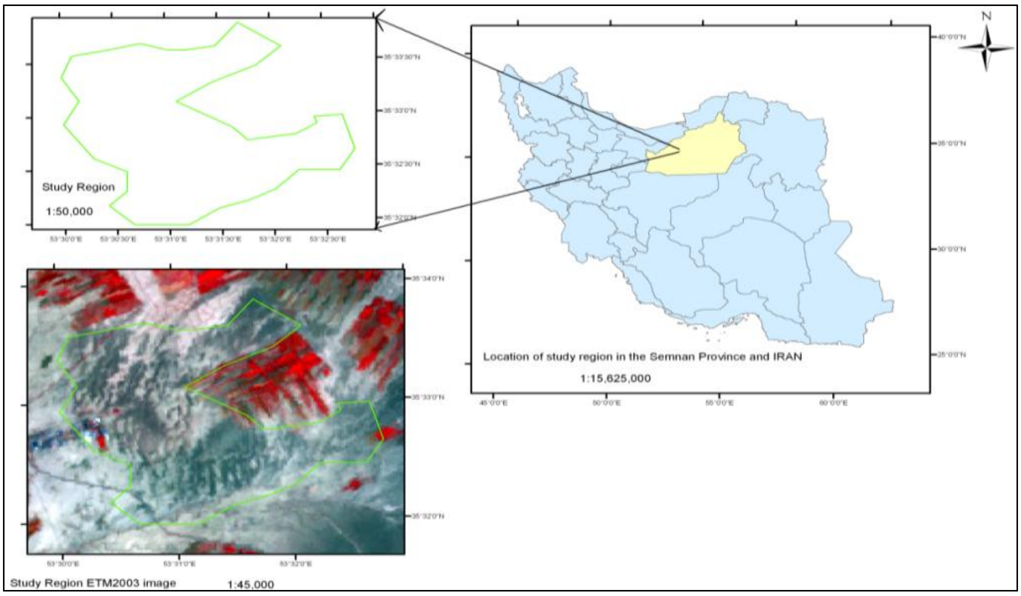

***Figure 2.** Map of the study area and its location in Semnan Province, Iran*

De Martonne's index (De Martonne, 1926) was used for climate classification based on mean annual precipitation and temperature through the following *Equation 1:*

$$I = P/T + 10 \qquad \text{(Eq. 1)}$$

where I, P, and T are de Martonne's aridity index, annual precipitation (in mm), and annual temperature (in °C), respectively. Using the previously mentioned values, I was calculated as 4.9 for the study area, i.e. the area had an arid climate during the study period.

Sampling method

In this study, vegetation variables were examined through a systematic random method with 10 nested plots. Six plots were used for each mass. The plot sizes were 10 × 10 m^2, 5 × 5 m^2, and 1 × 1 m^2 for tree, soil, and litter sampling, respectively (*Fig. 3*). Moreover, in order to reduce edge effects, a few rows around each mass were not sampled and the samples were mostly collected from the center of the masses. Both *Haloxylon* plantation and the adjacent lands (control area) were sampled. Five parallel transects with two square nested plots on each were placed on the study area. The first transect was established randomly and the subsequent transects were placed with a specific distance based on the area of the region. In the 10 × 10 m^2 plots, *Haloxylon* characteristics including diameter at breast height (DBH), tree height (H), height to crown (Hc), and the small and large diameters of the crown (D_s and D_l, respectively) were measured. Soil was sampled from 0-15 and 15-30 cm depths of the four corners of the 5 × 5 m^2 plots. The obtained samples were then mixed to form a single sample for each plot (Gao et al., 2007). Moreover, all the litter over the 1 × 1 m^2 plots of each mass

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was collected and weighed. Afterward, 20-g samples were packed in plastic bags and transported to the laboratory to determine moisture and carbon percentage (McDicken, 1997). Due to the absence of vegetation on the control area, only soil was sampled.

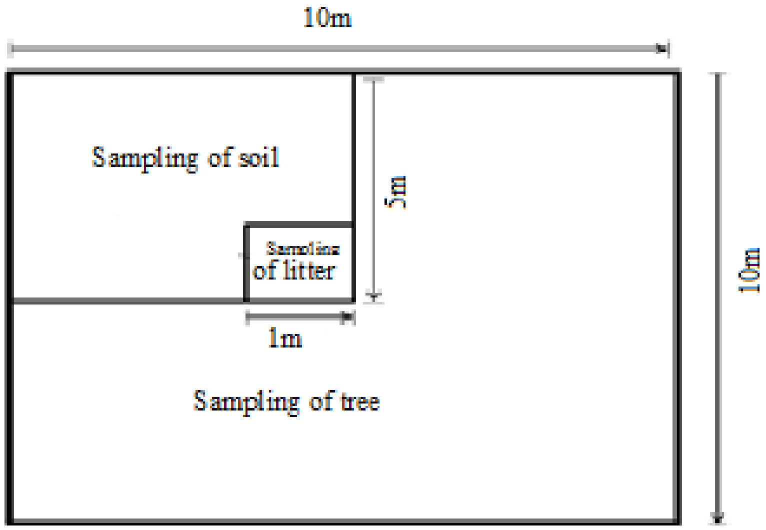

Figure 3. *The size of plots used to measure each of the plant variables*

Biomass calculation

Trunk volume, canopy structure and volume, and aerial and underground biomass of *Haloxylon* were calculated based on the method proposed by Hernandaz et al. (2004). First, *Equations 2, 3,* and *4* were used to obtain tree basal area, volume, and biomass (per kg), respectively:

$$A_b = \pi \times r^2 \quad \text{(Eq. 2)}$$

$$V = A_b \times H \times K_c \quad \text{(Eq. 3)}$$

$$Biomass = V \times WD \times 1000 \quad \text{(Eq. 4)}$$

where $\pi = 3.14$, r = radius of the tree (m), and $K_c = 0.54$. A_b, V, Kc, H, and WD are basal area of the tree (m^2), tree volume (m^3), tree height, and density (g/cm^3), respectively.

In order to save time and costs and to avoid destructive sampling methods for estimating root biomass of *Haloxylon spp*, root biomass of *H. aphyllum* and *H. persicum* species were calculated using *Equation 5* and considering 83% and 93% of aerial biomass as root biomass, respectively:

$$BGB = AGB \times K \quad \text{(Eq. 5)}$$

where AGB and BGB are aerial and underground biomass, respectively. K is percentage of aerial biomass.

Canopy volume of *Haloxylon spp*. was calculated using *Equation 6:*

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$$V\left(m^3\right) = \left(\pi \times D_b^{\,2} \times H_c\right)/12 \tag{6}$$

where D_b is $(D_l + D_s)/2$.

Laboratory methods

In the laboratory, samples of trunks, branches, roots, and litter were dried in the oven at 105°C for 24 h and their moisture content was determined. The samples were then thoroughly ground. Afterward, three 2-g quantities of each sample were combusted in an electric furnace (500-600°C) for three-four hours. The burnt samples were dried in a desiccator and their ash weight was recorded. As the initial and ash weights were both available, the organic carbon percentages of tree organs and litter were separately computed based on the ratio of organic carbon to organic materials in *Equation 7:*

$$OM = 1/2OC \tag{Eq. 7}$$

where OM and OC are organic material and organic carbon, respectively.

Organic carbon percentage was determined by Walkley-Black method (1934). Organic carbon was oxidized using potassium dichromate (K_2Cr_2O) in an acidic environment full of H_2SO_4. The remaining potassium dichromate was then titrated by 0.1 normal sodium thiosulfate ($Na_2S_2O_3$) in the presence of potassium iodide reagent (KI).

The experiments were performed in both the presence and absence of soil samples. Therefore, 0.5-2 g dried soil (depending upon the amount of organic matter in the sample) along with 16 cc concentrated H_2SO_4 and 10 cc potassium dichromate 1 normal were poured into 100-cc Florence flasks and heated for 90 min at 120°C. The remaining amount of potassium dichromate was determined using iodometry, i.e. 25 cc of the flask contents (diluted with distilled water to reach a volume of 100 cc) along with 2 g potassium iodide was poured into 250-cc Erlenmeyer flasks. After adding 2 g potassium iodide, the obtained mixture was titrated with 0.1 normal sodium thiosulfate. The amounts of thiosulfate used for samples and controls were recorded at each stage of the experiment. Organic carbon percentage was then calculated using *Equation 8:*

$$OC\% = 4\left(A-B\right) \times 100 \times 3/S \times 1000 = 0.12\left(A-B\right)/S \tag{Eq. 8}$$

where OC, A, B, and S are organic carbon, amount of sodium thiosulfate applied to controls (cc), amount of sodium thiosulfate applied to samples (cc), and sample weight (g), respectively. In this equation, multiplying A-B by four reflects the fact that the real samples (25 cc) constituted one-fourth of the tested samples as they were diluted with distilled water to reach a volume of 100 cc. The value 3 in the equation represents carbon equivalent.

Finally, soil organic carbon content (kg/ha) was computed using *Equation 9:*

$$OC = 1000 \times OC\% \times B_d \times E \tag{Eq. 9}$$

where OC, OC%, B_d, and E are organic carbon (kg/ha), organic carbon percentage, soil bulk density (g/cm^3), and sampling depth (cm), respectively.

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The economic cost of carbon capture and storage was estimated by considering $200 per ton of carbon as previously suggested by Varamesh (2009). All required diagrams were drawn in Microsoft Excel 2007.

Results

Soil carbon sequestration

According to our findings, planting *Haloxylon* in Ala region of Semnan Plain (during 1974-2012) significantly increased soil carbon sequestration. The total sequestered carbon in the study and control areas were 13.9 and 5.9 ton/ha, respectively. Thus, planting *Haloxylon* in this region increased carbon sequestration by up to 8 ton/ha compared to the control area (*Fig. 4*).

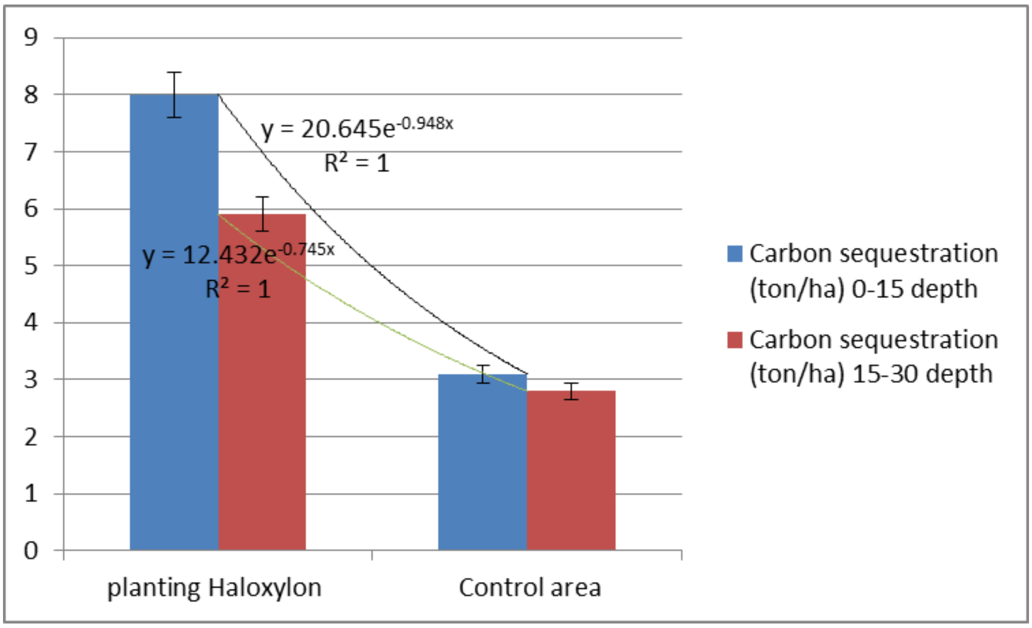

Figure 4. *Carbon sequestration at 0-15 and 15-30 cm depths in Haloxylon plantation and control area (statistical significance at the 0.05 level)*

Comparison of mean values obtained from 0-15 and 15-30 depths of soil showed the greatest amount of soil carbon sequestration at 0-15 cm depth of the *Haloxylon* plantation. Moreover, in both areas, carbon sequestration was higher at 0-15 cm depth than at 15-30 cm depth. However, the difference was not significant in either area (*Fig. 4*).

Carbon sequestration in plant biomass

Due to lack of vegetation in the control area, only plant biomass data related to the *Haloxylon* plantation is presented in *Table 1*. As it is seen, the highest and lowest amounts of plant biomass were detected in canopy and trunk, respectively. In addition, carbon sequestration in aerial biomass (total biomass of trunk and canopy) and litter was 7.1 and 3.5 ton/ha, respectively.

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Table 1. *Measurements of plant biomass (ton/ha) in the Haloxylon plantation (all values were equal to zero in the control area)*

Attribute	Value (ton/ha)
Trunk biomass	1.51
Canopy biomass	12.65
Root biomass	11.72
Total biomass	26.7
Litter biomass	8.1
Carbon sequestration in aerial biomass	7.1
Carbon sequestration in underground biomass	6.0
Carbon sequestration in litter biomass	3.5
Total carbon sequestration in plant biomass	16.6

Total carbon sequestration

Total carbon sequestration per unit area in the *Haloxylon* plantation and the control area (wasteland) was 30.36 and 5.9 ton/ha, respectively ($P > 0.05$) (*Table 2*). Therefore, the planted *Haloxylon* species increased carbon sequestration by up to 24.46 ton/ha.

Furthermore, the highest and lowest carbon sequestration levels were related to branches and trunks, respectively. In general, carbon sequestration in various plant parts summed to 16.6 (54%). On the other hand, soil carbon sequestration reached a total of 13.9 ton/ha (46%) in the first and second depths (0-15 and 15-30 cm) of the whole *Haloxylon* plantation. Hence, maximum and minimum carbon sequestration occurred in soil and litter, respectively (*Table 2*).

Considering insufficient vegetation in the control area, no atmospheric carbon sequestration had occurred in this area. Therefore, only 5.83 ton/ha carbon was sequestrated by soil. In each hectare of the control area (wasteland), 3.1 ton (53%) and 2.8 ton (47%) organic carbon were sequestrated at 0-15 and 15-30 cm depths of soil, respectively (*Table 2*).

Table 2. *The rate of carbon sequestration in different parts of the Haloxylon plantation and control area*

	Parameter		Carbon sequestration (ton/ha)	Carbon sequestration percentage
Haloxylon plantation	Plant part	Trunk	0.74	3
		Branch	6.16	21
		Root	5.83	19
		Litter	3.73	12
	Soil depth (cm)	0-15	8.00	26
		15-30	5.90	19
	Total		30.36	100
Control area	Soil depth (cm)	0-15	3.1	52.5
		15-30	2.8	47.5
	Total		5.9	100

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Discussion

Haloxylon species are halophytic, psammophytic, and xerophytic plants. They are in fact considered as the most compatible species among desert and semi-desert plants and are hence widely used in sand dune stabilization (Safarnezhad, 2005). The present study revealed that planting sand dunes of the Ala desert area with *Haloxylon* led to high potential for carbon sequestration and could increase soil carbon sequestration by 8 ton/ha compared to the control area. Likewise, Amani and Maddah Arefi (2003), Sarparast et al. (2013), and Su et al. (2010) confirmed that soil carbon sequestration increased in *Haloxylon* plantations.

In the current study, comparison of carbon sequestration in various plant parts and soil depths indicated that soil and underground plant parts were responsible for more than 70% of carbon sequestration in the *Haloxylon* plantation. In contrast, Amani and Maddah Arefi (2003) found no significant differences between underground and aerial plant parts in this regard.

Trees remove carbon dioxide from the atmosphere through the natural process of photosynthesis. They store carbon in their leaves, branches, stems, bark, and roots. Carbon comprises about half of the trees' biomass dry weight. Since one ton of carbon equals 3.67 ton of carbon dioxide (Johnson and Coburn, 2010), the total amount of carbon sequestration in aerial, underground, and litter biomass per unit area of *Haloxylon* plantation in the present study was 16.6 ton/ha (*Table 2*). With the total area being 766 ha, carbon sequestrated by *Haloxylon spp.* equaled 12715.6 ton which means 46666.25 ton of CO_2 gas had been absorbed.

The significant amount of carbon sequestration in the soils of the studied *Haloxylon* plantation (45%) highlights the importance of soil as a natural resource to control GHG emissions. Besides, we found 0-15 cm depth of soil to have maximum carbon sequestration potential. Similarly, Abbas Nejad and Khajodin (2012) reported larger levels of carbon sequestration in soil surface layers. They also found carbon sequestration to decrease with increasing depth. Sheidai Karkaj et al. (2013) introduced high carbon stock in higher depths of microsites to cause greater expansion of *Atriplex* roots compared to *Agropyron* roots. However, due to the presence of litter and thus storage of more carbon in the higher depths (0-15 cm) of soil, this part was more important for carbon sequestration. In other words, carbon content had an inverse relationship with depth.

The high level of carbon sequestration in soil implies that soil erosion will undoubtedly lead to carbon waste. Therefore, any sort of biological or mechanical operation to prevent soil regression and promote vegetation is certainly beneficial to carbon sequestration management (Izaurralde et al., 2007; Abdi, 2005). In such conditions, carbon sequestration adds to other values and uses of forest ecosystems and can be used as an indicator for assessing the sustainability of natural resources (Varamesh et al., 2011). Furthermore, atmospheric carbon filtration using synthetic methods will impose heavy costs, e.g. about $100-300 in the U.S. (Cannell, 2003). Carbon sequestration in the studied *Haloxylon* plantation was about 24.46 ton/ha higher than the control area. As the plantation covered an area of 766 ha, the total increase in carbon sequestration was 18,736.36 ton. By considering the minimum value per ton of carbon sequestration as $200 (Varamesh, 2009), the economic value of carbon sequestration in the *Haloxylon* plantation was approximately $3.74 million.

Carbon sequestration potential varies depending on plant species, location, and management methods (Mortenson and Schuman, 2002). Forozeh et al. (2008),

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Haghdoost et al. (2012), Abbas Nejad and Khajodin (2012), and Sheidai Karkaj (2013) showed that various plant species lead to different levels of carbon sequestration. Alizadeh et al. (2010) indicated grazing management to be able to alter carbon sequestration levels. Terakunpisut (2007) suggested differences in carbon sequestration in various regions of a forest.

This investigation confirmed the carbon sequestration potential of *H. aphyllum* and *H. persicum*. Moreover, gaps in the area (*Fig. 1*) indicated sharp drop in groundwater and the necessity of sand control with other species in the studied region.

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THE EFFECT OF MYCORRHIZAL SYMBIOSIS AND SEED PRIMING ON THE AMOUNT OF CHLOROPHYLL INDEX AND ABSORPTION OF NUTRIENTS UNDER DROUGHT STRESS IN SESAME PLANT UNDER FIELD CONDITIONS

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Abstract. Plants are exposed to environmental stresses during their growth. One of the most important stresses is drought stress, which can affect the absorption and transfer of nutrients to the plant. The use of advantageous microorganisms such as mycorrhizal fungi as well as seed priming are among the solutions that have been taken into consideration in many plants in recent years to mitigate the effects of water shortages and drought stress. In the present study, the effect of mycorrhizal symbiosis and seed priming on the amount of chlorophyll index and absorption of nutrients in sesame oilseed under drought stress was investigated during 2013 and 2014 at the farm of Hajiabad Agricultural Research Station in Hormozgan- Iran. The main drought stress factor included irrigation based on providing 100% of the plant's water requirement (normal irrigation), providing 70% of the plant's water requirement (mild stress) and providing 50% of the plant's water requirement (severe stress), Priming substrate was at three levels: no priming (control), hydro-priming and osmo-priming, and another sub-factor consisted of mycorrhiza fungi species: without inoculation mycorrhizal fungi (control), using *Glomus mosseae* and *Glomus intraradices*. The results of combined analysis of variance showed that the effects of drought stress and mycorrhizal symbiosis on leaf chlorophyll index, nitrogen (N), phosphorus (P), potassium (K), iron (Fe), zinc (Zn) and copper concentration (Cu) in leaf were significant. Sodium concentration was only significantly affected by drought stress and seed priming was only effective on Cu concentration. Interaction of irrigation × mycorrhizal symbiosis was significant on pand Cu uptake and interaction of irrigation × seed priming was only significant on iron concentration. Results showed that severe drought stress (providing 50% of plant water requirement) had the highest effect on decreasing amount of chlorophyll index and concentration of nitrogen and phosphorus elements in leaves, whereas concentrations of potassium, zinc, iron, copper and sodium increased with drought stress. Inoculation with mycorrhizal fungi increased the amount of chlorophyll index, nitrogen, phosphorus, potassium, zinc, iron and copper uptake compared with the absence of mycorrhizal fungi.

Keywords: *chlorophyll index, drought stress, mycorrhizal symbiosis, nutrients, seed priming, sesame*

Introduction

Sesame (*Sesamum indicum* L.) is an annual and diploid that grows strong. Direct sesame root system ,which is capable of robust and wide-permeable soils ,warm and moist to a depth of 2 m to penetrate. Depth development of roots in irrigated conditions is often less than 1 m, with the majority of the roots to a depth of 60 cm can be seen. Drought is one of the most common abiotic environmental stresses and the most important limiting factor for a successful crop producing, especially in arid and semiarid regions of the world (Kramer and Boyer, 1995). One of the most harmful effects of drought stress is disruption in the process, absorption and accumulation of nutrients that causes the reduction of grain and forage yield (Irannejad, 1991). Drought stress decreased total and b chlorophyll and leaf RWC in various Sesame genotypes (Hassanzadeh et al., 2009). The plant can withstand drought through various mechanisms, such as closing the stomata, thickening of the cuticle, reducing transpiration, preventing protein depletion and osmotic regulation (Premachandra et al., 2002).The mechanisms of absorption and transfer of nutrients in plants, such as mass flow, emission or absorption and transfer by osmotic phenomena, are all function of the moisture content of the soil and the expansion of the absorbing root, and in the case of reduced moisture or roots expansion, intensity and amount of nutrient uptake are undergoing changes (Taiz and Ezeiger, 1998). Sesame plants are adversely affected by continuous flooding conditions or environments severe drought (Menshah et al., 2006). Various approaches have been proposed to mitigate the effects of drought soils, such as disruption in nutrient uptake and reduction of chlorophyll content of leaves. Biological solutions, such as the use of microorganisms, like mycorrhiza fungi, are solutions that have recently attracted more attention. Application of *Glomus* spp. (VA mycorrhizae) significantly reduced wilt and root-rot incidence of sesame plants. *Lums* spp. (VA mycorrhizae) also significantly increased plant morphological characters such as plant height, number of branches and number of pods for each plant. Using *Glomus* spp. to protect sesame plants by colonizing the root system, significantly reduced colonization of fungal pathogens in sesame rhizosphere as well as pathogenic activity of fungal pathogens increased lignin contents in the sesame root system were also observed. Furthermore, mycorrhizae treatment provided selective bacterial stimulation for colonization on sesame rhizosphere. These bacteria belonging the *Bacillus* group showed highly antagonistic potential to fungal pathogens (Ziedan et al., 2011). Both mixed biofertilizer of *Pseudomonas geniculate* and *Alcaligenes faecalis* and foliar application of KCl had significant positive effect on the sesame yield, oil content and chemical constituents of sesame seeds under saline condition (Omer and Abd-Elnaby, 2017). Arbuscular mycorrhizal fungi play an important role in improving the nutrition and growth of plants under drought conditions (Singh et al., 1997). Mycorrhiza fungi, as one of the most important microorganisms in the soil, by coexistence with many plant species, improve the absorption of water and nutrients by host plants (Smith and Read, 1997). Today it is known that mycorrhizal fungi increase the nutrition of plants and, consequently, increase the growth of host plants by enhancing the absorption of nutrients and water (Feng et al., 2002). Studies show that mycorrhizal fungi contribute to plant growth under drought stress by increasing nutrients absorption and reducing stress (Ruiz-lozano and Azcon, 1996). Increasing the absorption of mineral elements, especially non-moving elements such as phosphorus in the host plant, is the most important effect of the symbiotic relationship with mycorrhizal fungi (Li et al., 1991; Bolan, 1991). The mycorrhiza inoculation could help in effective utilization of rock

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phosphate by changing it into available forms, which is later taken up by the sesamum plant for their better growth and development. The AM symbiosis optimized the Phosphorus solubilization from Rock Phosphate and affects microbial activity in the hyphosphere of *Sesamum indicum* L., oil yielding plants (Sabannavar and Lakshman, 2009). Mycorrhiza fungi hyphae can enter into very small pores that even root hairs cannot penetrate into them and cause more water absorption (Tisdall, 1991). Mycorrhizal inoculation significantly increased sesame root colonization under both sterile and nonsterile soil conditions compared to the control. Mycorrhizal inoculation significantly improved nutrient uptake of sesame particularly N, P, K, Ca, Mg, Na, Fe, Cu, Mn and Zn under both sterile and non-sterile soil conditions (Babajide and Fagbola, 2014). The indigenous AMF improved the growth and yield characters of sesame though their efficiency varied (Harikumar, 2013). Application of mycorrhizal fungi significantly increases leaf number and leaf area of Sesamum. The leaf area increased by 136% at the plants inoculated with *Glomus fasciculatum* and number of leaves by 70% at the plants inoculated with *Glomus mosseae*. Moreover, inoculation improved the root system by increasing volume and dry weight of roots (Boureima et al., 2007). Inoculation with mycorrhiza showed more efficiency, and were positively reflected in growth traits (plant height, leaf number, dry weight, tissue phosphorus and nitrogen) than addition of mineral phosphorus (Alsamowal et al., 2016). It is indicated that the reason for reducing the absorption of sodium, phosphorus and potassium from plant roots in dry soil is lower access of plants to these elements availability (Fatemy and Evans, 1986). Due to the fact that the absorption of nutrients changes with irrigation regimes, these changes affect the growth and yield of the plant, as well as the fact that mycorrhizal fungi have symbiotic relationship with the roots of most crops and with increasing water absorption, nutrient elements and resistance to environmental stresses cause growth and development of host plant. According to recent drought in Hajiabad region as well as relative resistance of Sesame against drought stress, the aim of this study was:

1. Evaluation the effects of mycorrhizal symbiosis on sesame oilseed
2. The effect of seed priming on sesame oilseed
3. The effect of drought stress on sesame oilseed in Hormozgan region.

Materials and methods

In order to evaluate the effects of mycorrhizal symbiosis, seed priming and drought stress on chlorophyll index and nutrient absorption, a split factorial based on randomized complete block design with 3 replications was carried out in Agricultural Research Station of Hajiabad county, Hormozgan province, during 2014 and 2015. The longitude of the experiment site was 55º 54’ and its latitude was 28° 19’, and the altitude is 920 m (*Fig. 1*), the mean annual precipitation and evaporation were 262.7 and 3200 mm, respectively, and climate is among warm and dry areas (*Figs. 4* and *5*). Some characteristics of the physical and chemical properties of the soil are presented in *Table 1*.

Table 1. *Physico-chemical soil properties of the experimental site*

Year	Soil depth (cm)	Texture	EC (ds/m)	pH	Organic carbon (%)	Available P (mg/l)	Available K (mg/l)
2014	0-30	Sandy-loamy	2.43	8.01	0.63	6.3	185
2015	0-30	Sandy-loamy	2.22	7.98	0.77	5.9	203

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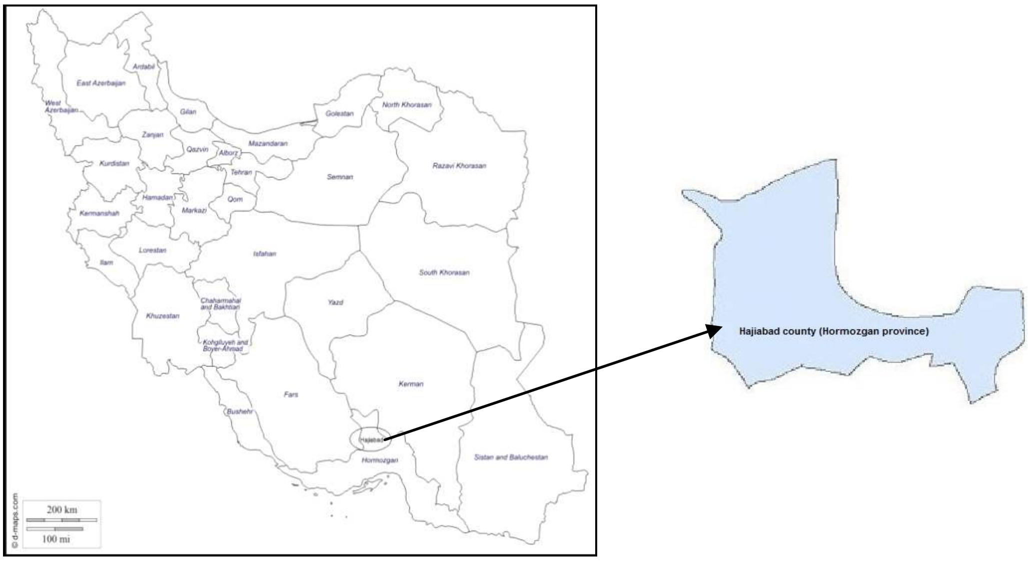

Figure 1. *Location of the experiment site within Iran*

The main plot of drought stress (*Fig. 2*) were as following: irrigation based on 100% water requirement (normal irrigation), providing 70% of the plant's water requirement (mild stress) and providing 50% of the plant's water requirement (severe stress) and sub plots of seed priming experiments were designed at three levels: no priming (control), hydro priming (24 h in distilled water and then air dried 24 h) and osmo-priming (using a solution of PEG 6000, 0.2 MPa and Placing seeds in a solution for 24 h and then air dry them for 24 h and another sub-treatments included different species of mycorrhizal fungi: Without incubation mycorrhiza fungi (control), incubation with *G. mosseae* and *G. Intraradices* species.

Figure 2. *Irrigation of drought stress treatments in sesame experiment site*

The used Mycorrhizal inoculum that was obtained from corn which planted in pot involved tiny pieces of symbiosis corn roots, contained hyphae, vesicles, arbuscular and fungal spores and soil with them. Seedbed preparation included plowing, disking and

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leveling in June and planting operations were performed in the first half of July for two years of experiment. Each plot consisted of 6 lines with a length of 5 m and a row spacing of 40 cm and 10 cm plant spacing on a row. In order to prevent mixing treatments effects, the sub-treatment space from each other was 1.5 m and the main treatments were 2 m and the space between repetitions was 3 m. At 2-3 leaf stage and complete plant development, all treatments were irrigated uniformly and after this stage, different levels of drought stresses were applied. To determine the amount of irrigation at desired level of irrigation, the results of the research about the determination of water requirement of the Guam reference plant (ETo) in the Hajiabad region that was determined by (Moradi-Dalini, 2012) and the amount of plant coefficient (Kc) of sesame at different stages of growth from the results Published by the National Institute of Soil and Water Research were used (Farshi et al., 1998). Finally, considering the effective rainfall, the amount of water for irrigation of sesame was calculated for complete irrigation (without stress) and according to that amount, the water used in each level of drought stress was calculated. These calculated values with the help of a volumetric flow of water were applied at intervals of once every five day and separately for each drought stress level. Irrigation method was drip-tape (type). On the other hand, due to the irrigation of the water through the pipe and the use of drip-tape method, the amount of waste water was considered to be negligible and equal to zero in the surface water and leachate. Measurement of plant pigmentation index was done at full flowering stage using SPAD (*Fig. 3*). To measure and determine the concentration of nitrogen, phosphorus, potassium, iron, zinc, copper and sodium, were sampled from all the plots from the fully developed leaves of the end parts of the plant and dry ash digestion method was used. In this method, 2 g of plant material was ashed into an electric furnace and dissolved in 10 cc of chloride. After filtration the volume brought to 100 ml. The amounts of potassium and sodium elements were read in the photometric instrument and phosphorus in the spectrophotometer. The amounts of phosphorus and potassium were calculated and expressed as percentage and the amount of Iron, Zinc, Copper and Sodium were calculated and expressed as gram per kilogram of dry matter according to the standard table. Nitrogen content was measured using Kjeldahl method and in and calculated and expressed as percentage. Also, in order to evaluate the reduction or increase of traits in stress and without stress conditions, the percentage of changes of measured traits was calculated.

Data were subjected to analysis of variance (ANOVA) using statistical analysis system (SAS) version 9.1 (SAS Institute Inc., Cary NC, USA). The means were separated using the LSD test ($P \leq 0.05$).

Results

Analysis of variance of traits

The results of combined analysis of variance showed that the effects of drought stress and mycorrhiza on leaf chlorophyll index and nitrogen, phosphorus, potassium, iron, zinc, copper contents in leaves were significant ($P \leq 0.01$). Sodium concentration in leaves was only significantly affected by ($P \leq 0.01$) drought stress and seed priming only affected the concentration of copper ($P \leq 0.05$). Interaction of irrigation × mycorrhiza on P concentration ($P \leq 0.01$) and on copper concentration ($P \leq 0.05$) and interaction of irrigation × priming was significant only on the iron concentration in leaf ($P \leq 0.05$) (*Table 2*).

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Figure 3. *The measurement of chlorophyll index by SPAD in sesame*

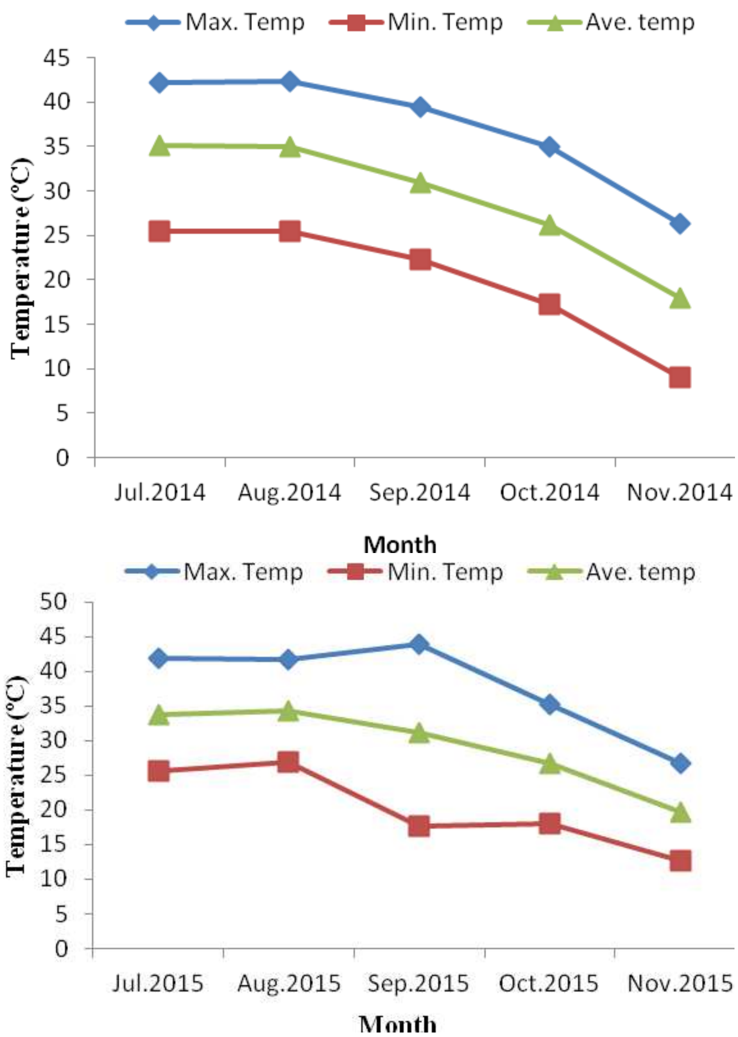

Figure 4. *Changes of Temperature during Sesame cultivation period (July to November)*

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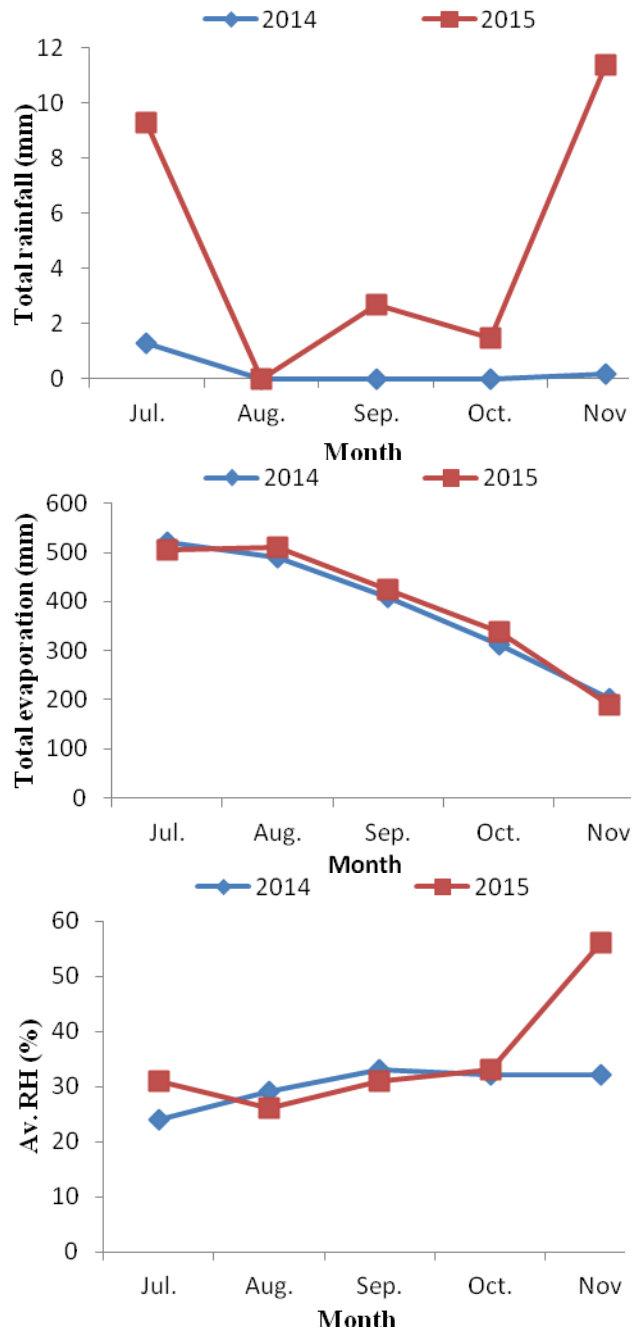

Figure 5. *Changes of rainfall, evaporation and relative humidity during Sesame cultivation period (July to November)*

Chlorophyll index

The results showed that with increasing drought stress the chlorophyll index decreased significantly. The mild drought stress reduced the chlorophyll index about 14.30% and 2.02%, respectively when compared with the optimum irrigation conditions. The highest chlorophyll index (41.67%) was obtained in the control (*Fig. 6*). Inoculation with mycorrhizal fungus of *G. mosseae* and *G. intraradices* improved the chlorophyll index by 2.93% and 2.07% compared to the (*Fig. 7*).

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Table 2. *Combined analysis of variance (mean squares) for plant characteristics of sesame in irrigation, priming and mycorrhiza treatments*

		(MS)							
S.O.V.	**D.F.**	**Chlorophyll index**	**N conc.**	**P conc.**	**K conc.**	**Fe conc.**	**Zn conc.**	**Cu conc.**	**Na conc.**
Replication(R)	2	5.34	0.1023	0.00001	0.00115	5.5740	0.3063	7.4413	0.01009
Year(Y)	1	0.79^{ns}	0.0015*	0.00038*	0.02907**	33.8000**	52.0540**	0.1643^{ns}	0.0053^{ns}
R×Y	2	0.60	0.0007	0.00008	0.00190	1.1296	0.0454	0.0252	0.0001
Irrigation(I)	2	562.3**	0.3948**	0.00882**	0.02270**	110.8889**	69.5238**	56.6487**	0.1148**
Y×I	2	0.31^{ns}	0.0097^{ns}	0.00042**	0.00114^{ns}	7.6296^{ns}	0.1218^{ns}	0.0098^{ns}	0.000009^{ns}
R×I	4	4.40	0.0139	0.00006	0.00102	9.2407	1.1948	2.2988	0.0063
Priming(P)	2	1.05^{ns}	0.0175^{ns}	0.00005^{ns}	0.00186^{ns}	2.9074^{ns}	1.4801^{ns}	5.0804*	0.0011^{ns}
Mycorrhiza(M)	2	19.48**	0.8642**	0.00332**	0.02807**	108.74074**	20.3769**	29.0744**	0.0030^{ns}
Y×P	2	0.24^{ns}	0.0040^{ns}	0.000001^{ns}	0.00062^{ns}	0.16667^{ns}	0.5632^{ns}	0.7283^{ns}	0.00004^{ns}
Y×M	2	0.55^{ns}	0.0313^{ns}	0.000018^{ns}	0.00078^{ns}	0.22222^{ns}	1.3492^{ns}	0.0231^{ns}	0.0001^{ns}
Y×I×P	4	0.09^{ns}	0.0299^{ns}	0.000059^{ns}	0.00133^{ns}	0.74074^{ns}	4.6022**	0.4924^{ns}	0.0045^{ns}
Y×I× M	4	0.17^{ns}	0.0268^{ns}	0.000089^{ns}	0.00204^{ns}	0.40740^{ns}	0.2492^{ns}	1.9194^{ns}	0.0022^{ns}
Y×P×M	4	0.23^{ns}	0.0174^{ns}	0.000079^{ns}	0.00042^{ns}	0.44248^{ns}	0.1542^{ns}	3.2489^{ns}	0.0006^{ns}
I×P	4	1.50^{ns}	0.0152^{ns}	0.000085^{ns}	0.00129^{ns}	7.79629*	0.3837^{ns}	2.6395^{ns}	0.0033^{ns}
I×M	4	0.24^{ns}	0.0153^{ns}	0.00086**	0.00126^{ns}	2.29629^{ns}	1.0016^{ns}	4.2674*	0.0017^{ns}
P×M	4	0.28^{ns}	0.0091^{ns}	0.00012^{ns}	0.00067^{ns}	3.59260^{ns}	0.7354^{ns}	3.5101^{ns}	0.0043^{ns}
I×P×M	8	0.82^{ns}	0.0109^{ns}	0.00011^{ns}	0.00187^{ns}	4.70370^{ns}	1.5807^{ns}	1.9562^{ns}	0.0014^{ns}
Y×I×P×M	8	0.16^{ns}	0.0246^{ns}	0.00012^{ns}	0.00315^{ns}	0.24074^{ns}	2.3478^{ns}	0.5035^{ns}	0.0006^{ns}
C.V. (%)	-	3.28	11.56	5.37	4.04	4.20	4.18	12.28	4.15

ns: non- significant; * and **: significant at 5% and 1% probability levels, respectively

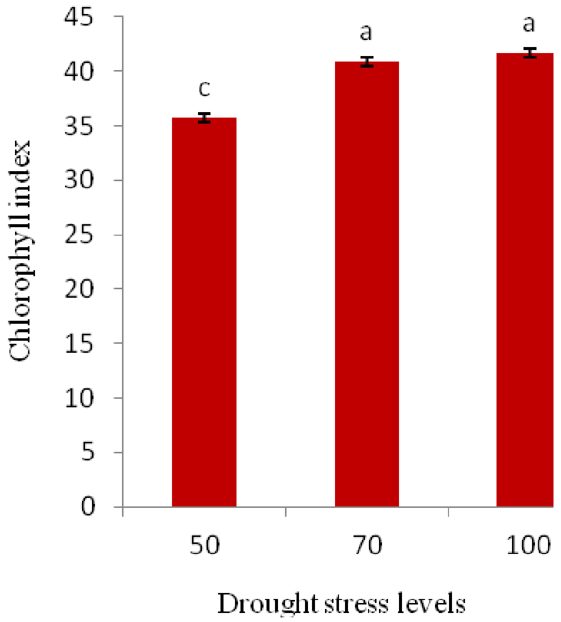

Figure 6. *Effect of drought stress levels on chlorophyll index*

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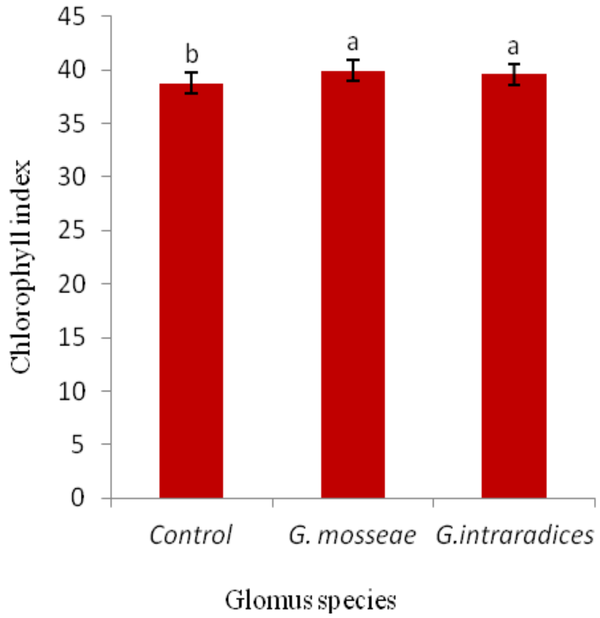

Figure 7. *Effect of Glomus species on chlorophyll index*

Nitrogen concentration

The results of this study showed that nitrogen concentration of leaf was decreased with increasing drought stress. According to the results, the highest nitrogen concentration was obtained for (control) with an average of 1.31%, while the lowest was obtained in severe drought stress conditions with an average of 1.14%. Sever and mild drought stresses reduced the nitrogen concentration by 12.81% and 8.67%, respectively, when compared with optimal irrigation. Soil water reduction in stress treatments caused nitrogen supply and reduced absorption and concentration of nitrogen in the plant (*Fig. 8*).

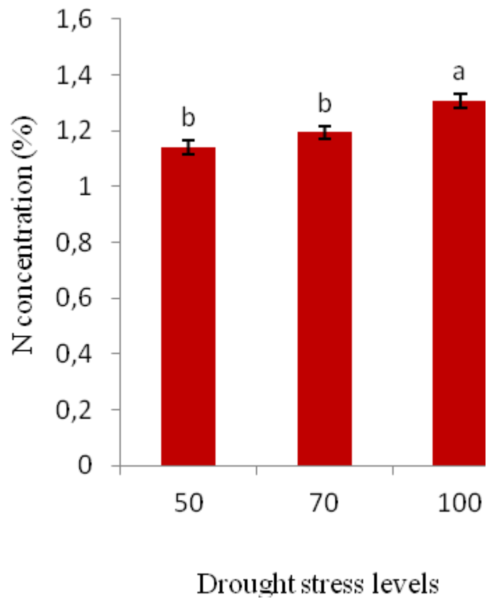

Figure 8. *Effect of drought stress levels on N concentration*

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DOI: http://dx.doi.org/10.15666/aeer/1601_335357

Inoculation with *G. mosseae* and *G. intraradices* mycorrhizal fungi increased nitrogen concentration to the 17.05% and 15.74% when compared with control (*Fig. 9*).

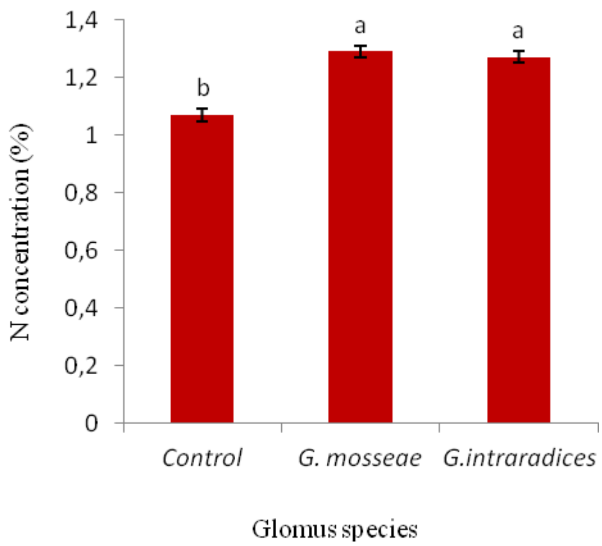

Figure 9. *Effect of Glomus species on N concentration*

Phosphorous concentration

Based on the results of this study, drought stress decreased phosphorus concentration in the leaves as well as the nitrogen. The highest concentration of P was obtained for control with an average of 0.16%, while the lowest was obtained in severe drought stress conditions with an average of 0.13%. Sever and mild drought stress, reduced phosphorus concentration to 15.96% and 6.96%, respectively when compared with optimal irrigation (*Fig. 10*).

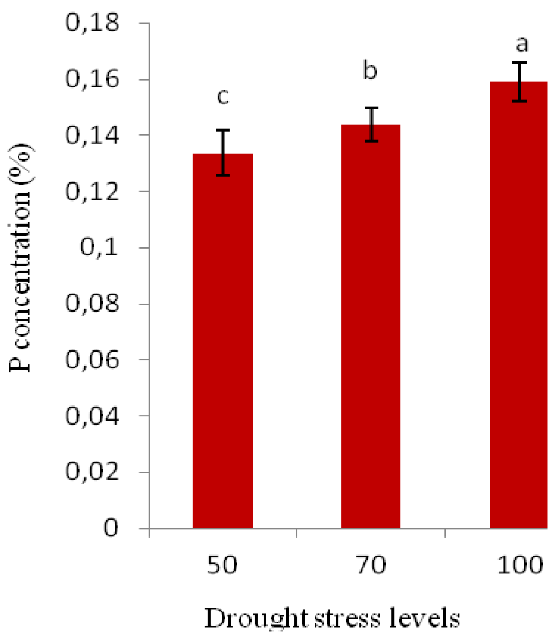

Figure 10. *Effect of drought stress levels on P concentration*

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Inoculation of soil with *G. mosseae* and *G. intraradices* mycorrhizal fungi increased the concentration of phosphorus to 9.54 and 8.54% when compared with the control (*Fig. 11*). The interaction of drought stress and mycorrhiza was significant for phosphorus concentration (P≤0.01) (*Table 2*). This indicates that the effect of fungi on the concentration of phosphorus is not independent from effect of stress and is affected by phosphorus. The highest concentration of phosphorus in irrigation conditions was related to the control and inoculation with *G. mosseae* species with an average of 0.17% and the lowest was obtained in severe drought stress and without usage of mycorrhizal fungus with an average of 0.12% (*Fig. 12*). The results also showed that there were no significant differences between two species of *G. mosseae* and *G. intraradices* for phosphorus concentration in leaf.

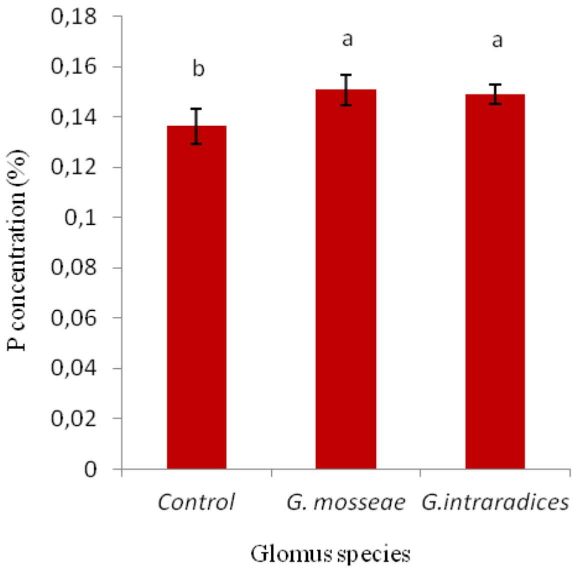

Figure 11. *Effect of Glomus species on P concentration*

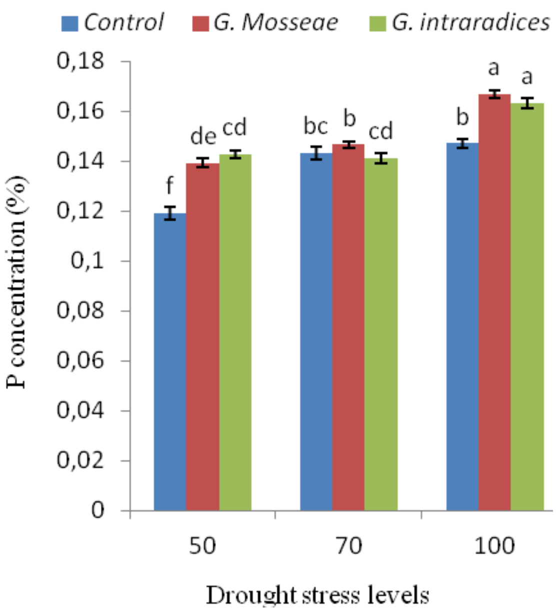

Figure 12. *Interaction effect of Glomus fungi and drought stress on P concentration*

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Potassium concentration

Drought stress increased potassium concentration in leaf. Based on the results the highest concentration of potassium in the leaf was obtained under severe stress conditions with an average of 1.07% and the lowest in control with a mean of 1.03%. Sever and mild drought stresses increased potassium concentration by 3.8% and 2.3% in comparison with optimal irrigation (*Fig. 13*). Inoculation with *G. mosseae* and *G. intraradices* mycorrhizal fungi increased the concentration of potassium to 4.06 and 3.19% in comparison with non-inoculated fungi (*Fig. 14*). The results also showed that there were no significant differences between two species of *G. mosseae* and *G. intraradices* for potassium concentration in leaf.

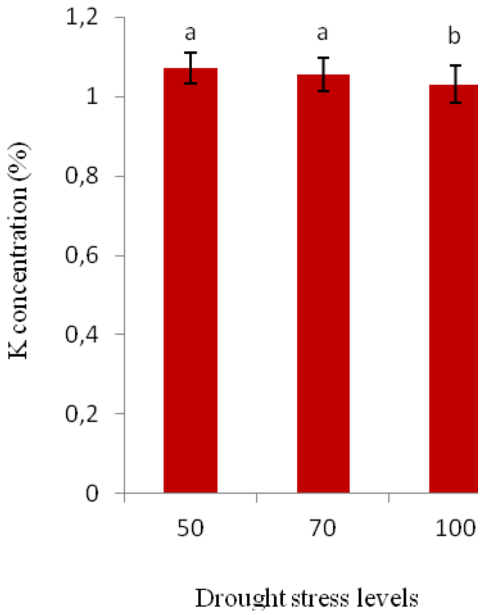

Figure 13. *Effect of drought stress levels on K concentration*

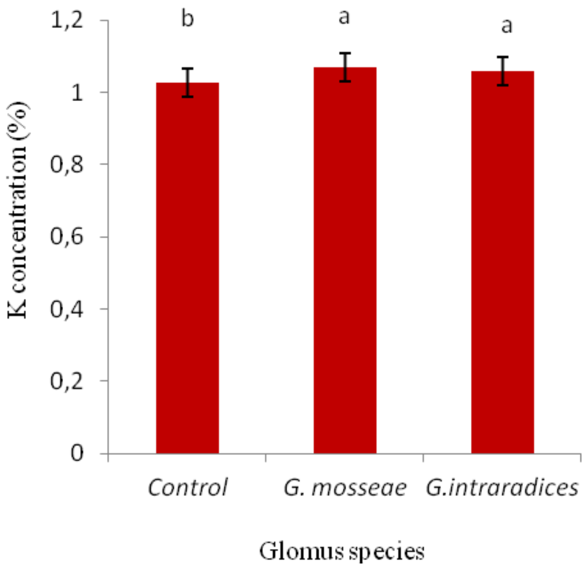

Figure 14. *Effect of Glomus fungi on K concentration*

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Iron concentration

The results showed that the concentration of iron in the leaf increased with drought stress applies. Based on the results the highest iron concentration in the leaf under severe drought stress was obtained with an average of 39.22 mg/kg leaf dry matter and the lowest in control with a mean of 36.44 mg/kg leaf dry matter. Intense and mild drought stresses increased the iron concentration to 7.09 and 5.2%, respectively compared with optimal irrigation (*Fig. 15*). Inoculation with *G. mosseae* and *G. intraradices* mycorrhizal fungi improved the iron concentration to 6.67 and 5.94% compared with non-inoculated mycorrhizal fungi (*Fig. 16*).

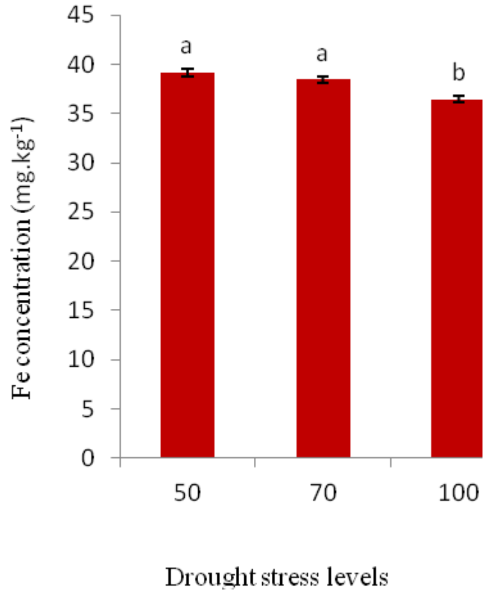

***Figure 15.** Effect of drought stress levels on Fe concentration*

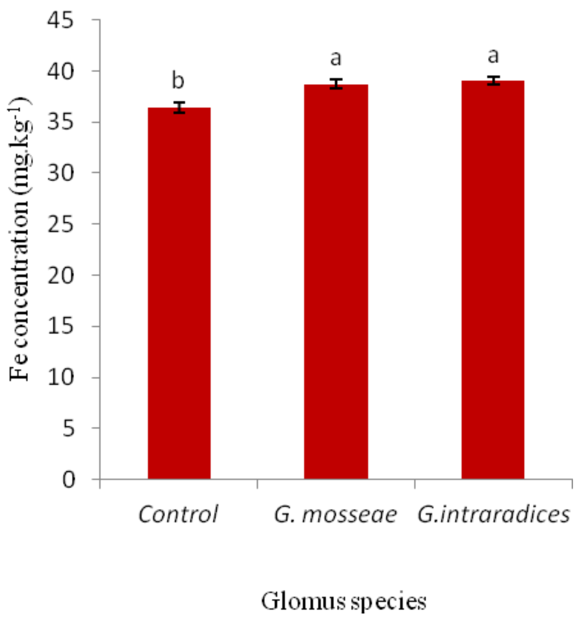

***Figure 16.** Effect of Glomus fungi on Fe concentration*

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The interaction of drought stress and priming on the concentration of Fe in leaf ($P \leq 0.05$) was significant. The highest iron concentration was observed under severe stress conditions (irrigation equivalent to 50% water requirement) and hydro-priming with an average of 39.50 mg/kg leaf dry matter and the lowest in control and without priming with mean of 35.55 mg/kg leaf dry matter (*Fig. 17*).

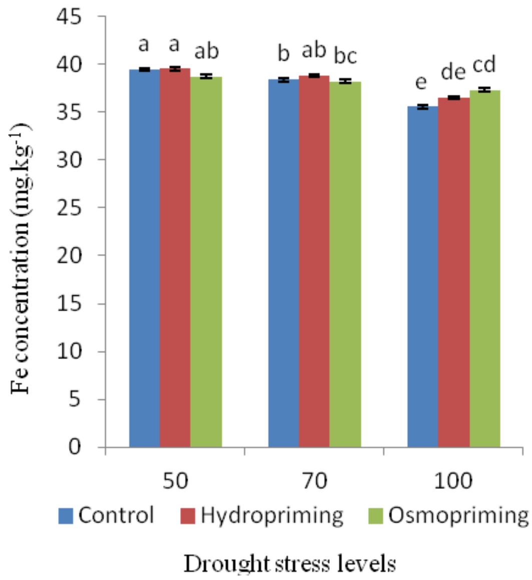

***Figure 17.** Interaction effect of Glomus fungi and drought stress on Fe concentration*

Zinc concentration

Drought stress increased zinc concentration in the leaves. Based on the results the highest zinc concentration was observed in severe drought stress conditions with an average of 26.95 mg/kg leaf dry matter and the lowest in control with an average of 24.73 mg/kg leaf dry matter. Severe and mild drought stress increased concentration of zinc in comparison with optimal irrigation by 8.24 and 5.75%, respectively (*Fig. 18*).

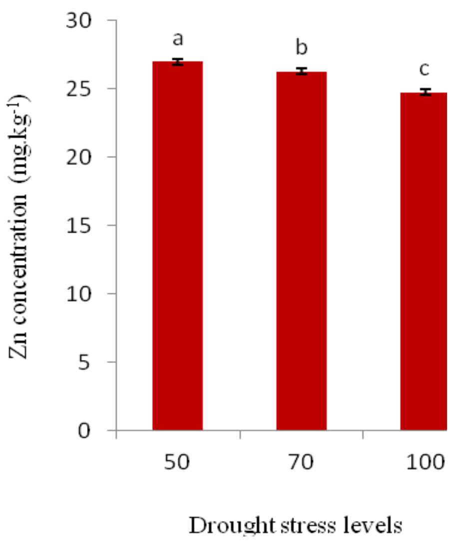

***Figure 18.** Effect of drought stress levels on Zn concentration*

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Inoculation with mycorrhizae of *G. mosseae* and *G. intraradices* increased zinc concentrations up to 4.14% and 3.95% compared to the non-inoculated with mycorrhizal fungi (*Fig. 19*).

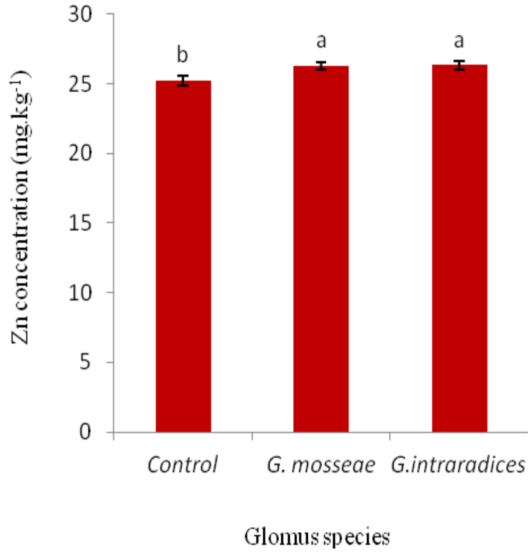

Figure 19. *Effect of Glomus fungi on Zn concentration*

Copper concentration

The results showed that the concentration of copper in the leaf increased with drought stress. Based on the results the highest concentration of copper in severe drought stress conditions was obtained with an average of 10.89 mg/kg leaf dry matter and the lowest in control with an average of 8.87 mg/kg leaf dry matter. Severe and mild drought stresses increased the copper concentration by 18.55% and 12.78%, respectively, as compared to the optimal irrigation (*Fig. 20*).

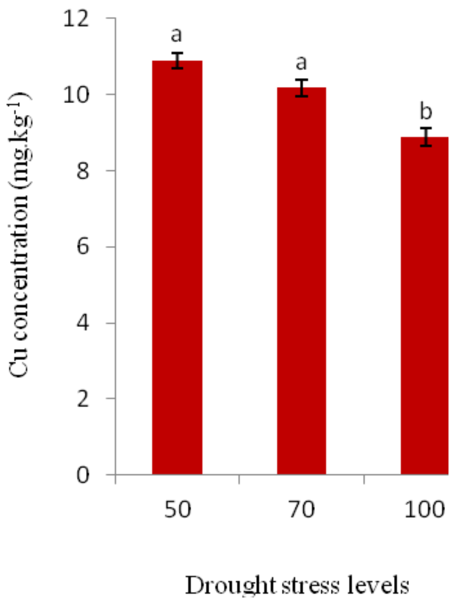

Figure 20. *Effect of drought stress levels on Cu concentration*

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Inoculation with *G. mosseae* and G. *intraradices* mycorrhizal fungi increased the copper concentration to 12.72% and 11.71% in comparison with non-inoculation with mycorrhizal fungus (*Fig. 21*).

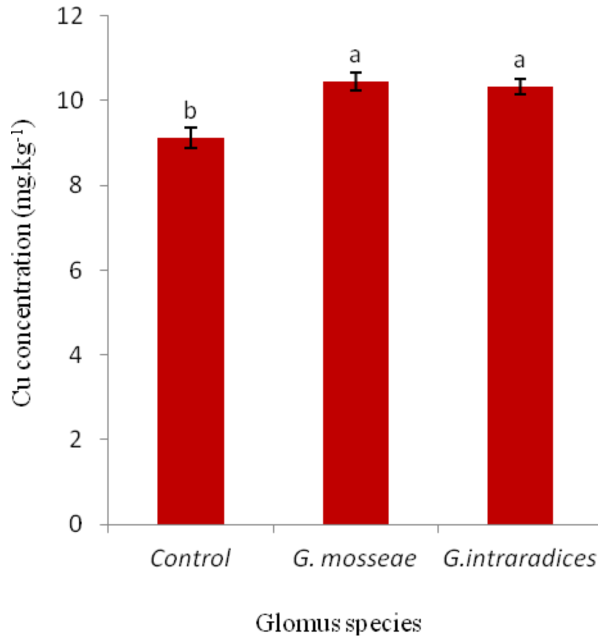

Figure 21. *Effect of Glomus species on Cu concentration*

Seed priming increased concentration of copper in the leaves to 3.49 and 2.48% than non-priming (*Fig. 22*).

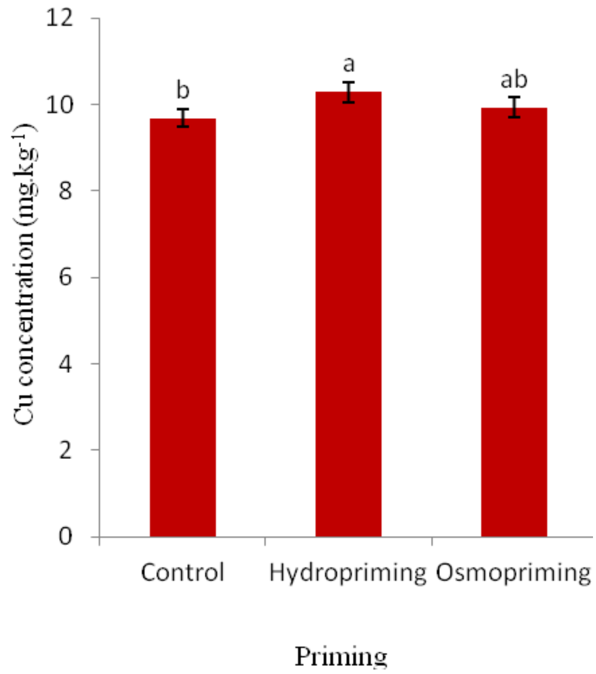

Figure 22. *Effect of priming levels on Cu concentration*

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The effect of drought stress and mycorrhiza on the concentration of copper in leaf was significant at 5% probability level. The highest concentration of copper was obtained in severe drought stress and inoculation with *G. mosseae* 11.64 mg/kg dry matter and the lowest in control and without usage of mycorrhizal fungus with an average of 8.08 mg/kg leaf dry matter (*Fig. 23*).

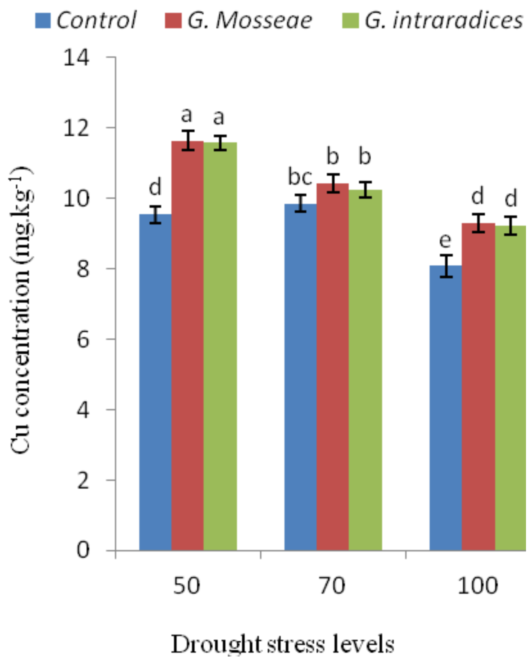

Figure 23. *Interaction effect of Glomus species and drought stress on Cu concentration*

Sodium concentration

The results showed that by applying drought stress, the concentration of sodium in the leaf was increased. Based on the results the highest concentration of sodium was obtained in the leaf under severe stress conditions with an average of 1.14 mg/kg leaf dry matter and the lowest in control with an average of 1.05 mg/kg leaf dry matter. Severe and mild drought stresses increased the sodium concentration in the leaf to 7.89% and 4.54% respectively when compared with optimal irrigation (*Fig. 24*).

Discussion

Under our conditions of experiment, the results showed that with increasing drought stress the chlorophyll index decreased significantly. Reduction of chlorophyll content in drought stress conditions has been reported in sunflower (Gholam-Hosseini and Ghalavand, 2008). Dehydration stress through chlorophyllase and peroxidase enzymes activities in plants lead to chloroplast destruction and chlorophyll content reduction (Misra and Sricastatva, 2000). The plant’s water conditions have important effects on leaf chlorophyll (Vidal et al., 1999). Reducing chlorophyll content due to drought stress is related to the increase of oxygen radicals in the cells (Schutz and Fangmeir, 2001). It seems that chlorophyll content reduction under drought conditions is due to chlorophyllase, peroxidase activities and consequently chlorophyll degradation

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(Ahmadi and Ceiocemardeh, 2004). The reduction of chlorophyll content in this study was consistent with the results of other researchers (Zhang et al., 2006; Sanchez-Blanco et al., 2006). The highest rate of photosynthesis and chlorophyll content in maze plant was obtained when inoculated with mycorrhiza and bacteria (Jahan et al., 2007).

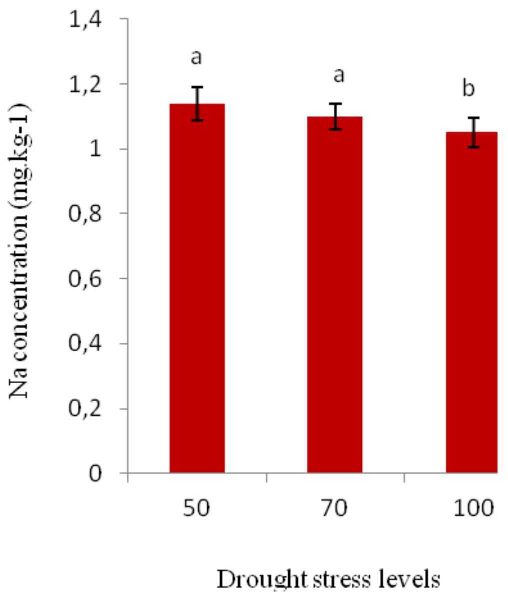

Figure 24. *Effect of drought stress levels on Na concentration*

The results of this study showed that nitrogen concentration of leaf was decreased with increasing drought stress. Under severe stress conditions, plant roots are exposed to water deficit and decreases nitrogen absorption from soil since nitrogen uptake is function of transpiration stream (Saneoka et al., 2004), which is consistent with the results of this experiment. Mass flow plays a dominant role in the supply of nitrogen (especially in the form of nitrate) to the root and its absorption by the plant. On the other hand, the amount of mass flow depends on the amount of soil water. Soil water reduction in stress treatments caused nitrogen supply and reduced absorption and concentration of nitrogen in the plant. One of the drought stress effects is modulation of root development. In this case, horizontal growth decreases and vertical root growth increases. It is mentioned that root growth is closely related to the absorption of phosphorus and nitrogen from soil (Fan and Mackenzie, 1994). Based on the results of this study, drought stress decreased phosphorus concentration in the leaves as well as the nitrogen. It seems that decrease of phosphorus concentration under drought stress is because of the low mobility of phosphorus in the soil because the supply of phosphorus to root is due to diffusion and the amount of soil moisture influences the rate of diffusion. Of course, it should be considered that soils differ in terms of the phosphorus availability and stabilizing for plants (Kafi et al., 2010). Inoculation of soil with *G. mosseae* and *G. intraradices* mycorrhizal fungi increased the concentration of phosphorus. These results are consistent with the results of (Auge, 2001). Inoculation with mycorrhiza fungi caused development of root system and increased the concentration of phosphorus in the leaf. The effect of mycorrhizal fungus on the growth of host plant under drought stress has been reported through improvement of phosphorus availability because access to the phosphorus decreases in dry soils

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(Subramanian et al., 2006). Thus, reducing soil moisture reduces nutrients, especially phosphorus from soil to the root. Therefore, mycorrhiza increases phosphorus uptake by plant roots under drought stress and without stress (Hetrick et al., 1996). Phosphorus uptake increased by absorption from roots (Smith and Read, 1997; Cui and Caldwell, 1996). The symbiosis with the *G. intraradices* fungus in pepper resulted in an increase in leaf area ratio, which is due to mycorrhizal effect on increasing phosphorus content in this plant (Demir, 2004). In general, the use of fungi increased the concentration of phosphorus in the leaf rather than its application. It can be stated that fungus developed root system through its mycelium and rays, and caused the plant roots to use rhizosphere more widely (Bolan, 1991).

Stress increased potassium concentration in leaf. Reports from various researchers also confirmed that potassium absorption increased during drought stress. During drought stress plants increase K concentration in the root due to the increased drought resistance. Increasing potassium adsorption has a positive effect on photosynthesis, growth and leaf area index, open and closing of stomata regulation, transpiration decrease (Abd-EL-Moez, 1996; Gonzales and Salas, 1995). The other reason which researchers have suggested for increase of potassium adsorption in plants under drought stress, is continuous dry and drying in the soil, that releases K from clay layers and this phenomenon increases potassium uptake (Logan et al., 1997). Inoculation with *G. mosseae* and *G. intraradices* mycorrhizal fungi increased the concentration of potassium. Mycorrhizal inoculation increased moisture availability and provided more access to nutrients. Some studies confirmed that mycorrhizal symbiosis improved the active root system to increase the absorption of water and nutrient (Kapoor et al., 2004). The results showed that the concentration of iron in the leaf increased with drought stress applies. Inoculation with *G. mosseae* and *G. intraradices* mycorrhizal fungi improved the iron concentration. It seems that increasing absorption of nutrients is mainly due to the release of mycorrhizal mycelia and the formation of an additional complementary absorption system to the root system of the plant, which makes it possible to use more volumes of soil that the feeder roots do not have access to. Mycorrhizal fungi control the problems of reducing water absorption under conditions of depleted moisture in the root environment by improving the hormonal status of the plants in controlling the opening and closing of leaf stomata and increasing the water absorption due to the spread of the hyphae network (Roldan-Fagardo et al., 1982). The interaction of drought stress and priming on the concentration of Fe in leaf ($P \leq 0.05$) was significant (*Table 2*). This suggests that the seed priming effect on the concentration of iron is dependent of effect of stress. Drought stress increased zinc concentration in the leaves. Limited reports have been published on zinc ion accumulation under stress conditions in plant aerial organs. In corn (Alizadeh, 2010) and in canola (Nasri et al., 2008), reported that dehydration stress increased zinc concentrations in plant aerial organs, which is consistent with the results of this study. Inoculation with mycorrhizae of *G. mosseae* and *G. intraradices* increased zinc concentrations. Mycorrhizal symbiosis increases the absorption of zinc by increasing the length of the roots and also increasing the absorption by the fungal roots (Kothari et al., 1991). The concentration of copper in the leaf increased with drought stress, inoculation with *G. mosseae* and G. *intraradices* mycorrhizal fungi and seed priming. In justifying the function of priming, we can point to the rapid and favorable establishment of the plant and its further use of nutrients, soil moisture and solar radiation. Primed seeds are germinated sooner, and their various biological stages are also more likely to result. This natural adaptation of stressful living factor changes with the phonological stages of the plant, and the damage to the plant will be reduced. Foreign mycelia of Mycorrhiza arbuscular fungi mainly contain hyphae and fungal spores. External hyphae extend in the soil and produce high absorption of copper (Li et al., 1991). In corn, mycorrhizal symbiosis increased the concentration of copper in the shoot but did not have a significant effect on underground organs (Kothari et al., 1991). Mycorrhizal fungi increased their efficiency in absorption of water and nutrients; in particular, phosphorus, zinc, and copper with an extensive hefy network and increased absorption rate of roots (Marschner and Dell, 1994). The results showed that by applying drought stress, the concentration of sodium in the leaf was increased. The accumulation of sodium in the tissue is due to more absorption by the root and more drainage from the stem to the leaves. Osmotic balance of plants carries out by absorbing more sodium that make plants enable to absorb more water (Munns and James, 2003). It was reported that with increasing stress, the amount of sodium accumulation in the leaf increased in wheat plant (Bagheri, 2009). In sugar beet, it was stated that under drought stress, sodium and potassium accumulated greatly in roots and stems (Ghoulam et al., 2002). The increase in sodium due to drought stress has also been reported by other researchers (El-Tayeb, 2006). This increase has been proposed as a defense mechanism that helps plants under stress to increase the amount of sodium in order to regulate the osmotic pressure of cells and tissues under stress, in order to improve the absorption of water from soil.

Conclusion

In general, the results of this study showed that drought stress reduced the concentration of phosphorus and nitrogen, but increased potassium, zinc, iron, copper and sodium in the leaves. Also, symbiosis with mycorrhizal fungi increased concentration of all of the nutrients in sesame except for sodium. Also, it was found that the positive effects of mycorrhizal symbiosis were not dependent to fungi species. Mycorrhiza absorbs and transports water and nutrients to the plant through the release of mycelia's in the micro porous pores, and also improves the plant's aquatic relationships which causes increasing turgor pressure. Therefore, under poor water conditions the use of mycorrhizal fungi, reduces water consumption and provides a suitable source for increasing drought tolerance in plants. Based on the results of this study it can be recommended, sesame farms in the country get inoculated by inoculum of mycorrhizal fungi arbuscular and benefit from positive effects of this symbiosis in yield increase and nutrient uptake, especially phosphorus, nitrogen, potassium, iron and copper and develop Plant growth conditions.

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EFFECTS OF NATURE TRAINING PROJECTS ON ENVIRONMENTAL PERCEPTION AND ATTITUDES

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Abstract. Environmental education aims to create positive and environmental-sensitive perceptions, attitudes and behaviours toward environmental protection. This education primarily focuses on changing the attitudes and behaviours of students, rather than transferring information. Nature training projects provide improving methods to impart information on nature and environment in formal education programmes and develop people that are sensitive to society and environment. In 2014, a project titled "Judas Trees Are Blooming" was implemented in Turkey with the participation of secondary school 6th grade students. The aim of the project was to create positive perception and attitudes regarding nature, forests and the environment. This study investigated and determined the effects of nature training projects on environmental perception and attitudes of secondary school students using the above-mentioned project as a case study. Students that participated in the project had more positive environmental perception and attitudes compared to those who did not participate according to questionnaires and observations given during the project. These positive environmental perceptions and attitudes are the benefits of forests, the importance of plants for our life, the mysteries of the worlds of plants and insects, nature and environmental protection and photosynthesis. Furthermore, the project provided positive contributions for raising awareness and improving the interest and knowledge of the participants regarding nature and the environment.
Keywords: *environmental education, practical training, environmental protection, nature conservation, Turkey*

Introduction

The environment is a physical, biological, social, economic and cultural setting in which people and other organisms sustain relationships throughout their lives and interact mutually. Environmental protection is of crucial importance and precious for the survival of organisms. Today, it is understood that the globalization of environmental problems can only be solved by changing people's behaviours. This can be accomplished by developing desired skills, knowledge, attitudes and behaviours in cognitive, affective and behavioural domains (Geray, 1998; Erten, 2005; Çimen and Yılmaz, 2014; Milton et al., 1995; Poortinga et al., 2004). Specifically, these behavioural changes can be achieved through environmental education and nature training (Güven, 2014).

In comparison, "shallow environmentalist" approaches that disregard human-nature relationships, do not adequately focus on formal and non-formal education, and ignore participation do not suffice in achieving an effective environmental and nature education (Tlert, 1998; Noughton et al., 2005). However, formal education programs in developing countries are also known to be inadequate in terms of nature-environmental

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education. This is the case even though considerably positive changes have been achieved in society through environmental/nature education (Malone and Tranter, 2003; Güler, 2009). The effects of nature training are popular contemporary themes being studied in developed countries (Ogurlu, 2016).

When provided in a natural setting, a well-structured environmental and nature education is important to ensure that people see life by establishing an emotional bond with nature. This approach increases their interest and develops desired behaviours (Palmberg ve Kuru, 2001; Phenice and Griffore, 2003; Atasoy, 2006; Güler, 2009).

Nature training projects offer significant opportunities to create an active mass of people that have the potential to contribute to the promotion and protection of nature and natural assets. These projects play an important role in improving conceptual and practical knowledge and awareness among participants. The projects aim at ensuring that events and facts are understood from a scientific perspective and engage people to emerge them from a passive position to the position of "doing-experiencing". This is done by assigning active duties to participants, which enables them to experience meaningful learning (Alkan and Ogurlu, 2014).

Primary and secondary school is the main legal institution that responsible for providing education to members of society and serves as a genuine criterion to assess the development level of countries (Harlen and Qualter, 2014). In other words, secondary school is the most effective institution that shapes the personality of a child, gives direction to her/his future and creates the infrastructure for a robust development (Yıldırım and Köklükaya, 2016). In this context, a well-structured environmental and nature education that focuses on the importance of secondary school education is important to raise consciousness in future generations.

Child-oriented environmental education can ensure that environmental information is transferred to the next generation and triggers targeted behavioural changes (Damerell et al., 2013). Studies show that current environmental education offered at schools in Turkey are insufficient (Soran et al., 2000; Yılmaz et al., 2002; Tanrıverdi, 2009; Zengin and Kunt, 2013; Alkan and Ogurlu 2014). Therefore, non-formal nature training projects are important for addressing this gap. The aim of this study was to determine the effects of nature training projects on the environmental perception and attitudes of secondary school students in Turkey.

Project description

The "Judas Trees Are Blooming" project was implemented in Isparta-Turkey with the support of The Scientific and Technological Research Council of Turkey (TUBITAK). The Judas tree (*Cercis siliquastrum*) is a small deciduous tree that can grow up to 4–5 m long and has a wide crown. The most important characteristic of Judas tree is that it bears flowers directly on its trunk. Its pink-purple flowers that bloom in spring can be recognized easily in nature (Davis et al., 1970; Mamikoglu, 2007). Secondary school education is one of the most important stages after primary education for the development of a child in all aspects. The behaviours acquired in this period can be permanent. Therefore, an effective environmental and nature education that is provided during secondary school will ensure the acquisition of permanent behaviours. Just as Judas trees are easily recognized in nature, students who receive nature training will be easily recognized in the society. For that reason, the title of the project was "Judas Trees Are Blooming". Sixty 6th grade students at the same secondary school who

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were randomly selected participated in the project. Under the project, training courses were delivered for seven days to groups of 30 students.

During the project, the participants were given instruction about the world of plants (recognition and classification, usage areas, endemic plants, etc.), the world of insects, ecosystems, importance of forests for our life, photosynthesis, importance of environmental protection and nature tourism. Throughout this curriculum, the goal was to create positive perception and attitudes regarding nature, forests and the environment. The training courses were delivered in practical form at the Botanic Garden and Herbarium Research and Applied Centre of Süleyman Demirel University and Isparta Kovadaçayı Arboretum. During the training courses, students were asked to draw pictures about plants and participated in activities that were held in a natural environment.

Methodology

Study data about environmental perception and attitudes were collected through questionnaires. Pre-evaluation and post-evaluation questionnaires were applied to test for change in the knowledge level of the students who participated in the project courses. The post-evaluation questionnaire contained questions about perceptions and attitudes regarding nature, forests and the environment. A three-point Likert scale (I agree, neutral, I disagree) was used to enable the students to answer the questions. The Cronbach alpha value was 0.824. The internal consistence coefficient was found to be greater than 0.8; therefore, the scale that was used had a statistically high reliability. Post-evaluation questionnaires were also given to students in the same class who did not participate in the training courses provided under the project. This was done to explore the effects of the training on changes in perception and attitudes regarding nature, forests and the environment.

Since the data from the questionnaires were non-parametric (Kolmogorov-Smirnov test, $p < 0.05$), the differences in the knowledge level of the students participating in the project before and after the training were found through a chi-square test. The Mann-Whitney U test was used to identify differences in the perception and attitudes of those students who received training and those who did not.

Result and Discussion

Characteristic of the students

The sex and age distribution of the students who participated in the training project and those who did not is presented in *Table 1*.

Table 1. *Student characteristics*

Characteristics		**A***	**B***
Gender	Girl	31	32
	Boy	29	28
	Total	60	60
Age	11	36	34
	12	24	26
	Total	60	60

*A: Students who participated in the project, B: Students who did not participate in the project.

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As understood from the table, the sex distribution of the students who participated in the project (A) and those who did not (B) is balanced. Students were between 11 and 12 years old.

Difference in knowledge levels before and after the nature training

The effect of the project on the knowledge level of the students was determined through the pre-evaluation and post-evaluation questionnaires given to the students who participated in the training project. Their knowledge levels with respect to knowing plant names, ability to classify plants, reproduction and growth styles and the concepts of ecosystem and endemic were compared.

Table 2 shows that the knowledge levels in the relevant areas improved at the end of the project. The increase was more pronounced in their knowledge level with respect to knowing plant names, ability to classify plants and the concepts of ecosystem and endemic (Chi-square test, $p < 0.05$). The difference in knowledge levels regarding plant reproduction was not statistically significant according to the chi-square test ($p > 0.05$). This was because the students had already received training on that subject matter prior to the project. The most important differences were found in the concepts of ecosystem and endemic. Previously, none of the students had heard about the concept of endemic. Similarly, the number of students who knew the meaning of ecosystem was low prior to the project (6.7%). Knowledge level for the concepts of endemic and ecosystem increased by 95% and 92%, respectively after the project (*Table 2*).

Table 2. *Difference in knowledge level before and after the nature training*

Subject	Time of questionnaire	Options (%)		Chi-square (X^2)	p
		Yes	No		
Know the names of plants in the environment	The end of the project	95.0 ▲	5.0 ▼	30.470	0.000*
	Before the project	50.0	50.0		
Able to classify plants	The end of the project	100 ▲	- ▼	26.939	0.000*
	Before the project	63.3	36.7		
Know how plants reproduce	The end of the project	98.3 ▲	1.7 ▼	2.807	0.103
	Before the project	91.7	9.3		
Know how plants grow	The end of the project	98.3 ▲	1.7 ▼	6.982	0.008*
	Before the project	85.0	15.0		
Know the concept of endemic	The end of the project	95.0 ▲	5.0 ▼	108.571	0.000*
	Before the project	0.0	100		
Know the concept of ecosystem	The end of the project	98.3 ▲	1.7 ▼	101.086	0.000*
	Before the project	6.7	93.3		

*$p < 0.05$, ▲ : increase, ▼: decrease

According to the evaluation regarding the efficacy of the training project, the students reported that the topics addressed in the project were sufficient by 96.7%. The students were also asked to evaluate the trainers and 98.3% of the students found that the trainers were competent in the training.

All of the students indicated that the plant species were explained adequately to them and that a sufficient number of plant sample was shown to them. The plant games were found to be sufficient by 98.3%.

While 28.3% of the students stated that the training project slightly changed their perspective and knowledge level about plants, 81.7% of the students indicated that their

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perspective and knowledge level completely changed. The training project satisfied all student expectations. All of the students stated that their knowledge about plants and nature improved and thus the project was useful for them. Those students who were highly satisfied with the project were observed to be willing and enthusiastic about taking part in other similar activities.

Perception and attitude changes created by the project

Forests provide wood and non-wood forest products and services to societies. While wood products were considered to be primary products in the past, it is now a fact that non-wood forest products are primary products in some forest areas (Shackleton and Pandey, 2014). A significant difference was found in this matter between those students who participated in the project and those who did not (*Fig. 1*). Students who participated in the project think that non-wood forest products and services play a more important role with respect to the benefits of forests. This difference was statistically significant (*Table 3*). The reason for this difference was that the benefits of forests were addressed thoroughly during the project and students learned it very well. Our results correspond with the findings of Zengin and Kunt (2013). Similarly, a majority of the students who participated in the project emphasized that plants played a crucial role in our daily life whereas there was a relatively higher number of students who did not participate in the project and were indecisive on this matter (*Fig. 1*). Another reason for that was the insufficient knowledge level of teachers on nature and the environment. Velempini et al. (2017) has reached the point that environmental education topics are not sufficiently included in the curriculum of education and that the teachers in these subjects are also inadequate. This issue can be tackled through training, especially of candidate science teachers about the environment and nature thru active learning techniques and methods (Alkan and Ogurlu, 2014; Uzun and Keles, 2012).

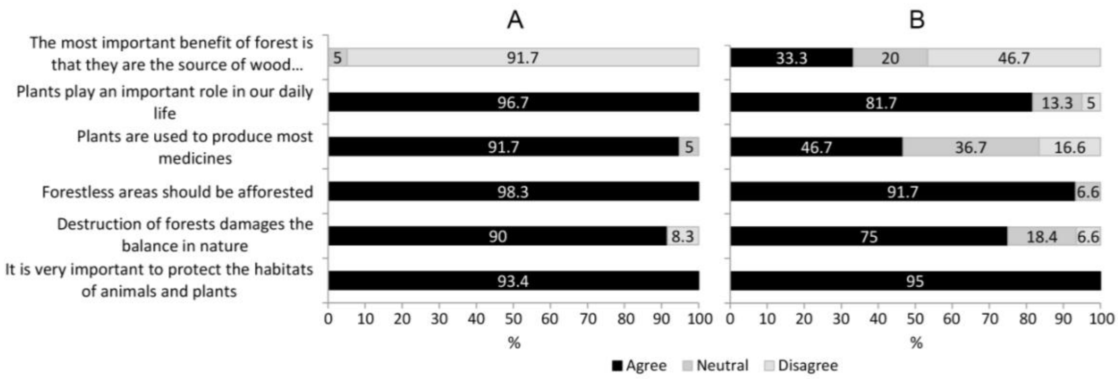

Figure 1. *Change in the level of knowledge and awareness about forest and nature (n_A=60, n_B =60, only values ≥5%)*

Students who participated in the project had a high knowledge level (91.7%) about the medical use of plants. More than half of the students who did not participate in the project was neutral (36.7%) or did not possess any knowledge (16.6%) about this about this matter (*Fig. 1*). Mann-Whitney U test results (*Table 3*) show that the differences between the answers of both groups were statistically significant ($p < 0.05$). The project curriculum included the topic of medical use of plants.

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Table 3. *Mann-Whitney U test results for changes in knowledge and awareness about forest and nature*

Subjects	Groups	n	M-Whitney U	Z	p
The most important benefit of forests is that they are the source of wood products such as timber and paper.	A B Total	60 60 120	972.000	-5.345	**0.000***
Plants play an important role in our daily life.	A B Total	60 60 120	1513.500	-2.519	**0.012***
Plants are used to produce most medicines.	A B Total	60 60 120	997.000	-5.191	**0.000***
Forestless areas should be afforested.	A B Total	60 60 120	1679.500	-1.675	0.094
Destruction of forests damages the balance in nature.	A B Total	60 60 120	1555.500	-1.941	0.052
It is very important to protect the habitats of animals and plants.	A B Total	60 60 120	1769.000	-0.401	0.689

* $p<0.05$

The students gave positive and similar answers to the following statements: “destruction of forests will damage the natural balance”, “afforestation” and “importance of protecting the habitats for wild life and plants” (*Fig. 1*). This finding indicates that student knowledge and awareness about nature and environmental protection was adequate. Mann-Whitney U test results (*Table 3*) reveal that there were not statistically significant differences between the students who participated in the project and those who did not ($p > 0.05$). This finding shows that prior knowledge regarding the importance of nature protection may raise such awareness. Similar results were reported by Ozbas (2013).

Both the students who participated in the project and those who did not stated that “listening to the voices of the organisms in the nature and smells in nature” gave one a peaceful feeling (*Fig. 2*). Students who participated in the project described the world of plants and insects as mysterious. There were statistically significant differences in the answers to questions on this matter given by the students who participated in the project and those who did not (*Table 4*). The world of plants and insects was addressed thoroughly during the project and likely facilitated this result.

One of the most important objectives of the project was to encourage the students to inquire and be curious about nature. Theoretical environmental education that is delivered in the form of note-taking is not successful (Ballantyne and Packer, 2002). For success, students should be enabled to have contact with natural and cultural elements, especially through experiential field visits (Jose et al., 2017) and outdoor activities (Jannah et al., 2013; Tesfai et al., 2016). According to Vural and Yilmaz (2016), only 19% of the positive behaviours of students about environment and nature are based on theoretical information. Practical training was delivered in this project.

A majority of the students who participated in the project agreed with the proposition, “I can walk for several kilometres in the forest to see an endangered plant”, while some of the students who did not participate in the project disagreed with this proposition (18.3%), and 26.7% was neutral (*Fig. 2*). The difference between the answers of both groups to this proposition was statistically significant (*Table 4*). Similarly, there was a statistically significant difference ($p < 0.05$) between the answers given to the proposition, "I would try to prevent anyone from damaging nature" (*Table 4*). This difference is due to the positively changing attitudes towards nature after the practical project training provided. Our results correspond with the findings of Tungac et al. (2017) and Tezel and Karademir (2014) who found that practical trainings given in nature training projects change the opinions of students about nature and forest positively.

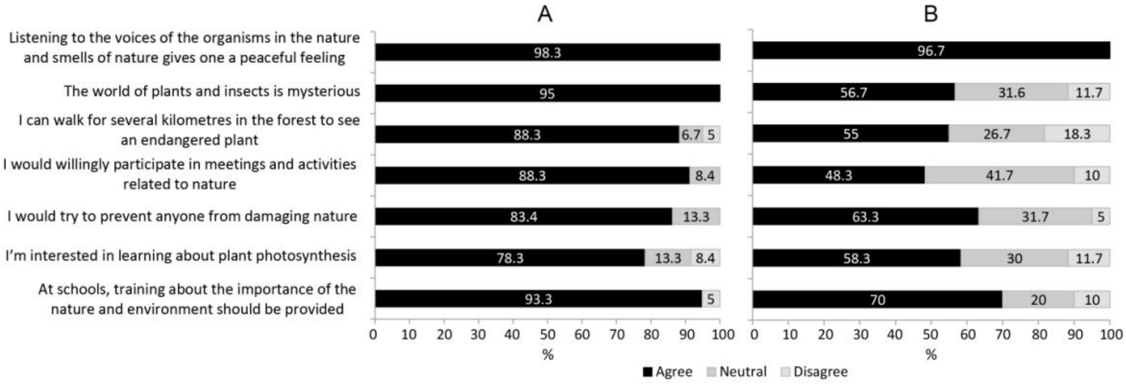

Figure 2. *Perception and attitude changes towards nature and environment (n_A=60, n_B =60, only values ≥5%)*

Table 4. *Mann Whitney-U test results for perception and attitude changes*

Subjects	Groups	n	M-Whitney U	Z	p
Listening to the voices of the organisms in the nature and smells of nature gives one a peaceful feeling.	A B Total	60 60 120	1770.000	-0.582	0.560
The world of plants and insects is mysterious.	A B Total	60 60 120	1125.500	-4.735	**0.000***
I can walk for several kilometres in the forest to see an endangered plant.	A B Total	60 60 120	1202.000	-3.968	**0.000***
I would willingly participate in meetings and activities related to nature.	A B Total	60 60 120	1090.000	-4.570	**0.000***
I would try to prevent anyone from damaging nature.	A B Total	60 60 120	1447.000	-2.404	**0.016***
I'm interested in learning about plant photosynthesis.	A B Total	60 60 120	1457.000	-2.200	**0.028***
At schools, training about the importance of the nature and environment should be provided.	A B Total	60 60 120	1395.000	-3.156	**0.002***

* $p<0.05$

A majority of the students who participated in the project (88.3%) stated that they would be willing to participate in training events and activities related to nature. However, the percentage of the students who did not participate in the project and

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agreed with this proposition was low (*Fig. 2*). The difference between the two groups was significant (*Table 4*). This finding is important because it indicates that the students participating in the project were satisfied with the training they received and were enthusiastic about participating in future nature and environmental training activities. Willingness can be used as an opportunity to raise motivated and consciousness. Importantly, this will result in students who are determined to respect the environment for a sustainable future (Zsóka et al., 2013).

Education regarding nature and the environment that is provided at schools is inadequate and thus is an important issue. Environmental education is currently incorporated into the science education at schools (Tesfai et al., 2016). However, science education deviates from the goal of developing knowledge and skills related to environmental matters and creating environmentalist behaviours (Wals et al., 2014). The relationship between science and environmental education is characterized as a distant, competitive, predator-prey and host-prey relationship (Gough, 2002). Furthermore, environmental education is transferred superficially and theoretically and thus it cannot ensure the desired behavioural and attitude changes.

Most of the students who participated in the project and those who did not agreed with the proposition, “More comprehensive education regarding the importance of the nature should be provided at schools”. This indicates that the education at school was insufficient (*Fig. 2*). Moreover, the percentage of the students participating in the project who agreed that current environmental education was insufficient was even higher. The difference between the two groups was statistically significant ($p < 0.05$).

A majority of the students who participated in the project (78.3%) was interested in photosynthesis. This rate was lower for the students who did not participate in the project. The difference between the two groups was statistically significant ($p < 0.05$). The most important reason for this was that photosynthesis was explained thoroughly both theoretically and in practical terms during the project and students gained a strong understanding about the importance of how oxygen is produced for our world.

Conclusions

TUBITAK supports many training projects under the scope of non-formal education. Similar to several other projects, this project was also useful for creating positive perceptions and attitude towards nature and the environment. Therefore, we recommended that such projects should be continued.

The current literature and the findings of this project show that training content, selection of training themes according to the level of the students, duration of the training, competence of the trainers, training methods and effective delivery of the training are important. Additionally, taking necessary steps toward environmental protection and solution of environmental problems and factors play a role in developing positive attitudes and behaviours in individuals. These are important goals and outcomes of environmental education and nature training.

Such training events are very important for promoting environmental awareness and attitudes and need to be developed and planned in terms of content, duration, trainers and practice. In this project, the trainers were selected with due diligence according to the training topics and level of the students.

When students have an opportunity to come into contact with natural objects, this plays a crucial role in creating positive changes regarding nature and the environment in nature

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training projects. In this project, the students were alone in a natural environment for seven days, during which time they engaged with nature and environment utilizing all their senses.

The main success factor for the project came about by presenting the theoretical education provided at schools in a natural environment in the form of practical training, which raised curiosity and interest. Today, due to urbanization, children who are not raised in a rural environment no longer experience the pleasure of communing with nature.

According to the research findings, the selection of sixth grade students as the target group was appropriate. Students who participated in the project developed more positive environmental awareness and attitudes compared to those who did not participate. Moreover, the project also contributed to the raising of awareness, curiosity and improvement of knowledge level regarding nature and the environment.

Subsequent generations can be made more conscious about nature and the environment through similar projects. This would be especially the case if nature and environment-oriented practical training is incorporated into the formal education curricula. Importantly, it is critical to monitor and evaluate the training to identify long-term effects.

Teachers are among the most important elements of the teaching-learning process and should also be trained regarding environmental education. This training of teachers will disseminate the effect of the projects with a multiplying effect. In other words, the environmental education at schools will become more effective in this way. Teachers who are willing to learn about plants, animals, and habitats will transfer such knowledge to their students.

Further research should be conducted on how students' environmental attitudes are reflected in their behaviours. In addition, projects and researches should be carried out to raise awareness about current environmental problems.

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COUPLING DEGREE MODELING BETWEEN SOIL AND SPECTRAL CHARACTERISTICS OF CROPS BASED ON VEGETATION INDICES AND ENTROPY THEORY

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Abstract. Quantitative inversion of soil quality is a hot topic in soil science and environmental science research, but it is difficult to obtain high-precision soil spectrum information without attachment interference. Therefore, we carried out pot experiment after testing the soil. And then based on the synergistic changes between soil quality and plants, the entropy theory and spectral vegetation indices were used to construct a parameter model of soil environmental factors and plant spectrum at different growth stages with the aid of the spectral integration of hyperspectral imaging and visualization. Parameters of plant spectrum characteristics were used to achieve the goal of indirectly indicating soil quality. The research finds that the characteristic spectral bands of plants lie near 450, 500, 520, 550, 670, 730 and 800nm. As the plant growth progressed, its spectral reflectivity gradually decreased, the red edge slope and red edge position of plant also manifested a blue shift phenomenon. Inversion is better conducted during the jointing period and MCARI/OSAVI is the optimal vegetation index. The model based on the entropy theory ($r = 0.917$, sig < 0.01) has higher inversion accuracy than the average spectral vegetation index model ($r = 0.829$, sig < 0.05) which indicates that the dual judgment model based on entropy theory and spectral vegetation index better facilitates the remote sensing monitoring research on soil quality. This research is the preliminary application of indirect inversion soil quality and condition through hyperspectral imaging technique and a result from potting and monoculture. Thus, the model still need to be tested further in order to improve its universality.
Keywords: *soil environmental factors, disaster land, MCARI/OSAVI, hyperspectral imaging, vegetation spectral features*

Introduction

On May 12, 2008 (Beijing time), the Ms8.0 major earthquake occurred in Wenchuan County, Sichuan Province, China. Earthquake and its secondary geological disasters caused damage in 73,300 hectares of cultivated land, loss of 17,700 hectares of arable land and enormous change in soil physicochemical properties and nutrient cycling in Sichuan Province (Yang et al., 2014; Zhong et al., 2016; Ebrahimi et al., 2017; Hejazi et al., 2017). Besides, it resulted in the loss of production capacity of massive cultivated land as well as agricultural use value. Furthermore, it exerts an influence on people's production and life, and restricts the speed of post-disaster recovery and reconstruction. As land is the basis for the survival and development of mankind, to restore and dynamically monitor the resources of lands damaged by disaster is the focus and hotspot of the current research (Yang et al., 2010; Wei et al., 2012; Gao et al., 2017; Hashemi,

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2017). Rapid and accurate grasp of quality conditions of soil in the damaged land is of great significance to adjust and improve the restoration program of damaged lands, the scientific management of land resources, as well as to speed up the pace of post-disaster reconstruction. The traditional soil quality monitoring is mostly carried out by methods such as field sampling and laboratory chemical analysis, etc. Although highly precise, they are time-consuming and costly, unable to monitor dynamically from the macroscopic point of view. However, hyperspectral image technology integrates optics, electronics, information processing with computer science and technology. And combined with the traditional two-dimensional imaging technology and spectral technology, it is characterized by numerous bands, high spectral resolution and image-spectrum emerging, which can directly or indirectly acquire a large number of continuous high-resolution spectral image data, so as to meet the extensity of space and the continuity of time well (Yu, 2012; Hassan et al., 2017; Radan et al., 2017). Hyperspectral imaging stores data in the form of three dimensions, and each pixel is attached to its own spatial location information and single complete radiation or emission spectral information formed by a set of consecutive wavelengths. Based on the spectral information, the physiochemical characteristics of the object can be identified (Ma, 2015). At present, a lot of research results have been achieved on the application of hyperspectral imaging technology in these aspects such as quantitative inversion of soil environmental factors, crop growth monitoring under stress, plant identification and classification and extraction of mineral alteration substance, which provides technical means for dynamic monitoring of soil quality. But these studies mainly focus on the soil in the exposed areas (Vazdani et al., 2017). And the quantitative inversion of the soil environmental condition in the vegetation-covered area is still in the exploratory stage. The main reason is that it is difficult to remove the interference of the attachments when the soil spectrum information is obtained in the vegetation-covered areas and is hard to get the soil spectral information with high-precision (Fernández-Buces et al., 2006; Tilley et al., 2007; Wun et al., 2017).

As the plant leaf tissue changes with the soil nutrient status, biomass and phenological period, spectral characteristics change as well. The spectral reflectance of the plants at 400-700 nm, 680-760 nm and 780-1300 nm has high correlation with surface index, leaf pigment and substance content (Gu et al., 2008; Xiao et al., 2017), thus the plants physiological parameters have been used to indirectly invert soil indicator factors. Walburg et al. (1982) studied the spectral reflectance characteristics of maize (*Zea mays* L.) canopy under four different nitrogen stress conditions and found that it is feasible to monitor crop growth through the analysis of maize canopy spectral characteristics. After studying the hyperspectral reflectance, red edge parameters and physiological and ecological parameters of winter wheat (*Triticum aestivum* L.) under different water stress, found that the red edge area decreased with the increase of drought stress, therefore the hyperspectral characteristics and red edge parameters of winter wheat can help decide the growth of winter wheat and the degree of water stress (Gu et al., 2008). Lu et al. (2007) studied the heavy metal stress of vegetation in Dexing Copper Mine with hyperspectral data. It was found that heavy metal stress caused the phenomenon “red edge” blue shift of vegetation reflectance spectrum. The amount of blue shift is closely related to the degree of heavy metal stress: the more the blue shifts, the more heavy metal content in vegetation canopy there will be and the deeper the vegetation is poisoned. Therefore, the amount of blue shift can indicate the status of soil environmental quality in mining areas. Chen et al. (2012) found that there was a

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difference in the spectral absorption depth of plants under heavy metal stress; therefore, a multiple regression model of heavy metal and absorption depth between 550 and 760 nm was established (Yang et al., 2017). And they considered it is feasible to use the hyperspectral remote sensing to evaluate the distribution and enrichment of heavy metal elements in vegetation-covered areas. Jia and Zhang (2014) studied the spectral characteristics of sunflowers (*Helianthus annuus* L.) at different growth periods on soils alkalized to different degrees. According to the study, the reflectance of canopy spectra varied with growth periods. The red edge position and red edge slope showed the blue shift phenomenon as soil alkalinity rose, and the wavelength and amplitude reached the maximum during the flowering period. The correlation coefficient between the red edge position and the soil alkalinity and pH was 0.768 and 0.681 respectively, which showed a significant positive correlation and the soil alkalization was indicated by the red edge feature of the vegetation spectrum.

A vegetation index is composite spectral information composed of multiple bands, which not only has higher sensitivity than a single band, but also reduces the background error (Zhang et al., 2011). Therefore, vegetation indices have been widely used in the inversion of hyperspectral remote sensing quantification. Bunnik (1978) used a great number of vegetation indices to invert LAI（Leaf Area Index）, confirming the possibility of remote sensing technology in extracting vegetation cover and LAI. Zhang et al. (2011) obtained the hyperspectral images of maize in potted trial and in field using near-ground imaging hyperspectral spectrometer, and established the relationship model between vegetation indices of TCARI (Transformed chlorophyll absorption in reflectance index), OSAVI, CARI (chlorophyll absorption in reflectance index) and maize chlorophyll content. The results showed that MCARI/OSAVI was remarkably correlated with chlorophyll content of potted and field maize. Liu et al. (2011) studied the relationship between the spectral vegetation indices of *Suaeda salsa* and soil chemical properties. It was found that NDVI (Normalized Difference Vegetation Index) could be an elementary indicator of the changes in soil total phosphorus and pH, vegetation indices could well reflect the change of soil salinity's content.

Information entropy is proposed by Shannon in 1948 based on probability theory. It is a measure of the uncertainty of random variables (Shannon 1949). It is widely used in data set segmentation, image quality evaluation, classification decision-making and total pollutant distribution. As a measure of the uncertainty or amount of information of a random variable, the entropy function can be used to calculate the correlation between spatially discrete variables, so as to evaluate the balance of the system. The greater the differences between individuals, the smaller the information entropy and the more imbalanced the system, so information entropy can be used to evaluate crop growth. Influenced by soil environmental factors, crop leaves are a collection of non-equilibrium systems. The worse the soil quality is, the more unstable the crop growth environment will be (Zhang et al., 2005; Wang et al., 2009).

In conclusion, hyperspectral imaging and spectral vegetation index have made a lot of research results in quantitative inversion of soil environmental factors, but the vegetation spectral characteristics are used less to indirectly inverse soil quality and condition and even less especially when combined with remote sensing images after a series of mathematical transformation treatments to indirectly inverse the soil quality. In this paper, the soil samples of regional damaged land were collected from the Duba River basin in Beichuan County, and the contents of pH, available nitrogen, available potassium, available phosphorus and content of organic matter in soil samples were

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analyzed and tested. In addition, the hyperspectral imaging techniques were used to collect maize canopy and single leaf spectral information, based on the "red edge" effect and spectral variation characteristics, the hyperspectral imaging technique was utilized to construct the relationship model of soil environmental factors in disaster-stricken land and crop spectrum characteristics from the perspective of the entropy theory and spectral vegetation indices. Through the analysis of their coupling relationship, this paper tries to indirectly determine the soil environmental quality in disaster-stricken land by means of the physiological characteristics of the crops, which may facilitate rapid and real-time access to the degree of damage so as to offer a study method for the restoration and utilization of disaster-stricken land as well as the soil quality monitoring.

Materials and methods

Overview of the research area

Located in the eastern part of Beichuan County and seated in the Longmen Mountains, the Duba River Basin is selected as the typical investigation area (*Figure 1*).

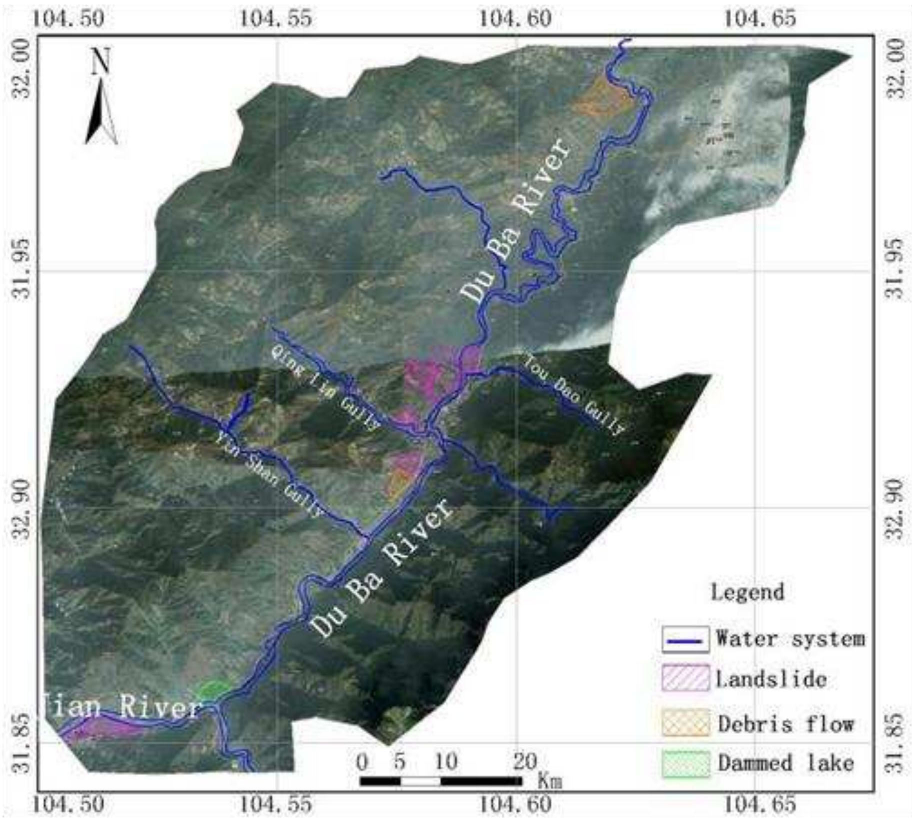

***Figure 1.** Distribution map of disaster-stricken land. The blue line represents the river distribution, the purple area indicates the land damaged by landslide, the yellow area represents the land damaged by debris flow, the green area represents the land damaged by the barrier lake.*

Geographical coordinates are 104° 30' 37.01" ~ 104° 37' 39.09" E, 31° 51′ 35.88″ ~ 31° 59' 30.03" N. Characterized by high mountains and deep valleys, the territory has complex underlying lithology and geological structure and therefore has strong tectonic

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movements. The study area is mainly mountain, with north subtropical humid monsoon climate. The average temperature for years is 15.6 °C, and the average precipitation is 1300 mm. Vegetation mainly include *Cupressus funebris*, *Pinus massoniana*, and *Pteridium aquilimum*, etc. Soil contains *loess*, *brown earth* and *yellow brown earth*. Crops in this area mainly include maize and Sweet potatoes (*Ipomoea batatas* L.). Due to the Wenchuan earthquake and secondary geological hazards, multiple landslides, debris flows, dammed lakes, collapse and other geological disasters are distributed across the watershed, which result in serious water loss and soil erosion in this region, also cause bad ecological environment, soil fertility decline, loss of farming ability to some cultivated land, and intense relationship between land and people. This area is seriously affected by Wenchuan earthquake, having the typical and common characteristics of land damaged by disaster, therefore it is chosen as the study area.

Soil sampling and testing

The checkerboard pattern sampling method is adopted to collect multipoint mixed soil samples along the river basin, including landslides, debris flows and dammed lakes. *Table 1* represents the sample location located by GPS.

Table 1. *Soil profile and central coordinates in sampling area*

Sampling point	Soil texture	Damage type	Central coordinates
1#	Sandy soil	Landslide	104°37′9.54″ E, 31°59′15.61″ N
2#	Sandy soil	Landslide	104°37′12.09″ E, 31°59′16.82″ N
3#	Sandy soil	Landslide	104°37′16.5″ E, 31°59′19.05″ N
4#	Clay	Landslide	104°35′31.13″ E, 31°56′5.16″ N
5#	Clay	Landslide	104°35′32.04″ E, 31°56′17.12″ N
6#	Sandy soil	Landslide	104°35′9.3″ E, 31°56′41.44″ N
7#	Sandy soil	Landslide	104°35′14.98″ E, 31°56′33.65″ N
8#	Clay	Landslide	104°35′19.89″ E, 31°56′33.90″ N
9#	Sandy soil	Landslide	104°34′53.85″ E, 31°55′22.31″ N
10#	Sandy soil	Landslide	104°34′55.05″ E, 31°55′25.2″ N
11#	Sandy soil	Debris flow	104°34′46.59″ E, 31°55′1.04″ N
12#	Clay	Debris flow	104°34′48.72″ E, 31°54′46.81″ N
13#	Clay	Debris flow	104°34′40.34″ E, 31°54′38.77″ N
14#	Sandy soil	Dammed lakes	104°32′30.97″ E, 31°52′16.62″ N
15#	Clay	Debris flow	104°31′19.81″ E, 31°51′48.87″ N
16#	Clay	Landslide	104°35′13.75″ E, 31°56′9.55″ N

The Analysis and Determination Center of Sichuan Provincial Academy of Agricultural Sciences is entrusted to conduct test for the 5 environmental factors of the soil samples. The available nitrogen is measured with Alkali-diffusion Method according to the forestry industry standards of the People’s Republic of China standard LY/T 1228-2015. The available phosphorus is measured by inductively coupled plasma atomic emission spectrometry according to LY/T 1232-2015. The available potassium is measured by atomic absorption spectrophotometer after the sample soil is extracted by ammonium acetate of 1mol/L according to LY/T 1234-2015. The organic matter is measured

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according to NY/T 1121.2-2006: first to oxidize the organic carbon with the excessive potassium dichromate sulfuric acid solution, then to titrate by ferrous sulfate standard solution, so to calculate the amount of organic carbon by the consumption amount of potassium dichromate by oxidation correction coefficient, then to multiply with the constant 1.724. pH is measured by pH meter according to NY/T 1121.2-2006 (*Table 2*).

Table 2. *Soil environmental factor content of damaged land*

Environmental factor	Max	Min	Mean	Standard deviation
pH-x_1	7.98	6.69	7.58	0.34
Available N-x_2 （*mg/kg*）	151.00	15.00	56.65	40.82
Available K-x_3 （*mg/kg*）	325.00	39.20	93.25	64.06
Available P-x_4 （*mg/kg*）	72.00	3.20	23.11	22.62
DOM-x_5 （*g/kg*）	43.70	3.50	16.36	9.58

Experimental design

Experimental design is based on improved previous studies (Zhang et al., 2011; Yang et al., 2015). The experiment was carried out in the teaching and research base of the Department of Landscape Architecture in Chengdu University of Technology. First, pH, available nitrogen, available phosphorus, available potassium and dissolved organic matter (DOM), altogether five environmental factor content in the sample soil were measured in the Analysis and Determination Center of Sichuan Academy of Agricultural Sciences (*Table 1*). Then soil samples of the same quality were taken for the cultivation of potted maize plants, and each soil sample was divided into two, one of which was added with 60 g of farmyard manure as a control (Zhang et al., 2015), thus total 2 groups (the original group and the control group). Antai 5000 was chosen as the maize variety. In order to eliminate the system error, three maize seeds were planted on each pot, and the planting time was May 17th, 2016. The corn was watered regularly and sprayed pesticides to prevent pests and diseases without any fertilizer in the process of growth. The hyperspectral image data of maize leaves were collected at the jointing period and tasseling period respectively.

Hyperspectral data acquisition and processing

The spectral data were collected from near-ground imaging by the hyperspectral imaging system of the imaging spectrometer Hyspex of the Norwegian company NEO. The spectral range was 400-1000 nm and the number of bands was 108. Hyperspectral images of maize canopies and single leaves were collected in two periods (jointing and tasseling). The last three single leaves were collected during the jointing period while the last four were gathered during the tasseling period, producing a total of 128 hyperspectral images.

Since the acquired hyperspectral images were noisy, the original images must be corrected in black and white, and the correction formula is as follows (*Eq. 1*).

$$R = \frac{R_{img} - R_{dark}}{R_{ref} - R_{dark}} \quad \text{(Eq. 1)}$$

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where R is the corrected hyperspectral image, R_{img} is the original hyperspectral image, R_{dark} is the black calibration image obtained by turning off the camera lens, and R_{ref} is the whiteboard calibration image obtained from the standard white calibration plate.

First-order differential processing was applied to the original spectrum, and the formula is as follows (*Eq. 2*).

$$\rho' = \frac{\mathrm{d}\rho(\lambda_i)}{d\lambda_i} = \frac{\rho(\lambda_{i+1}) - \rho(\lambda_{i-1})}{2\Delta\lambda} \qquad \text{(Eq. 2)}$$

where i is the spectral channel, λ_i is the wavelength of each band, $\Delta\lambda$ is the interval of wavelength from λ_{i-1} to λ_i, $\rho(\lambda_i)$ is the reflectance of band λ_i, and ρ' is the first-order differential spectrum of λ_i.

Information entropy calculation

Shannon first introduced the concept of entropy into the theory of information and defined it as information entropy. For an uncertain system, X is the state feature. For an discrete random variable, set the value of x as $X = \{x_1, x_2, x_3, \ldots x_n\}$ ($n \geq 2$), the corresponding probability of each value is denoted by P, $P = \{p_1, p_2, p_3, \ldots p_n\}$ ($0 \leq p_i \leq 1$, $i = 1, 2, 3, \ldots n$, and $\sum_{i=1}^{n} p_i = 1$). Then there is the formula (*Eq. 3*):

$$S = -\sum_{i=1}^{n} p_i \ln p_i \qquad \text{(Eq. 3)}$$

Results and discussion

Analysis of curve spectral characteristics

The hyperspectral images were processed to derive 64 average hyperspectral curves of 16 groups in two periods. Noise was found in the original spectrum, and it was the most obvious after 900 nm (Zou et al., 2011). In addition, the edge of the blade would overlap with the background due to the high spectral energy after 850 nm and supersaturated image information, so the range of the study was determined to be 400-900 nm. *Figure 2 (a), 2 (b)* are curves of noise reduction and differential treatment of the original spectral curves respectively. *Figure 2 (a)* shows a significant reflection peak near 550, 800 and 880 nm, a significant absorption valley near 670 and 820 nm, a sharp rise in reflectance between 670 and 750 nm, and a higher overall reflectance after 750 nm. These kinds of conclusions are also found in the research on pumpkin (*Cucurbita moschata*) leaves by Zhao et al. (2014). *Fig. 2 (b)* shows that during the jointing period, there are reflection peaks near 450, 520, 600, 730 and 850 nm, especially in the vicinity of 520 and 700 nm while there are three absorption valleys near 580, 655 and 810 nm. During the tasseling period, there is no obvious reflection peak near 450 and 850 nm, the absorption valley at 810 nm also disappears.

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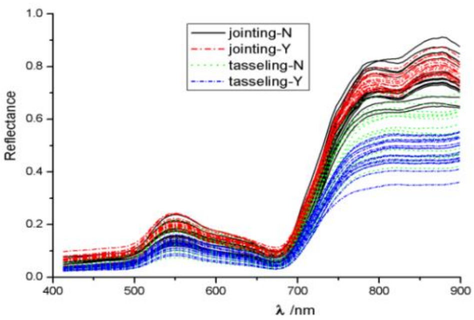
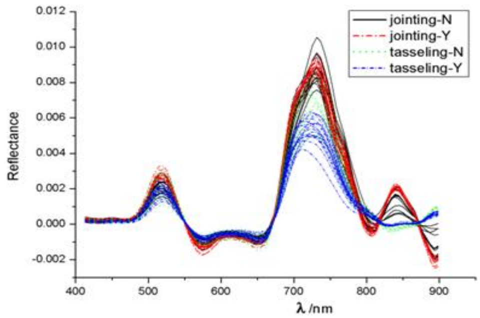


(a) The original spectral reflectance curve of blade

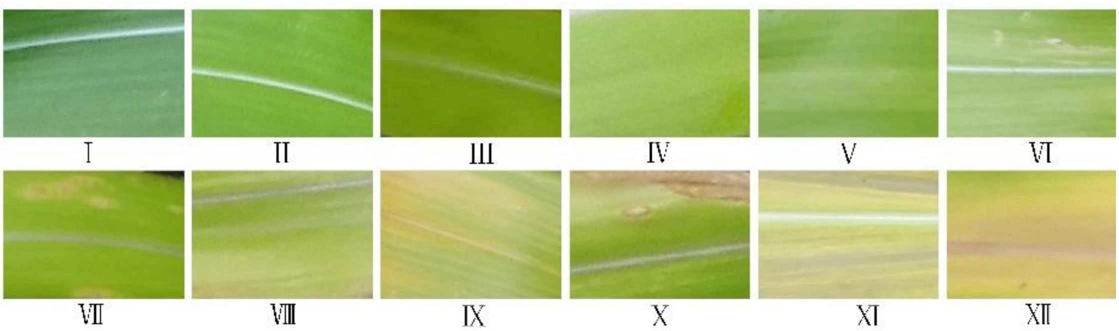


(b) First-order differential reflectance curve of blade

***Figure 2.** Spectral reflectance curve of blade during jointing and tasseling. The black line indicates the spectral curve of unfertilized corn leaves in the jointing stage jointing-N. the red one represents the spectral curve of fertilized corn leaves in the jointing stage jointing-Y. the green one demonstrates the spectral curve of unfertilized corn leaves in the tasseling stage tasseling-N. and the blue one represents the spectral curve of fertilized corn leaves in the tasseling stage tasseling-Y.*

A comparison of the four groups of spectral curves during the two periods (jointing period fertilization group and non-fertilization group, tassel period fertilization group and non-fertilization group are represented by jointing-Y, jointing-N, tasseling-Y and tasseling-N, respectively) revealed that during the jointing period, the reflectance of the fertilization group was significantly higher than that of the untreated group, especially after 730 nm, while the difference was less obvious during the tasseling period. The “red edge” position in the first-order differential spectrum was found near 730 nm, and the red edge position as well as the red edge slope of the tasseling period showed a significant blue shift.

Figure 3 (a) is a selection of maize leaves of different growth conditions (or morbid leaves) at different periods and different leaf positions in different soil environments. *Figure 3 (b)* and *Figure 3 (c)* are corresponding reflectance curves and first-order differential transformation spectral curves. By comparison we found that the reflection peak near 450 nm and the absorption valley near 800 nm has disappeared. The worse the crop grows, the more gentle the curve near 550-670 nm is with no obvious reflection peak, in the 670-740 nm “red edge” rising area, the less obvious the red edge feature is with obvious blue-shift phenomenon, which is mainly due to differences in chlorophyll content. A composite analysis of *Figure 2* and *Figure 3* shows that the relevant bands may be near 450, 500, 520, 550, 670, 730 and 800 nm.



(a) Leaves of different conditions

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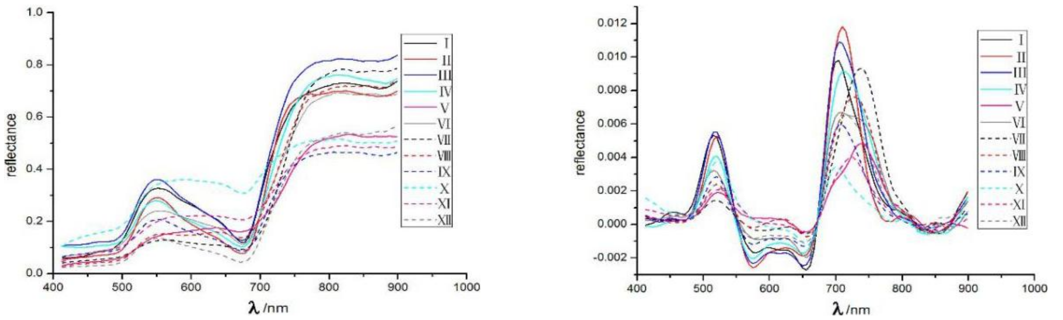

(b) The original spectral reflectance curve of leaves with different conditions

(c) First-order differential reflectance curve of leaves with different conditions

Figure 3. *Comparison of spectral reflectance of leaves in different conditions. Curves I-XII. in Figure (b, c) are the original and first-order differential spectral curves corresponding to the leaves I-XII. in Figure (a).*

Analysis of the correlation between average spectral vegetation indices and soil environmental factors

After reviewing the relevant literature and analyzing characteristic bands, six vegetation indices with better predictive effects were preliminarily screened from multiple spectral vegetation indices for the study, as shown in *Table 3*.

Table 3. *Spectral vegetation indices and references*

NO.	vegetation indices	Reference
1	NDVI(y_1)=(R_{800}-R_{670})/(R_{800}+R_{670})	Buschmann and Nagel, 1993
2	SIPI(y_2)=(R_{800}-R_{450})/(R_{800}+R_{450})	Penuelas et al., 1995
3	PRI(y_3)=(R_{550}-R_{531})/(R_{550}+R_{531})	Gamon et al., 1992
4	MTCI(y_4)=($R_{753.75}$-$R_{708.75}$)/($R_{708.75}$-$R_{681.25}$)	Dash and Curran, 2004
5	PSNDa(y_5)=(R_{800}-R_{680})/(R_{800}+R_{680})	Blackburn, 1998
6	MCARI/OSAVI(y_6)=[(R_{700}-R_{670})-0.2×(R_{700}-R_{550})]×(R_{700}/R_{670})/[1.16×(R_{800}-R_{670})/(R_{800}+R_{670}+0.16)]	Daughtry et al., 2000

Linear regression models of each vegetation index and soil environmental factors show that the correlation between a single environmental factor and vegetation index is small, and it is difficult to quantitatively invert or indicate the content of some soil environmental factor through vegetation indices. However, the correlation between the five environmental factors and vegetation indices is greatly improved, and it is proved that the crop growth is not controlled by a certain environmental factor, but is the result of the interaction of various environmental factors, as shown in *Table 4*.

It can be seen From *Table 4* that the correlation between vegetation indices and soil environmental factors during the jointing period was the highest, and the correlation between improved groups was low, indicating that fertilization affects the crop growth by changing the environment and thus changes the physiological spectrum of the crop. but the difference between jointing period and tassel period is obvious, which is similar to the results of the researches by others (Yang et al., 2017; Pan et al., 2015). The correlation during the jointing period was higher than that of the tasseling period, mainly because as the crop growth progresses, the demand for nutrients increases while

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nutrients in the soil are reduced, meanwhile, the effects of other factors in the growing environment play an increasingly significant role, leading to crop morbidity. The three vegetation indices of SIPI (Structure Insensitive Pigment Index), MTCI (MERIS terrestrial chlorophyll index) and MCARI/OSAVI were best correlated with soil environmental factors, and the correlation coefficient r was 0.812, 0.823 and 0.829 respectively, especially for non-fertilization group during the jointing period at a level of 0.05. Therefore, the three vegetation indices were identified as the optimal vegetation indices for further study.

Table 4. *Coupling relationship model and evaluation of average spectral vegetation indices and environmental factors*

Vegetation indices	Growing period	r	Linear regression model	Sig.
NDVI(y_1)	Jointing-N	0.581	y_1=-0.002x_1-0.007x_2-3.838×$10^{-5}$$x_4$+0.036$x_5$+0.849	0.456
	Jointing-Y	0.475	y_1=0.003x_1+0.005x_2-0.02x_5+0.775	0.714
	Tasseling-N	0.611	y_1=0.022x_1-0.004x_2+0.04x_5+0.66	0.38
	Tasseling-Y	0.502	y_1=0.022x_1-6.637×$10^{-5}$$x_2$+0.001$x_4$+0.015$x_5$+0.659	0.652
SIPI(y_2)	**Jointing-N**	**0.812**	**y_2=-0.003x_1-2.34×$10^{-7}$$x_2$+8.99×$10^{-5}$$x_3$+5.94×$10^{-5}$$x_4-0.003x_5$+ 0.85**	**0.033**
	Jointing-Y	0.745	y_2=-0.003x_1+4.6×$10^{-5}$$x_2$-3.94×$10^{-5}$$x_3$-1.54×$10^{-6}$$x_4-0.001x_5$+1.05	0.103
	Tasseling-N	0.566	y_2=-0.001x_1+0.001x_2+5.08×$10^{-5}$$x_3$+6.89×$10^{-5}$$x_4-0.008x_5$+1.03	0.494
	Tasseling-Y	0.567	y_2=-0.006x_1-2.965×$10^{-5}$$x_2-0.004x_5$+1.072	0.492
PRI(y_3)	Jointing-N	0.47	y_3=-0.005x_1+2.327×$10^{-5}$$x_3$-1.843×$10^{-5}$$x_4$+0.001$x_5$+0.850	0.723
	Jointing-Y	0.684	y_3=-0.001x_1+4.636×$10^{-5}$$x_3$-3.759×$10^{-5}$$x_4-0.002x_5$+0.038	0.209
	Tasseling-N	0.186	y_3=5.94×$10^{-8}$$x_3$-3.451×$10^{-5}$$x_4-0.001x_5$+0.052	0.995
	Tasseling-Y	0.234	y_3=-0.003x_1+1.62×$10^{-5}$$x_2$-1.6×$10^{-5}$$x_3$-1.91×$10^{-5}$$x_4$+0.001$x_5$+0.034	0.986
MTCI(y_4)	**Jointing-N**	**0.823**	**y_4=-0.09x_1-0.08x_2-0.003x_3-0.001x_4+0.479x_5+2.909**	**0.025**
	Jointing-Y	0.499	y_4=0.186x_1+0.025x_2-0.015x_5+0.515	0.66
	Tasseling-N	0.719	y_4=0.644x_1-0.114x_2-0.007x_3-0.003x_4+0.986x_5-2.8927	0.143
	Tasseling-Y	0.643	y_4=0.123x_1+8.173×$10^{-5}$$x_2-0.004x_3$+0.009$x_4$+0.115$x_5$+1.179	0.3
PSND$_a$(y_5)	Jointing-N	0.592	y_5=-0.002x_1-0.007x_2+3×$10^{-5}$$x_4$+0.036$x_5$+0.833	0.427
	Jointing-Y	0.501	y_5=-0.022x_1+0.001x_4+0.015x_5+0.652	0.655
	Tasseling-N	0.611	y_5=0.022x_1-0.004x_2+0.039x_5+0.657	0.38
	Tasseling-Y	0.501	y_5=0.022x_1-7.662×$10^{-5}$$x_2$+0.001$x_4$+0.015$x_5$+0.652	0.655
MCARI/OSAVI (y_6)	**Jointing-N**	**0.829**	**y_6=0.004x_1+0.001x_2+4.26×$10^{-4}$$x_3$+6.299×$10^{-5}$$x_4-0.065x_5$ +0.228**	**0.022**
	Jointing-Y	0.6	y_6=-0.045x_1+0.001x_2-0.016x_5+0.665	0.407
	Tasseling-N	0.713	y_6=-0.056x_1+0.014x_2+0.001x_4-0.108x_5+0.207	0.154
	Tasseling-Y	0.597	y_6=-0.01x_5+0.235	0.415

Image visualization and analysis

Band Math of ENVI was used for band operations and the results were graded according to *Table 5,* and leaf visualization distribution was generated through the image processing technology. As shown in *Figure 4,* the grading distribution of SIPI were more disorderly with an overall lower grade on the left side of the veins and the leaf edge while a higher grade on the right side, the whole leaves were dominated by

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grade 4, MTCI presented an upgrade from the blade edge to the center area with more distinctive leaf grade distribution, and most parts were classified as grade 5.

Table 5. *Grading table of vegetation indices*

Vegetation indices	**Grade**				
	1	**2**	**3**	**4**	**5**
SIPI	<1.00	1.00～1.05	1.05～1.10	1.10～1.15	>1.15
MTCI	<4.06	4.06～7.52	7.52～10.98	10.98～14.44	>14.44
MCARI/OSAVI	<0.17	0.17～0.28	0.28～0.39	0.39～0.50	>0.50

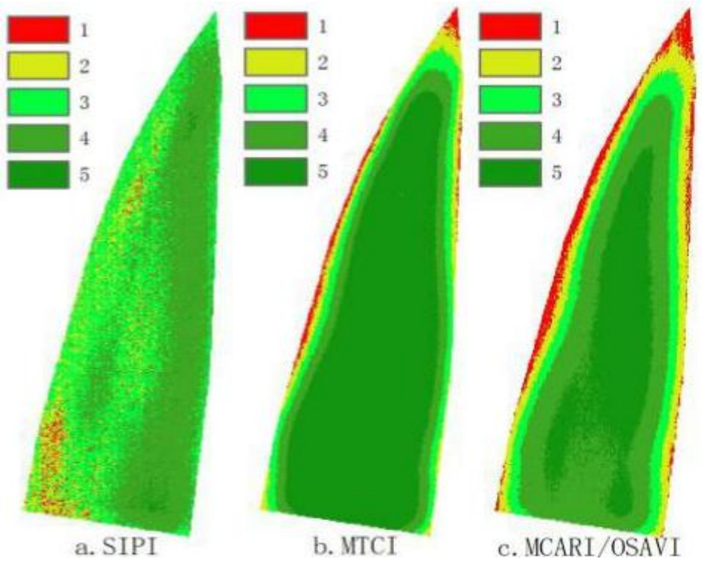

Figure 4. *Comparison of classification results. The following are three grading results comparison figures: a. the grading results through band operation by SIPI; b. the grading results through band operation by MTCI; c. the grading results through band operation by MCARI/OSAVI.*

When studying the chlorophyll content in the leaves of cucumber (*Cucumis sativus* L.) and pumpkin, other scholars found that the chlorophyll content in the leaf edge was significantly lower than that in other regions. More healthy the leaves are, more uniform the chlorophyll distributes (Zhou et al., 2014). MCARI/OSAVI had certain similarities with MTCI such as clearer distribution, lower grade on the blade edge, higher grade at the center yet dominated by grade 4 and 5. Finally, the ENVI tools were used for statistics of all grades.

Correlation analysis of entropy and soil environmental factors

Calculate the entropy (*S*) of each group, as shown the results in *Table 6,* the entropy values varied with growth periods. The standard deviation of the fertilization group was lower than that of the non-fertilization group based on the three vegetation indices, indicating that differences of spectral reflectance among the fertilization group was smaller. Changes in soil environmental factor content have an effect on crop growth process, which can be well indicated by the differences in spectral characteristics and entropy.

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Table 6. *Calculation results of S*

Vegetation indices	Growing period	Max	Min	Mean	Standard deviation
SIPI	Jointing-N	0.686	0.172	0.382	0.140
	Jointing-Y	1.088	0.486	0.754	0.102
	Tasseling-N	1.108	0.415	0.806	0.195
	Tasseling-Y	2.112	0.576	0.975	0.168
MTCI	Jointing-N	0.950	0.365	0.657	0.151
	Jointing-Y	0.600	0.300	0.429	0.102
	Tasseling-N	1.469	0.654	0.910	0.210
	Tasseling-Y	1.121	0.523	0.728	0.168
MCARI/OSAVI	Jointing-N	2.318	2.107	2.239	0.057
	Jointing-Y	2.301	2.022	2.216	0.046
	Tasseling-N	2.302	1.840	2.146	0.129
	Tasseling-Y	2.299	1.860	2.100	0.125

The linear regression model of entropy and soil environmental factors was established, as shown in *Table 7,* the correlation between crop spectral characteristics and soil environmental factors based on entropy principle was higher than that calculated in *Table 4,* MCARI/OSAVI, in particular, reached 0.917 (Sig < 0.01), much greater than the previous 0.829 and more stable. In the study of the relation between maize chlorophyll and spectral vegetation indices, it is found that MCARI/OSAVI is the best vegetation indices in modeling (Zhang et al., 2011). Based on all of the three vegetation indices, the correlation between jointing-N and the soil environmental factors was higher than that of other groups, which is in agreement with the analysis results in Results section, indicating that the jointing period is the best inversion period. pH, available nitrogen and organic matter are most closely related to crop growth according to the correlation model.

Table 7. *Correlation model and assessment of S and soil environmental factors*

Vegetation indices	Growing period	r	Linear regression model	Sig.
SIPI	**Jointing-N**	**0.824**	$\mathbf{y_1=0.023x_1+0.004x_2+0.002x_3+2.84\times10^{-4}x_4-0.023x_5+0.187}$	**0.025**
	Jointing-Y	0.386	$y_1=-0.002x_1-0.001x_2+0.013x_5+0.7$	0.870
	Tasseling-N	0.764	$y_1=-0.023x_1+0.003x_2+0.005x_4-0.016x_5+1.022$	0.077
	Tasseling-Y	0.359	$y_1=-0.223x_1+0.002x_2+0.001x_3-0.005x_4-0.014x_5+2.803$	0.904
MTCI	**Jointing-N**	**0.758**	$\mathbf{Y_4=-0.235x_1-0.004x_2-9.09\times10^{-4}x_3+4.06\times10^{-4}x_4+0.015x_5+2.555}$	**0.085**
	Jointing-Y	0.495	$Y_4=0.025x_1+0.002x_4-0.001x_5+0.242$	0.668
	Tasseling-N	0.721	$Y_4=0.023x_1-0.005x_2-0.003x_3-9.271\times10^{-5}x_4+0.03x_5+0.769$	0.139
	Tasseling-Y	0.629	$Y_4=-0.249x_1-0.002x_2-2.312\times10^{-5}x_3+0.007x_4-0.006x_5+2.673$	0.127
MCARI/OSAVI	**Jointing-N**	**0.917**	$\mathbf{Y_6=0.081x_1+0.002x_2+4.26\times10^{-4}x_3-4.42\times10^{-4}x_4-0.008x_5+1.618}$	**0.001**
	Jointing-Y	0.575	$Y_6=-0.042x_1+1.772\times10^{-5}x_4+0.003x_5+2.56$	0.472
	Tasseling-N	0.660	$Y_6=0.014x_1+0.003x_2+0.002x_3-0.002x_4-0.014x_5+2.018$	0.262
	Tasseling-Y	0.652	$Y_6=0.134x_1-0.002x_2+0.001x_3-0.004x_4+0.008x_5+0.992$	0.280

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Figure 5 is the linear fitting of Soil Environmental Composite Index (SECI) and entropy (*S*) according to the correlation model during the jointing period based on vegetation index MCARI/OSAVI with a calculated correlation of 0.917. It can be seen that the composite index of soil environmental factors was positively correlated with the entropy. The maximum composite index was 2.312 when the corresponding pH of the soil samples was 7.49, the optimum pH value of crop growth, and the remaining four soil environmental factors were 151, 325, 72 mg/kg and 43.7 g/kg respectively, all reaching the respective maximum in collected soil samples, and the soil fertility levels reached the first grade. The minimum composite index was 2.126 when the corresponding pH of soil samples was 6.85, meeting the pH requirements for crop growth as well, but the other four soil environmental factors were 21, 97.8, 2.6 mg/kg and 15.9 g/kg respectively, smaller than other soil samples, and soil fertility levels were classified as grade Ⅳ. The comparison of the entropy, soil environmental composite index and contents of five soil environmental factors once again proves that the crop spectrum vegetation indices are more accurate in indicating soil quality in disaster-stricken land.

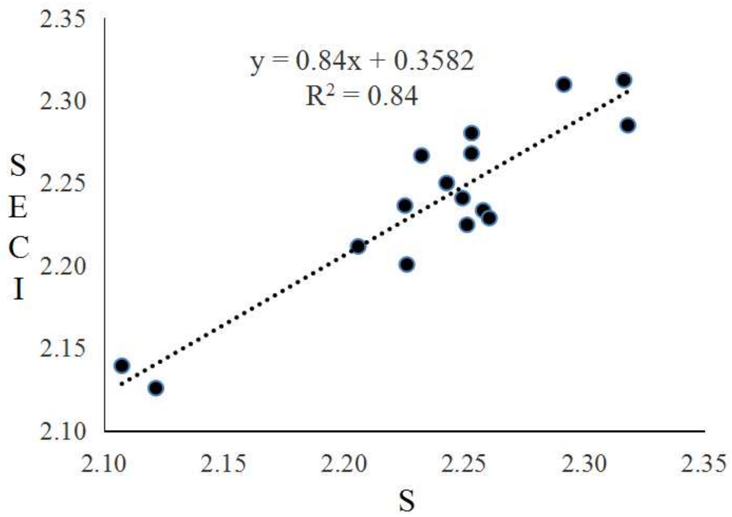

Figure 5. *Linear fitting of entropy S and SECI*

Conclusions

Through the analysis of original reflectivity spectral curves and first-order differential spectral curves, we found that the characteristic spectral bands of the crop were near 450, 500, 520, 550, 670, 730 and 800 nm, and the red edge was near 730 nm. As the crop growth progressed, its spectral reflectivity gradually decreased, and the red edge position together with red edge slope of crop showed a blue shift phenomenon. Taking characteristic spectral bands into consideration, six vegetation indices, such as SIPI and MCARI/OSAVI, were selected to establish the linear regression model of average spectral vegetation index and soil environmental factors, and the differences between the fertilization group and the non-fertilization group were significant, which indicates that the physiological characteristics of the crop were related to the soil environmental factors. The correlation coefficients of the three average spectral vegetation indices of SIPI, MTCI and MCARI/OSAVI in jointing-N group were 0.812, 0.823 and 0.829, respectively, pointing to a high coupling degree. Based on the entropy

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theory, a linear regression model of entropy and soil environmental factors was constructed by using visualization technology to generate pseudo-color vane images from which we learn that MCARI/OSAVI is the optimal vegetation index, and the model based on the entropy theory ($r = 0.917$, sig < 0.01) has higher inversion accuracy than the widely used average spectral vegetation index model ($r = 0.829$, sig < 0.05). Then the SECI-S linear regression model was further established and verified against the measured values one by one. It was found that the SECI was positively correlated with the S, and the predicted results were highly coupled with the actual condition of soil quality. Therefore, the model based on the entropy theory is more accurate, which may well contribute to the study of indirect indication of soil quality through crop physiological spectral variations. The study is based on the double decision model of entropy theory and the spectral vegetation indices, employing the hyperspectral imaging techniques to indirectly invert the soil quality, which is different from the previous research methods. But the result is deduced from potting and single crop varieties, so the model needs to be tested in field experiments and multiple crop varieties to improve the universality of the model. We hope to expand the advantages of the application of hyperspectral imaging in soil quality monitoring in the following study, to serve the agricultural production and post-disaster reconstruction.

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COMPARISON BETWEEN THE LINEAR MODEL AND K-NEAREST NEIGHBOR METHOD FOR PREDICTING MACROINVERTEBRATE ASSEMBLES IN A CITY RIVER IN BEIJING, CHINA

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Abstract. Benthic macroinvertebrates play an important role in materials and energy flow in river ecosystems. In this paper, we built models, a linear model and k-nearest neighbor method, for predicting biodiversity of macroinvertebrates in a city river using the data from Wenyu River. Both Shannon-Wiener index and Simpson index were considered for measuring the biodiversity of macroinvertebrates. The observed data of macroinvertebrates and 12 water quality indicators in Wenyu River, from 2010 to 2012, were applied in building and validating the predicted models. The results indicated that 1) The validity of the linear model was, though not perfect, better for predicting macroinvertebrates diversity using water quality indicators than k-nearest neighbor method in a city river; 2) Simpson index was more robust and accurate than the other biodiversity index to act as the variable of predicting benthic macroinvertebrates in a city river. There were 89.47% observations within the 99% confidence intervals. The developed predictive model was a useful tool for assessing river health, especially city river health, without taking into account the abundances of invertebrates.
Keywords: *macroinvertebrates, biodiversity, predicting models, city river, Beijing*

Introduction

Rivers are suffering biodiversity loss, water quality deterioration, hydrological changes, and channelization etc. (Davies et al., 2010; Pan et al., 2012). River restoration has become one of the important water environmental management problems. Benthic macroinvertebrates are proved to be valuable in conservation and ecological restoration of river ecosystems (Heino et al., 2003; Bae et al., 2005). Because of their confinement to the bottom, limited movement abilities and the long-life cycles, benthic macroinvertebrates are considered to be appropriate indicators for the evaluation of environments' long-term changes (Barbour et al., 1999; Timm and Mols, 2012; Pan et al., 2012; Hejazi et al., 2017). Consequently, they are widely used in stream bio-monitoring, restoration, and predictable to human influences on aquatic systems (Morse et al., 2007; Chen et al., 2013; Adugna and Alemu, 2017).

Many efforts are dedicated to modeling the benthic macroinvertebrate community based on the environment factors. The mathematical modeling with expressions of community dynamics (Gersteva et al., 2004), the hierarchical Bayesian model (Wyatt, 2003), the neural network model (Olden et al., 2006), Decision trees (D'heygere et al., 2003), STELLA model (Li and Yakupitiyage, 2003), RIVPACS-style models (Wright, 1995; Hawkins et al., 2000; Davy-Bowker et al., 2008), AUSRIVAS model (Simpson and Norris, 2000) are all applied to the studies. Most of these modeling aim at solving certain function- and process- oriented questions. Some of them are limited to lots of environment variables or available data. Despite all of these studies, the impact of river water quality on the macroinvertebrates community is not clear thoroughly. And the predictive accuracy of the models is inadequate. It hooks the predictive models in using widely. Therefore, we try two models in this study in order to dig the relationship between river water quality and macroinvertebrates deeply, and achieve the satisfactory predicting accuracy (Halim et al., 2017).

Many studies are conducted on the relations of macroinvertebrate communities to environmental factors, using abundance, richness, diversity variables (Clarke et al., 2003; Wyatt, 2003; Bonada et al., 2006; Mereta et al., 2012; Pan et al., 2012; Chen et al., 2013; Jiang et al., 2014; Sarkar et al., 2017). Results of previous studies have indicated that the environmental factors such as conductivity (Mesa, 2010), water temperature (Camur-Elipek et al., 2010), total nitrogen (Couceiro et al., 2007), total phosphorus (Maul et al., 2004), dissolved oxygen (Kaller and Kelso, 2007) and chemical oxygen demand (Song et al., 2009) are the important environmental factors impacted on macroinvertebrate assemblages.

Although, lots of studies on the relations between macroinvertebrate assemblages and environmental factors in aquatic ecosystems are carried out, the scarce of those in city river ecosystems still exists (Hashemi, 2017). Moreover, the prediction of macroinvertebrate assemblages should also be conducted more and deeply, better providing more useful implications for conservation and management of river and stream ecosystems. Thus, it is necessary to carry out quantitative studies on the relations of macroinvertebrate assemblages to hydro-environmental factors in urban rivers.

Therefore, the present study applies two procedures, linear model and k-nearest neighbor method, to predict the biodiversity of macroinvertebrate assembles in a city river, using the water quality indicators. The purposes of this work were: 1) to build macroinvertebrate biodiversity predicted models; 2) to compare the validities of linear model and k-nearest neighbor method.

Materials and methods

Study area

Wenyu River is the only one originating from Beijing urban area. It flows into North Canal through the Beiguan gate dam, located in Tongzhou District (*Figure 1*). There are three tributaries, Dongsha River (flowing through Changping District), Beisha River (flowing through Changping District) and Nansha River (flowing through Haidian District), which conflow at the Shahe Reservoir located in Changping District to form the upstream of Wenyu River with the drainage area of 1099 km^2 (Meng et al., 2010; Xiao et al., 2017; Radan et al., 2017). The segment after Shahe gate dam is described to "Wenyu River", flowing southeast into Beiguan gate dam, through Changyang District and Shunyi District. It is 47.5 km long, with a drainage area of 2478 km^2 (Meng et al., 2010; Vazdani et al., 2017). The segment from Shahe gate dam to Lutong gate dam is the middle reaches of Wenyu River, with a length of 23 km. Lingou River is the main tributary of the middle reaches. The segment from Lutong gate dam to Beiguan gate dam is called the downstream of Wenyu River, with a length of 24.5 km. Qing River, Ba River and Xiaozhong River contributes the main tributaries of the downstream

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(*Figure 1*). The mainstream and the associated riparian areas of Wenyu River are intensively affected by urban developments. Wenyu River is a typical urban river in China, with its segment flowing through many urban lands. The problem of channelization in the river is very severe.

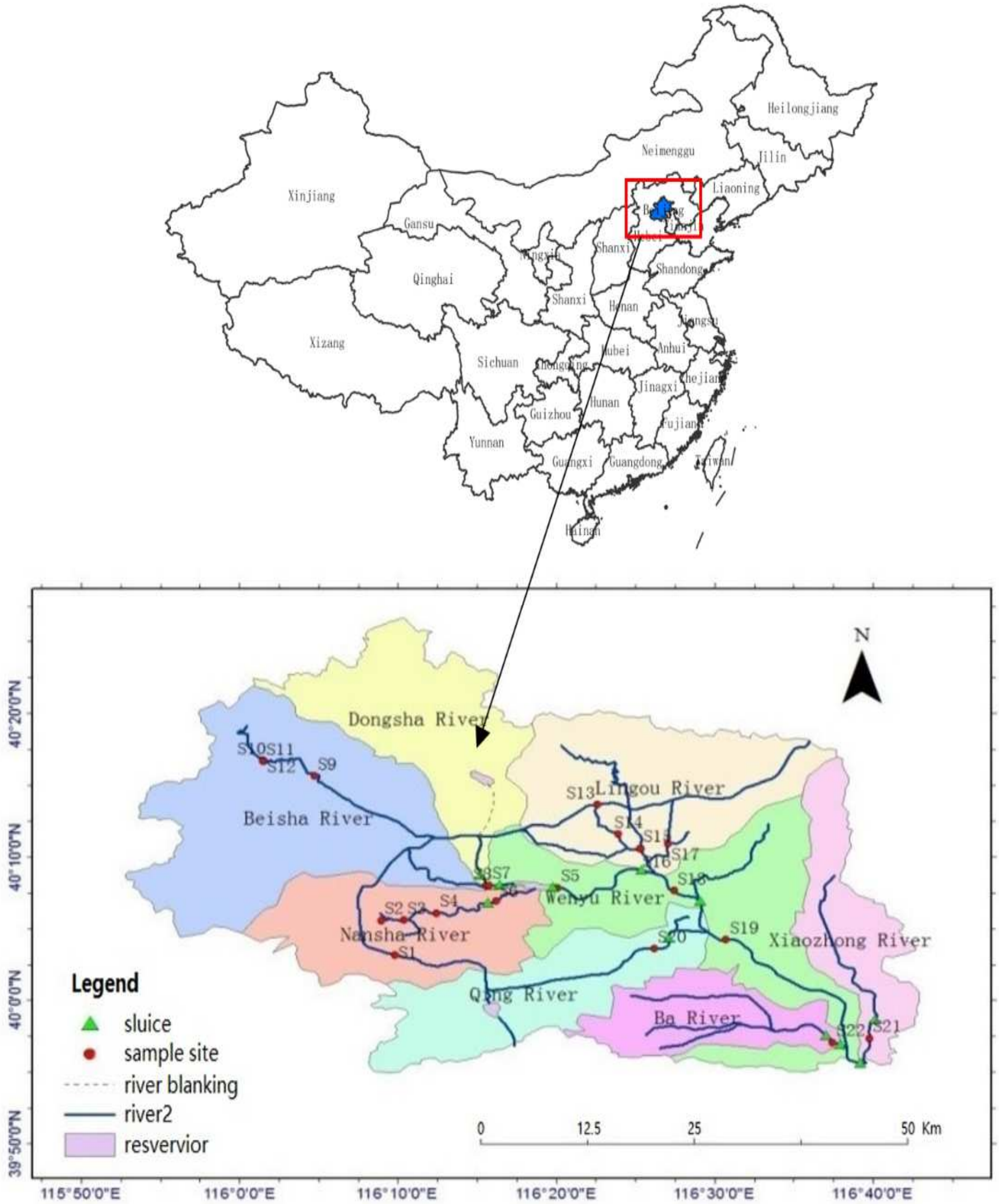

Figure 1. *Locations of the study area and the sample sites in Wenyu River, Beijing*

The drainage of Wenyu River belongs to temperate zone and the climate is continental monsoon climate. The rainfall varies greatly both between years and within one year. The mean annual rainfall is almost 600 mm, with 80% falling in wet season

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from June to September. The mean annual runoff is almost 350 million m^3 with 60%~70% coming from wastewater (Yang et al., 2014; Xiao et al., 2017).

Sample sites

A total of 22 sampling sites (abbreviated as S1 to S22) are monitored from the upstream tributaries to the downstream (*Figure 1*). Eleven sites are selected from the upstream tributaries (sites S1, S2, S3, S4 and S6 located in Nansha River, sites S7 and S8 located in Dongsha River, sites S9 to S12 located in Beisha River). Seven sites are selected from the middle reaches (sites S13 to S17 located in Lingou River, sites S5 and S18 located in Wenyu upper mainstream). Four sites are selected from the downstream reaches (site S19 located in Wenyu lower mainstream, site S20 located in Qing River, site S21 located in Xiaozhong River, site S22 located in Ba River) (*Figure 1*). The sampling sites selection is restrained by some construction and agricultural activities. For example, S12, located in the upstream reaches, are fenced and no entering because of the villager's fishing or paving cement at the bottom of the river.

Data collection

Water and macroinvertebrates samples are collected in every autumn (October to November) from 2010 to 2012, in each sampling site. Three macroinvertebrates samples are taken by a Peterson grab dredger (1/16 m^2) in each site. The samples are sieved by a 500 μm mesh sieve in situ. The animal individuals are selected from sediment manually on a white porcelain plate and conserved in 75% ethanol for identification. The organisms are identified to species level using a stereoscopic dissection microscope (magnification 10-75×) and counted (Zhou and Chen, 2011; Wang and Wang, 2011; Yang et al., 2014; Yang et al., 2017). Wet weight of macroinvertebrates is determined by an electronic balance after being blotted. The population density (ind/m^2) and biomass density (g/m^2) of each species in each sampling site are calculated respectively.

According to the literature, 12 physical and chemical variables are measured and sampled before macroinvertebrate sampling. Temperature (MYRONL ULTRAMETER Ⅱ6PFC), conductivity (MYRONL ULTRAMETER Ⅱ6PFC), pH (MYRONL ULTRAMETER Ⅱ6PFC), turbidity (HACH 2100N Turbidimeter) and dissolved oxygen (DO) (HACH HQ30d) are measured on site at each sampling site. Water samples for chemical variables analyses are collected by a water sampler and are conserved in 500ml polyethylene bottle at each sampling site. All the water samples are put in an ice chest at 4 °C and are analyzed within 24 h after collection. The total nitrogen (TN) and total phosphorus (TP) are analyzed by UV spectrophotometer. The biochemical oxygen demand (BOD_5) is determined by dilution inoculation method. The chemical oxygen demand (COD_{Mn}) is analyzed by potassium permanganate method. Ammonia nitrogen (NH_3-N), Nitrate nitrogen (NO_3^--N) and Nitrite nitrogen (NO_2^--N) are analyzed by gas phase molecular absorption spectrum method.

Linear model and k-nearest neighbor method

Two methods are utilized to build the relationship model between the biodiversity indices of macroinvertebrates and water quality concentrations, the linear model and the k-nearest neighbor method. Data in 2010 and 2011 are used to build the models and Data in 2012 are used to test the validity of models. The Shannon-Wiener index and

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Simpson index are both used in the linear model and the k-nearest neighbor method where the water quality indicators are the explainable variables. The biodiversity indices for the years 2010, 2011 and 2012 are both calculated by R software version 3.1.1, using the collected macroinvertebrate taxa data. We obtain 57 observations after eliminating the default (got samples but had no macroinvertebrates) and empty sample sites (do not obtain samples) (*Table 1*).

Table 1. *Numbers of observations in each sample site*

Sample sites	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**
Numbers of observations	3	3	2	2	3	2	3	3	3	3	3
Sample sites	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**	**S21**	**S22**
Numbers of observations	1	3	3	2	3	3	3	3	2	2	2

Results

Correlations between biodiversity indices and water quality indicators

We firstly compute the correlation matrix of biodiversity indices (Shannon-Wiener index and Simpson index) and the concentrations of 12 water quality indicators (*Table 2*). As far as Shannon-Wiener index is concerned, seven water quality indicators were significantly correlated with it (p-value ≤ 0.05), pH, DO, conductivity, NH_3-N, TP, COD_{Mn} and BOD_5. Whereas for Simpson index, less water quality indicators show significant correlations (p-value ≤ 0.05), only 5 of 12, DO, conductivity, NH_3-N, TP and COD_{Mn} (*Table 2*).

Table 2. *Correlation matrix of biodiversity indices and water quality concentration*

Water quality indicators	**pH**	**DO**	**Temperature**	**Turbidity**	**Conductivity**	**TN**
Shannon-Wiener index	0.276*	0.431*	-0.167	-0.216	-0.498*	-0.187
Simpson index	0.138	0.322*	-0.175	-0.108	-0.415*	-0.156
Water quality indicators	**NH_3-N**	**TP**	**COD_{Mn}**	**BOD_5**	**NO_3^--N**	**NO_2^--N**
Shannon-Wiener index	-0.413*	-0.423*	-0.457*	-0.318*	0.175	-0.056
Simpson index	-0.306*	-0.322*	-0.410*	-0.168	0.171	0.045

*Significant under the significance level of 0.05

Linear model for Shannon-Wiener index

Linear model

Shannon-Wiener index were transformed by $log\left(x+1\right)$, the nature logarithm transformation, since they are nonnegative numbers originally. We then used the R function lm() to fit the model (*Eq. 1*), which is:

$$\begin{aligned}\log(\mathrm{y}_i+1) = \beta_0 + \mathrm{p}H_i \times \beta_1 + DO_i \times \beta_2 + Temperature_i \times \beta_3 + Turbidity_i \times \beta_4 + \\ Conductivity_i \times \beta_5 + TN_i \times \beta_6 + NH_3 - N_i \times \beta_7 + TP_i \times \beta_8 + \\ CODmn_i \times \beta_9 + BOD_{5i} \times \beta_{10} + NO_3^- - N_i \times \beta_{11} + NO_2^- - N_i \times \beta_{12} + \varepsilon_i\end{aligned} \quad \text{(Eq. 1)}$$

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where y_i is the Shannon-Wiener index of observation, $i = 1, 2, \cdots, 38$, $\varepsilon_i i.i.d \quad N(0, \sigma^2)$ and σ^2 is unknown.

There are 12 variables and 38 observations in the model. Considering that not all the predictor variables are correlated to the response, we select the variables by AIC in a stepwise algorithm which is implemented by R function step, then we had the linear model (*Eq. 2*).

$$\begin{aligned} \log(y_i + 1) = 1.506 - 0.014 \times Temperature_i - 0.454 \times Conductivity_i - \\ 0.025 \times COD_{Mn_i} - 0.027 \times NO_3^- - N_i + 0.185 \times NO_2^- - N_i + \varepsilon_i \end{aligned} \quad \text{(Eq. 2)}$$

Figure 2 shows the observed values and fitted values:

$$\begin{aligned} (\log(\mathrm{y}_i + 1) = 1.506 - 0.014 \times Temperature_i - 0.454 \times Conductivity_i - \\ 0.025 \times CODmn_i - 0.027 \times NO_3^- - N_i + 0.185 \times NO_2^- - N_i) \end{aligned}$$

for Shannon-Wiener $\log(y_i + 1)$ of 38 observations. The variance estimation of the residual $\sigma^2 = 0.142$. The regression model's adjusted $R^2 = 0.588$. The more the adjusted R^2 is, the better the fitness of linear model is. The fitted results show the moderate correlations.

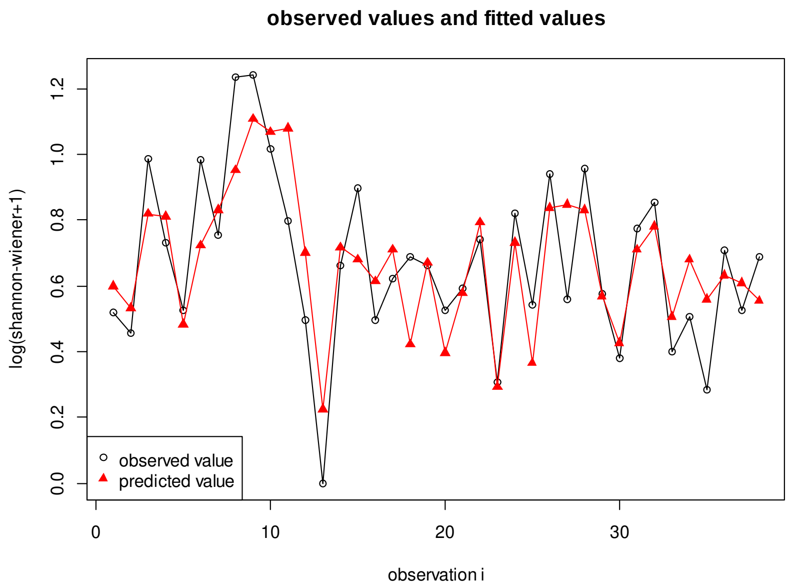

Figure 2. *Plot of observed values and fitted values of Shannon-Wiener index by linear model*

Validation of the prediction model

Applying the linear model (*Eq. 2*), Shannon-Wiener index of 22 sample sites are predicted by 12 concentrations of water quality indicators in Wenyu River monitored in 2012. They are compared to those computed by macroinvertebrate assembles samples collected at the same period (*Figure 3*). Since the Shannon-Wiener index takes non-negative numbers, the fitted values and 0 are assigned by max (*Eq. 3*). The 99%

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confidence intervals of the predicted Shannon-Wiener index are presented in *Figure 3*. It shows that there are 68.42% observations within the 99% confidence interval. For a given new sample, the predicted value is:

$$y_{new,i} = \max(\exp\{1.506 - 0.014 \times Temperature_{new,i} - 0.454 \times Conductivity_{new,i} - 0.025 \times CODmn_{new,i} - 0.027 \times NO_3^- - N_{new,i} + 0.185 \times NO_2^- - N_{new,i}\} - 1, 0)$$
$$\hat{y}_{new} = \max \qquad \text{(Eq. 3)}$$

where, $\hat{y}_{new}$ is the predicted Shannon-Wiener index of 22 sample sites in Wenyu River in 2012, $i = 1, 2, \cdots, 22$.

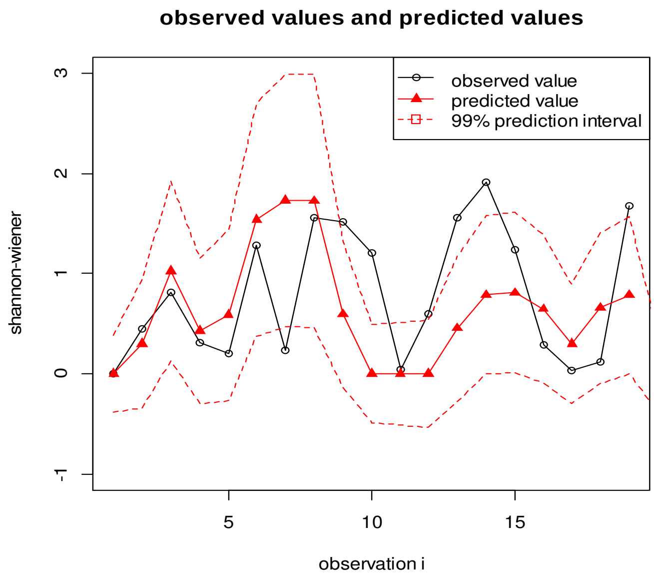

Figure 3. *The 99% prediction interval for Shannon-Wiener index using Linear model*

K-nearest neighbor method for Shannon-Wiener index

K-nearest neighbor method

The k-nearest neighbor method uses the points being close to the point of interest to do the training and predicting, where the Mahalanobis distance (*Eq. 4*) is used to evaluate the quantity of the closeness.

$$D(z_1, z_2) = \sqrt{(z_1 - z_2)^T S^{-1} (z_1 - z_2)} \qquad \text{(Eq. 4)}$$

where, z_1 and z_2 are p-dimensional column vectors, and S is the covariance matrix of z_1 and z_2. Here p=12.

The mean of Shannon-Wiener index of the observations is used as the predictive result. Denoted the set of the points that are closed to the points of interest by $\Omega(x_0)$. Let x_i denotes the observations of the above 12 features. Then the predicted value of the Shannon-Wiener index is calculated by (*Eq. 5*):

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$$y(x_0) = \frac{1}{\#\Omega(x_0)} \sum_{i \in \Omega(x_0)} y(x_i) \quad \text{(Eq. 5)}$$

where, $\#\Omega(x_0)$ denotes the number of the points in the set $\Omega(x_0)$. For a given is δ with $\delta > 0$, $\Omega(x_0)$ is calculated by (*Eq. 6*):

$$\Omega(x_0) = \{i : D(x_i, x_0) \leq \delta\} \quad \text{(Eq. 6)}$$

We use the cross validation method to find the optimal *k*, the number of points in $\Omega(x_0)$. The whole 38 observations are randomly partitioned into 5 subsamples, and the l^{th} subsample has n_l observations. A subsample is retained as the testing data for testing the model, and the remaining 4 subsamples are used as training data for fitting the model for each time. Then we obtain the predicted value for each observation, and use the mean squared prediction error to determine the optimal *k* that makes the mean squared prediction error being the smallest.

We obtain the predicted values by use of different *k* (1,2,…,20). Furthermore, we estimate the mean prediction error by *Equation 7* and get the line graph (*Figure 4*).

$$MSPE = \frac{1}{38} \sum_{l=1}^{5} \sum_{i=1}^{n_l} \left(y_{li} - y_{li} \right)^2 \quad \text{(Eq. 7)}$$

where, y_{li} and y_{li} denotes the original values and the predicted values at the *i*th site in l^{th} subsample.

According to *Figure 4,* the mean prediction error is least when *k* is 7.

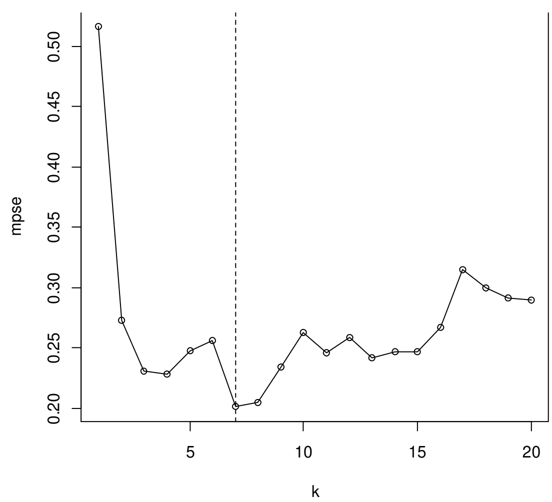

Figure 4. *The optimal number k of nearest neighbors based on MSPE using cross validation*

Prediction error of the method

Therefore, we set $k = 7$ to estimate the Shannon-Wiener index of the 22 sample sites of 2012, using the data of years 2010 and 2011. The 99% prediction interval for

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Shannon-Wiener index using k-nearest neighbors is given in *Figure 5*. It showed that there are 42.11% observations within the 99% confidence interval. The prediction validity is obviously not good.

Linear model for Simpson index

Linear model

We use the same analysis for Simpson index. The Simpson index and Shannon-Wiener index are significantly positive correlated (Corr(simpson, Shannon) = 0.886).

Using the data of 2010 and 2011, the fitted model is estimated by (*Eq. 8*):

$$\begin{aligned}\log(y_i+1) = \beta_0 + pH_i\times\beta_1 + DO_i\times\beta_2 + Temperature_i\times\beta_3 + Turbidity_i\times\beta_4 + \\ Conductivity_i\times\beta_5 + TN_i\times\beta_6 + NH_3-N_i\times\beta_7 + TP_i\times\beta_8 + COD_{Mn_i}\times\beta_9 + \\ BOD_{5i}\times\beta_{10} + NO_3^- - N_i\times\beta_{11} + NO_2^- - N_i\times\beta_{12} + \varepsilon_i,\end{aligned} \quad \text{(Eq. 8)}$$

where, y_i is the Simpson index of observation of the number i. $\varepsilon_i i.i.d \quad N\left(0,\sigma^2\right)$, σ^2 is unknown.

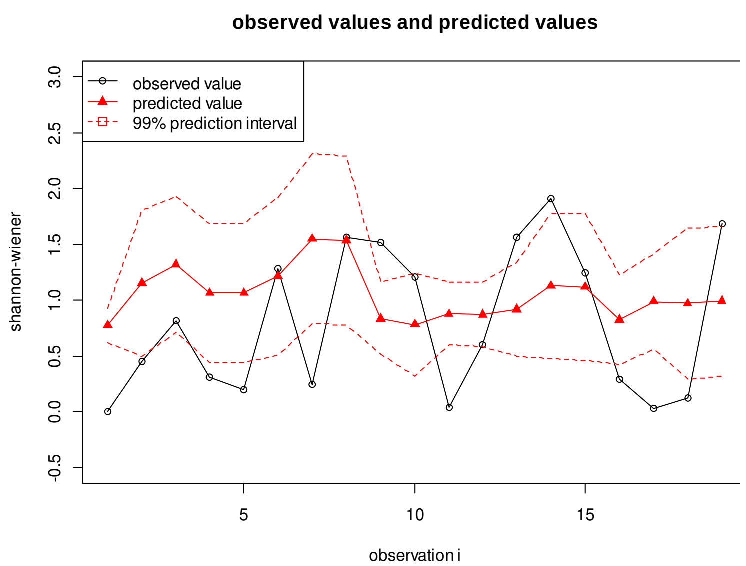

Figure 5. *The 99% prediction interval for Shannon-Wiener index using k-nearest neighbors*

We also select the variables by AIC in a stepwise algorithm which is implemented by R function, then we have the linear model (*Eq. 9*), considering that not all the predictor variables are correlated to the response.

$$\begin{aligned}\log(y_i+1) = 0.819 - 0.344\times Conductivity_i - 0.009\times COD_{Mn_i} - 0.020\times NO_3^- - \\ N_i + 0.160\times NO_2^- - N_i\end{aligned} \quad \text{(Eq. 9)}$$

where, y_i is the Simpson index of observation, $i=1,2,\cdots,38$. $\varepsilon_i i.i.d \quad N\left(0,\sigma^2\right)$, σ^2 is unknown.

We get the comparison plot of observed values and fitted values:

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$$(\log(\ y_i + 1\ ) = 0.819 - 0.344 \times Conductivity_i - 0.009 \times COD_{Mn_i} - 0.020 \times NO_3^{\ -} - N_i + 0.160 \times NO_2^{\ -} - N_i)$$

for Simpson $\log(y_i + 1)$ of 38 observations (*Figure 6*). The variance estimation of the residual $\sigma^2 = 0.016$. The regression model's adjusted $R^2 = 0.394$.

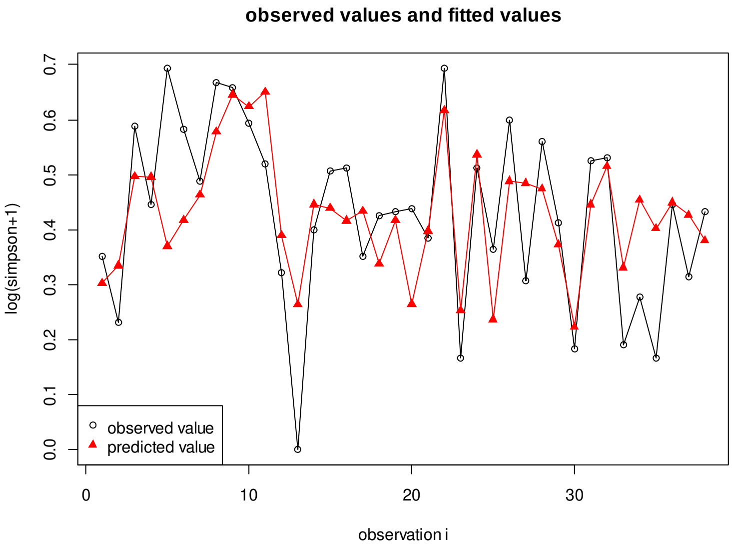

Figure 6. *Plot of observed values and fitted values of Simpson index by Linear model*

Validation of the prediction model

Applying the linear model (*Eq. 9*), Simpson index of 22 sample sites are predicted by 12 concentrations of water quality indicators in Wenyu River monitored in 2012. They are compared to the actual measured Simpson index at the same period (*Figure 7*).

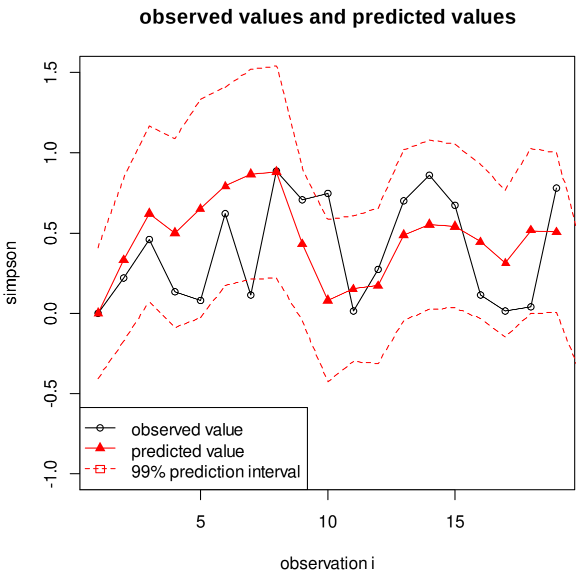

Figure 7. *The 99% prediction interval for Simpson index using Linear model*

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Since the Simpson index is also non-negative numbers, the fitted values and 0 are assigned by max again (*Eq. 10*). A 99% confidence interval of the predicted Simpson index is presented in *Figure 8*. There are 89.47% observations within the 99% confidence interval.

$$y_{new,i} = \min\{\max(\exp\{0.819 - 0.344 \times Conductivity_i - 0.009 \times COD_{Mn_i} - 0.020 \times NO_3^- - N_i + 0.160 \times NO_2^- - N_i\} - 1, 0), 1\} \quad \text{(Eq. 10)}$$

where, $y_{new,i}$ is the predicted Simpson index of 22 sample sites in Wenyu River in 2012 $i = 1, \cdots, 22$.

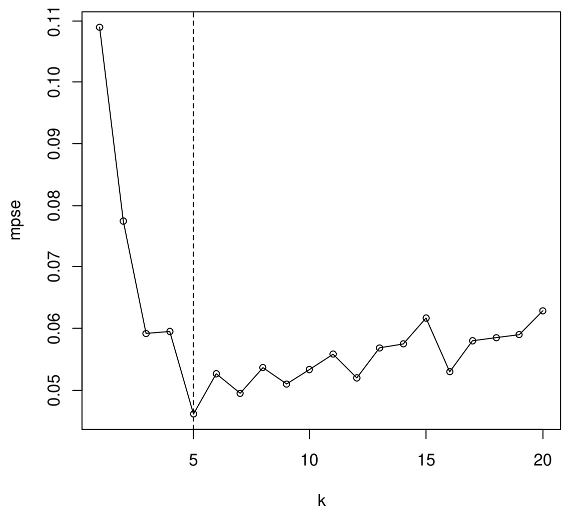

Figure 8. *The optimal number k of nearest neighbors based on PSME using cross validation*

K-nearest neighbor method for Simpson index

Similar with Shannon-Wiener index, we use the cross validation method to find the optimal *k* for Simpson index. The whole 38 observations are randomly partitioned into 5 subsamples, and the l^{th} subsample has n_l observations. We obtain the predicted value for each observation, and use the mean squared prediction error to determine the optimal *k* for which the mean squared prediction error is the smallest.

We obtain the predicted values by using different *k*(1,2,…,20). Furthermore, we estimate the mean prediction error by *Equation 7* and get the line graph (*Figure 8*). According to *Figure 8,* the mean prediction error is found to be the smallest when *k* is 5.

Therefore, we set $k = 5$ when we estimate the Simpson index of the 19 sample sites of 2012, using the data of two former years. The observed values and fitted values are compared to test the validity of 5-nearest neighbor method. A 99% confidence interval of the predicted Simpson index by 5-nearest neighbors is presented in *Figure 9*. There are 21.05% observations within the 99% confidence interval.

Comparisons of different simulated methods and biodiversity index

We put the predicted values by linear model and k-nearest neighbor method together in one plot, in order to compare the validities of two methods (*Figure 10*). According to *Figure 10,* the result of the linear model is better than the other method for Shannon-

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Wiener index. The test results of 99% prediction intervals also show the same conclusion. There are 68.42% observations within the prediction intervals by linear model, while only 42.11% are within the prediction intervals by k-nearest neighbor method.

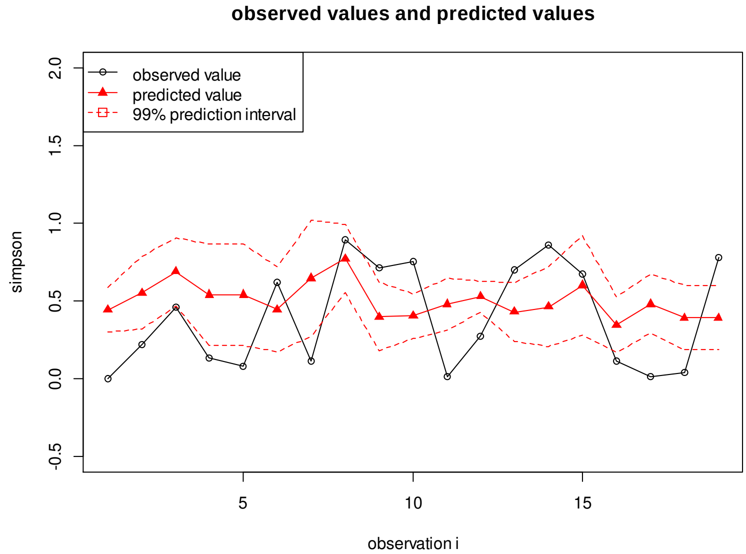

Figure 9. *The 99% prediction interval for Simpson index using k-nearest neighbors*

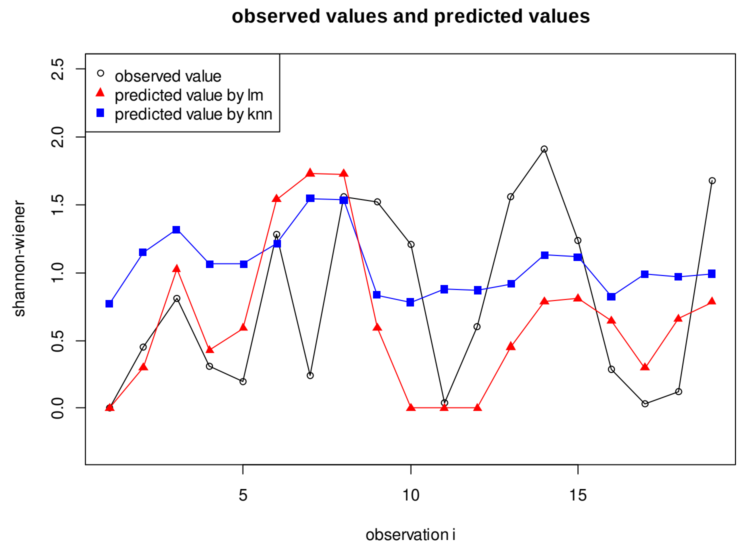

Figure 10. *Plot of Shannon-Wiener index predicted values by linear model and k-nearest neighbor method*

Similar conclusion is found for Simpson index prediction. The linear model (89.47% within the prediction intervals) is more suitable for predicting Simpson index than k-nearest neighbor method (21.05% within the prediction intervals) in Wenyu River (*Figure 11*).

As for the different biodiversity indices, Simpson index show more appropriate than Shannon-Wiener index for predicting macroinvertebrate assembles using water quality indicators in Wenyu River, a typical city river (*Figure 10, Figure 11*). There are 89.47%

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observations within the 99% confidence interval for Simpson index, whereas 68.42% for Shannon-Wiener index.

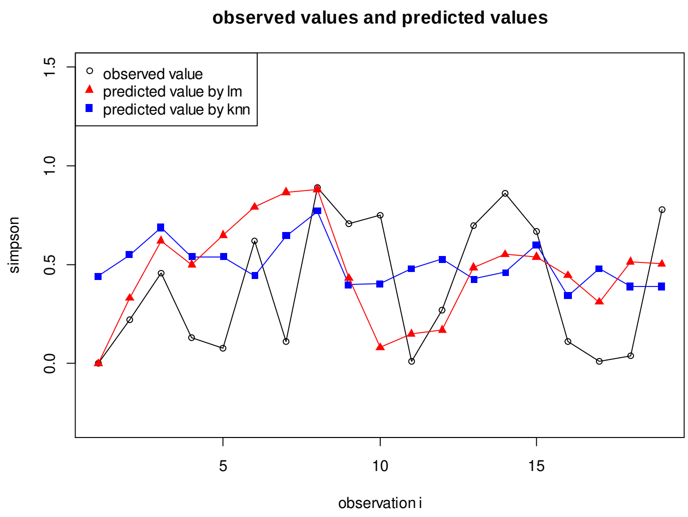

Figure 11. *Plot of Simpson index predicted values by linear model and k-nearest neighbor method*

Discussion

Biomass of macroinvertebrates

We chose the biodiversity index as the variable of macroinvertebrates. However, the abundance and biomass are often applied to researches conducted on the relationship between water quality and benthic macroinvertebrates in river systems. We also try to make macroinvertebrates prediction model using abundance and biomass variables. It is a pity that these two common variables show almost the same depressing predicted results. Abundance and biomass, thereby, are abandoned in this study. Considering the article’s length, we only take biomass as an example to explain the depressing result.

y_{it} is the total benthic macroinvertebrate biomass of the number t sampling of the i^{th} sample site in Wenyu River. $pH_{it}, \cdots, NO_2^- - N_{it}$ is the concentration of 12 water quality indicators of the number t sampling of the i^{th} sample site, $i = 1, 2, \cdots, 22, t = 1, \cdots, n_i$.

P value is less than 0.01 when y_{it} is in the normality of test. Therefore, y_{it} is made a transformation by box cox, $\lambda = 0.107$, which is close to 0, similar to the transformation by $\log(y)$. We get the histogram of $\log(y_{it})$ (*Figure 12*). We find $\log(y_{it})$ to be following the normal distribution approximately.

The relation model of the total biomass of macroinvertebrates and water quality indicators is found by (*Eq. 11*):

$$\begin{aligned}\frac{\log(y_{it}) - \overline{y}}{S_y} = {} & \beta_0 + pH_{it} \times \beta_1 + DO_{it} \times \beta_2 + Temperature_{it} \times \beta_3 + Turbidity_{it} \times \beta_4 + \\ & Conductivity_{it} \times \beta_5 + TN_{it} \times \beta_6 + NH_3 - N_{it} \times \beta_7 + TP_{it} \times \beta_8 + \\ & CODMn_{it} \times \beta_9 + BOD_{5it} \times \beta_{10} + NO_3^- - N_{it} \times \beta_{11} + NO_2^- - N_{it} \times \beta_{12} + \\ & pH_{it}^2 \times \beta_{13} + DO_{it}^2 \times \beta_{14} + \cdots + NO_3^- - N_{it} \times NO_2^- - N_{it} \times \beta_{90} + u_i + \varepsilon_{it}\end{aligned} \quad \text{(Eq. 11)}$$

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where, $\bar{y}=\frac{1}{260}\sum_{i=1}^{22}\sum_{t=1}^{n_i}\log(y_{it})$, $S_y^{\ 2}=\frac{1}{259}\sum_{i=1}^{22}\sum_{t=1}^{n_i}(\log(y_{it})-\bar{y})^2$. y_{it} is the total benthic macroinvertebrate biomass of the number t sampling of the i^{th} sample site in Wenyu River. $pH_{it},\cdots,NO_2^{\ -}-N_{it}$ - the concentration of 12 water quality indicators of the number t sampling of the i^{th} sample site, $i=1,2,\cdots,22, t=1,\cdots n_i$.

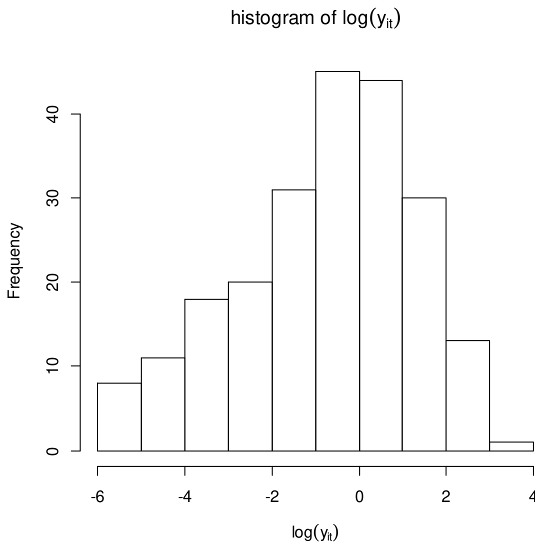

Figure 12. *Histogram of the normality of test of* $\log(y_{it})$. y_{it} *- Total benthic macroinvertebrate biomass of the number t sampling of the* i^{th} *sample site in Wenyu River*

We set values for λ from 0 to 26 (when $\lambda=26$, all the variables are not considered in the model), and maximized equation (*Eq. 12*). The Akaike information criterion (AIC) is the minimum when $\lambda=10$ (*Figure 13*). We get the non-negative variable β_0= -0.0538 and the final line mixed model (*Eq. 13*).

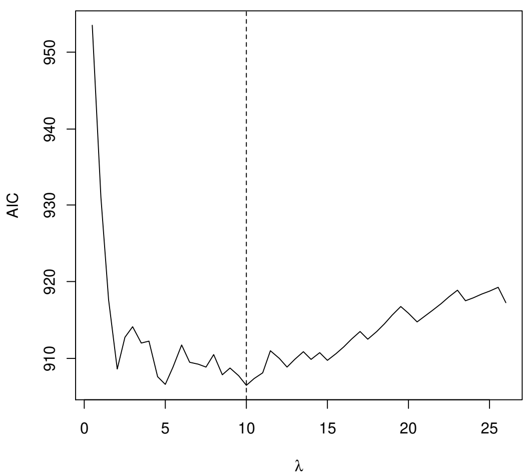

Figure 13. *The line graph of the model AIC and* λ

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$$Q(\beta,\sigma_u,\sigma_\varepsilon)=\frac{1}{2}\log(\det(V))+\frac{1}{2}(y-X\beta)^T V^{-1}(y-X\beta)+\lambda\sum_{k=1}^{90}|\beta_k| \qquad \text{(Eq. 12)}$$

$$\begin{aligned} y_{it} = &-0.14446+0.0793PH_{it}-0.0006NH4_{it}^{\ 2}-0.0018PH_{it}\times NH4_{it}+ \\ &0.003DO_{it}\times Temp_{it}+0.0199DO_{it}\times Cond_{it}-0.0002Tureb_{it}\times COD_{Mn_{it}}- \\ &0.0002NH4_{it}\times COD_{Mn_{it}}+0.0155NO3_{it}\times NO2_{it}+u_{it}+\varepsilon_{it} \end{aligned} \qquad \text{(Eq. 13)}$$

where, $u_i i.i.d \quad N(0,0.5486)$, $\varepsilon_{it} i.i.d \quad (0,2.8843)$.

The Tukey-Anscombe residual plot (*Figure14*) and QQ plot of the residuals (*Figure 15*) show that the residuals of the linear mixed model accorded with normal distribution.

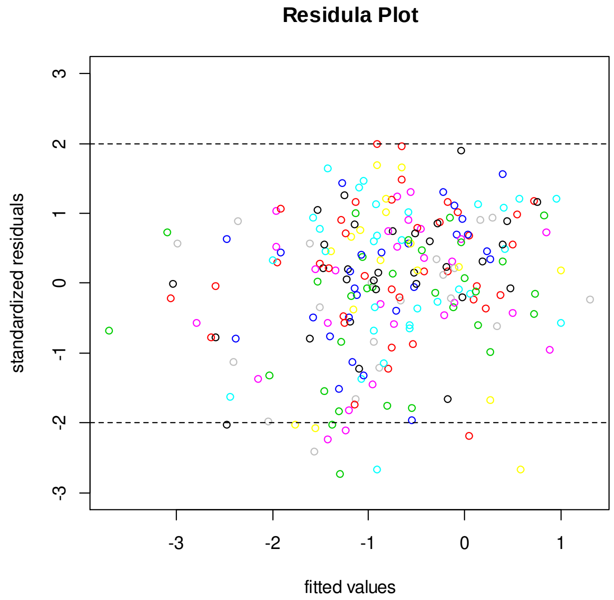

Figure 14. *Tukey-Anscombe residual plot of fitted values*

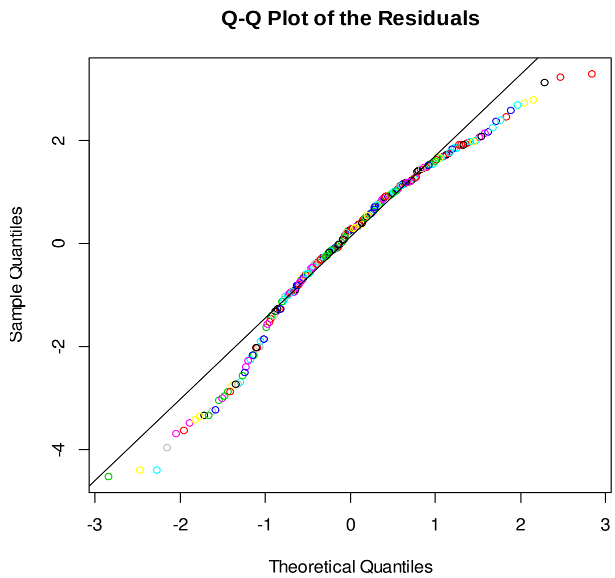

Figure 15. *QQ plot of the residuals*

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Normal distribution is also presented in the QQ plot of the standardized random effects (*Figure 16*). However, the prediction displayed an inaccurate result, contrast to the observed biomass values, by the line mixed model (*Figure 17*). Therefore, the biomass index is given up. The reason of this is yet not clear. It is perhaps concerned with the unpleasant water quality all through the river.

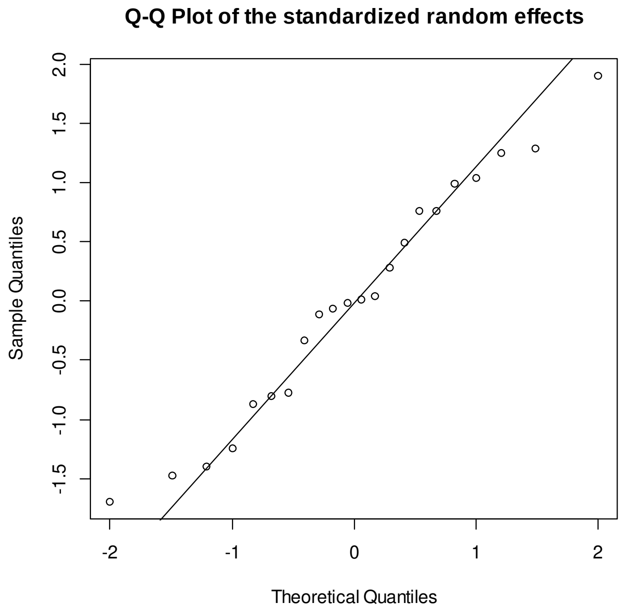

Figure 16. *QQ plot of the standardized random effects*

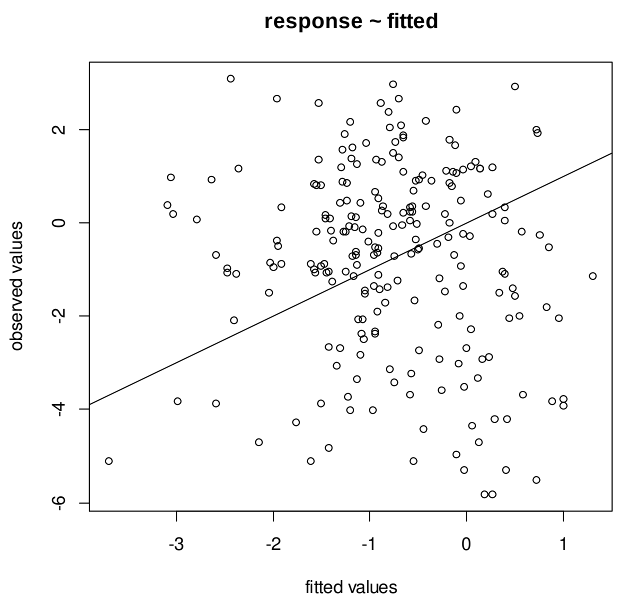

Figure 17. *Plot of the observed and predicted values of biomass by the line mixed model*

Simpson index

Simpson index shows a better predicted result than Shannon-Wiener index in this study. We know that Simpson index is more sensitive to the evenness index of a

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community while Shannon-Wiener index is more sensitive to the abundance index (Ma et al., 1995). The abundance of benthic macroinvertebrates in each sample site varies from 1 to 14, which 1 and 2 species most frequently appeared in sample sites. The abundance index changing distinctively accounted for Simpson index's better than Shannon-Wiener index.

Another reason about this could perhaps found in the research of Magurran (1988). He claims that Simpson index is more sensitive to the dominant species the Shannon-Wiener index. It seems the case in our study. For example, there are two species of macroinvertebrates in the sample site S19. One is *Limnodrilusclaparedianus*, the other is *Branchiurasowerbyi*. The individuals of the former are 3216 whereas the latter is 1. The similar status appears in most of the sample sites. The dominant species have apparent superiority of the amount and thus could affect the result of biodiversity prediction.

Limitations

We conduct a study to predict biodiversity of macroinvertebrates in a city river using two biodiversity index and 12 water quality indicators. Unfortunately, there are only 57 observations used in total, in which 38 are used for model training and 19 for validation. The poor data quality maybe affects the accurate conclusion about the prevalence of the linear model over the KNN. We should accumulate more and more observations during the next years for the supplement comparison study of these two models.

We use 12 water quality indicators to get the correlations with the biodiversity index of benthic macroinvertebrates. However, riverbed substrate and flow velocity have also important effect on macroinvertebrates (Damanik-Ambarita et al., 2016; Berger et al., 2017). Since the flow velocity of the observations in Wenyu river has little difference from each other, there is no significant correlation between flow velocity and biodiversity index in Wenyu river. Riverbed substrate types should be discussed in the future studies.

Conclusion

In this study we build two models, a linear model and k-nearest neighbor method, to predict biodiversity of macroinvertebrates in Wenyu river from the measured data of water and macroinvertebrates. Furthermore, the predicting ability of these two models are compared. We find the linear model is better for predicting macroinvertebrates diversity using water quality indicators than k-nearest neighbor method. For biodiversity indicators, Simpson index appears more robust and accurate than Shannon index for predicting benthic macroinvertebrates in a city river. The developed predictive model indicates a useful tool for assessing river health, especially city river health, since there were 89.47% observations within the 99% confidence intervals. The results of this paper could do some help to river health assessment and management.

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THE EFFECT OF ROOT REINFORCEMENT EXEMPLIFIED BY BLACK ALDER (*ALNUS GLUTINOSA* GAERTN.) AND BASKET WILLOW (*SALIX VIMINALIS*) ROOT SYSTEMS – CASE STUDY IN POLAND

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Abstract. This paper is focused on evaluation of the effect of roots of two species – black alder (*Alnus glutinosa* Gaerin.) and basket willow (*Salix viminalis*) on the increase in soil shear strength (root cohesion). The second aim of the paper was to compare the results of calculating root cohesion using the classical Wu/Waldron model and selected fiber bundle models, especially in the context of determining the influence of the criterion of distribution of stresses on calculation results. The research on the studied plant species was conducted on a seven-year plantation of energy crops located in Krakow, Poland, where the test area was modified by covering the natural site by anthropogenic soil. In order to determine the root cohesion site investigations, which included measurements of root area ratio, and laboratory tests, which included determination of root tensile strength, were conducted. The results of root cohesion calculation revealed that the studied species have relatively low values of this parameter. It was also shown that fiber bundle models yield lower values of root cohesion than the classic Wu-Waldron model. Among the fiber bundle models, the lowest values of the investigated parameter were obtained using the deformation model that takes into account the function of probability distribution of root tensile strength.
Keywords: *root reinforcement, slope stability, fiber bundle models*

Introduction

Research on the effect of plant root systems on slope stability is an issue that scientists have been investigating since the second half of the 20th century. Results of these investigations show significant importance of roots in soil stabiliazation. The range of soil stabilization depends on the architecture and strength of the root system, and these features are determined by specific features of plants and by environmental conditions (Bischetti et al., 2007, 2009). These relations are the reason why knowledge concerning mechanical (strength-related) characteristics of root systems is relatively scarce and requires further research (Mattia et al., 2005; Bischetti et al., 2009), especially in terms of the effect of bioengineering techniques on slope stability. The main goal of the paper was to evaluate the effect of roots of two species – black alder (*Alnus glutinosa* Gaerin.) and basket willow (*Salix viminalis*) on the increase in soil shear strength. These species are used in reclamation of degraded lands (Jaworski, 1995; Stachowski, 2006), they are tolerant to high soil moisture, and even to periodical floodings, which makes them potentially useful for stabilization of

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riverbanks or for the improvement of slope stability in landslide-prone regions. We hypothesized that soil reinforcement of root system of both species will not be high because of their age, but there will be a significant difference between species.

In non-rooted soil shear strength can be described using classical Mohr-Coulomb theory (*Eq. 1*):

$$\tau_f = \sigma \cdot \tan \phi' + c' \qquad \text{(Eq. 1)}$$

where: τ_f – shear strength of soil, σ – normal stress, ϕ' – effective angle of internal friction, c' – effective cohesion.

In rooted soil its displacement mobilize tension development within roots, which provides increase of soil shear strength, described as an additional cohesion (*Eq. 2*):

$$\tau_f = \sigma \cdot \tan \phi' + c' + c_r \qquad \text{(Eq. 2)}$$

where: τf, σ, ϕ', c', cr – the increase of cohesion due to presence of roots (or root cohesion; (Mattia et al., 2005; Bischetti et al., 2009).

Reinforcement of soil by presence of root, commonly called root cohesion (Bischetii et al., 2009), is determined by conducting direct field investigations (Mickovski and van Beek, 2009; Comino and Marengo, 2010; Rai and Shrivastva, 2012) and using calculation methods (Schmidt et al., 2001; Mattia et al., 2005; Burylo et al., 2011) or using back analysis (Sonenberg et al., 2010). Particularly useful is the second group of methods, which involves determination of the root area in the soil profile and determination of the root tensile strength or root pull-out resistance, followed by calculation of root cohesion. The advantage of this type of solution is a considerable reduction in the scope and costs of field work compared to in-situ tests and relatively low invasiveness of the method. The classical way of determining the effect of root systems on the increase in soil shear strength was proposed by Wu et al. (1979) and Waldron (1977). This model assumes that, during shearing, full mobilization of the tensile strength of all roots in the bundle takes place. As the results of direct investigations (Docker and Hubble, 2008; Pollen and Simon, 2005) indicate, using the Wu/Waldron model usually leads to overestimation of the influence of plant root systems on soil shear strength. Therefore, in recent years, the range of root cohesion has been determined using fiber bundle models (Pollen and Simon, 2005; Mao et al., 2012; Schwarz et al., 2012; 2013). These models assume irregular mobilization of maximum tensile strengths in individual parts of the root bundle, but these models differ in assumptions concerning the manner of distribution of root tensile strengths and root elasticity. That is why the second goal was to compare the results of calculating root cohesion using the classical Wu/Waldron model and selected fiber bundle models, especially in the context of determining the influence of the criterion of distribution of stresses on calculation results. We expected that fiber bundle models will give different calculation results and it would be possible to indicate the model which provides the most conservative values of root cohesion.

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Materials and methods

The research area

The research on the studied plant species was conducted on a seven-year plantation of energy crops located at the premises of the Faculty of Production and Power Engineering of the University of Agriculture in Krakow, Poland (N 50°4'54, E 19°52'2). The research area was located in an area subjected to anthropogenic modification of its morphology. The modification consisted in bringing silty sand soil (0.5-0.6 m thick) consisting also industrial wastes (pieces of furnace slag, bricks, concrete) onto the plot surface. Natural soil also includes sandy silty soil which is characterized by dark-gray color and low porosity. During the research, presence of plants from the *Equisetaceae* family from was found in this soil, which (in relation to land morphology) indicates that before the modification the natural land had been waterlogged. Apart from the studied species, test area was also covered by herbaceous species. In case of black alder dominant species were ground elder (*Aegopodium podagraria* L.) and common nettle (*Urtica dioica* L.). In the vicinity of basket willow prevailed Canadian goldenrod (*Solidago canadensis*), but giant goldenrod (*Solidago gigantea*), ground elder (*Aegopodium podagraria* L.), cock's-foot (*Dactylis glomerata* L.), reed canary grass (*Phalaris arundinacea* L.) and Tanacetum boreale (*Chrysanthemum vulgare* L.) were also found.

Field investigations

Measurements of the root area ratio (Ar/A) were carried out using a vertical trench profile wall method (Böhm, 1979) for six plants from each species. Root area ratio was defined as the fraction of the soil cross sectional area occupied by roots per unit of the excavated area (Comino et al., 2010) and it was assumed that roots are circular in cross-section. Excavations were executed (1.0 m wide and at least 0.80 m bgl) at a distance of approximately 0.5 m from the tree. The depth of excavation was dependent on the presence of roots, and when they occurred the excavations were done at least 0.1 m deeper than the deepest-occurring roots. The distance of excavation from the tree was established based on preliminary tests which showed that there are only single roots at 1.0 m from the tree. After cleaning the excavation wall surface, measurements of root diameters (at vertical spacing of 0.1 m) were taken. The analysis omitted roots smaller than 1 mm and bigger than 10 mm in diameter. Fine roots are difficult to distinguish and determine, and coarse roots are so stiff that they do not cooperate with soil in accordance with the assumptions of basic calculation models (Bischettii et al., 2009). Roots of the tested species were distinguished from the others on the basis of color, smell and texture.

Field investigations of soils involved determining their volumetric density using the cutting ring method and determining the natural moisture content using the drying and weighing method (weight moisture) and using time domain reflectrometry (TDR) (volumetric moisture).

The analysis of the research results consisted in determining the significance of differences in the amount of roots and their relative area in distinguished layers of the profile between both plant species. Normality of data distribution was analyzed first (using the Shapiro-Wilk test), and then, due to lack of normality of distribution of the analyzed parameter, the U Mann-Withney test was used.

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For quantitative description of root distribution in the profile, the Gale and Grigal's equation (1987; *Eq. 3*) was used:

$$Y = 1 - B^D \qquad \text{(Eq. 3)}$$

where: Y – cumulative number of roots in the soil profile, expressed as the percentage of roots, D – depth, B – the fitted coefficient.

Laboratory tests

The scope of the tests included determination of tensile strength of root samples of both plant species using a Hounsfield H50KS tensile testing machine. Before testing, the root samples were submerged in water for approximately 1 day in order to obtain their maximum possible saturation. The total length of the samples was approximately 20 cm, and effective distance between machine jaws was 10 cm. On the other hand, the tension speed was 10 mm min^{-1}. The test was conducted until the sample broke or came out of the jaws. The diameter of the sample was measured at the breaking point, at the same time noting the way it was destroyed. Then, taking into account root diameter and the value of tensile force, root tensile strength was calculated. The statistical analysis of the research results consisted in establishing the relationship between tensile force and root diameter and the relationship between tensile strength and root diameter. This analysis was conducted in Microsoft Excel, using the least squares method, and only the samples broken between machine jaws were used for the analysis.

On the other hand, the significance of differences in tensile strength between the studied plant species was analyzed in Statistica using the analysis of covariance (ANCOVA). In this analysis, root diameter was the covariate, and the value of tensile force was the dependent variable. Before commencing the analysis, values of both parameters had been logarithmized, followed by verification of normality of their distribution using the Shapiro-Wilk test for significance level 0.05.

Calculations of root cohesion

The value of root cohesion (c_r) was determined based on the measurement results of the root area ratio and determination results of root tensile strength, using the classic Wu-Waldron model (Waldron, 1977; Wu et al., 1979) and five fiber bundle models. In the first model (W-W), root cohesion was calculated according to the following equation:

$$c_R = k' \cdot k'' \cdot \sum_{i=1}^{N} T_{Ri} \cdot \left(\frac{A_{ri}}{A} \right) \qquad \text{(Eq. 4)}$$

where:

k' – root orientation factor, assumed usually within the range 1.0-1.3; the calculations assumed k'=1.2, k" – correction factor, proposed by Preti (2006); k"=1.0 was assumed, T_{Ri} – root tensile strength, A_{ri} – root area ratio in the soil, A – area of the studied section.

The disadvantage of Wu-Waldron model is the assumption that soil shearing is accompanied by mobilization of full tensile strength of all roots in the system. Such an assumption is the reason why the effect of root systems on root cohesion is overestimated (e.g. Pollen and Simon, 2005). That is why Preti (2006) suggested that

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values of root cohesion calculated using *Equation 4* be corrected by introducing the k" factor to the original formula of Wu-Waldron model.

The fiber bundle model (FBM) assumes that breaking of roots during soil shearing takes place successively. Initially, soil load is distributed evenly to all roots until breaking of the weakest root. As a result, tensile force is transmitted onto a system with the smallest number of roots (n-1), which means that there is an increase in the force transmitted to a single root. There are several criteria describing the mechanism of destruction (breaking) of the root system. Five calculation models were used in this paper. One group includes the so-called static models, which take into account mainly information on dimensions of roots and their tensile strength. These models assume that the amount of tensile strength can be distributed to roots proportionally to their section area (FBM1), to their diameter (FBM2) or their number in a bundle (FBM3). In the first case (FBM1), it is assumed that each root is subjected to the same tensile stress, and the value of root cohesion is calculated from the following dependence (*Eq. 5;* Mao et al., 2012):

$$c_R = k' \cdot \left(T_{rj} \cdot \sum_{n=1}^{j} \frac{A_{rn}}{A} \right) \qquad \text{(Eq. 5)}$$

where:
k' – assumed as in formula (4), T_{rj} – "j" root tensile strength, A_{rn} – cross-section area of intact roots in a bundle, A – as in *Equation 4*.

In the second case (FBM2), the value of force transmitted to roots is proportional to root diameter, according to the proposition made by Pollen and Simon (2005), and the value of root cohesion is determined from the following dependence (*Eq. 6;* Mao et al., 2012):

$$c_R = k' \cdot \left(T_{rj} \cdot \frac{A_{rj}}{A} \cdot \frac{\sum_{n=1}^{j} d_n}{d_j} \right) \qquad \text{(Eq. 6)}$$

where: k' – assumed as in *Equation 4,* T_{rj} – tensile strength of a root with diameter d_j, A_{rj} – cross-section area of a root with diameter d_j, d_n – root diameter, d_j – diameter of the root with the lowest tensile strength in the bundle.

On the other hand, according to the third criterion (FBM3), tensile force transmitted by individual roots is identical, and its value is proportional to the number of roots. The calculations were made using the following formula (*Eq. 7;* Mao et al., 2012):

$$c_R = k' \cdot \left(T_{Rj} \cdot \frac{A_{Rj}}{A} \cdot j \right) \qquad \text{(Eq. 7)}$$

where: k' – as above, T_{Rj} – tensile strength of the weakest root in the bundle, A_{Rj} – root area in soil, j – number of unbroken roots in the system, A – as above.

The drawback of the above-described models is the assumption of identical elasticity of all roots in the bundle. Schwarz et al. (2012) proposed a modification to fiber bundle model – Root bundle model (RBM1). The modification consisted in the assumption that each root has its own distinct geometric and strength-related properties. To calculate the

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properties of individual roots, the authors of this model proposed the following equations (*Eqs. 8, 9* and *10*):

$$L(d) = L_0 \cdot \left(\frac{d}{d_0} \right)^{\gamma} \quad \text{(Eq. 8)}$$

$$E(d) = E_0 \cdot \left(\frac{d}{d_0} \right)^{\beta} \cdot r \quad \text{(Eq. 9)}$$

$$F_{\max}(d) = F_0 \cdot \left(\frac{d}{d_0} \right)^{\xi} \quad \text{(Eq. 10)}$$

where: L – length of a root with diameter "d", L_0 – empirical characteristic length of a root with diameter d_0, γ, ξ, β – exponent, d_0 – diameter of a characteristic root, usually assumed as d_0=1 mm (in order to obtain dimensionless term of *Equations 6-8* occurring in parenthesis, E(d) – Young's module for a root with diameter "d", E_0 – empirical characteristic Young's module for a root with diameter d_0, r – dimensionless factor introduced in order to take into account the effect of root tortuosity on its behavior under tension (assumed as r=0.4), F_{max} – maximum tensile force, F_0 – empirical characteristic tensile force of a root with diameter d_0.

For the purpose of the analysis, the L_0, γ and r values were assumed based on data provided by Schwarz et al. (2012). On the other hand, Young's module and β values were estimated based on test results on tensile strength, being aware, however, of the fact that these were approximate values with no significant effect on the results of calculations of the value of the force distributed by roots.

By integrating the above-mentioned equations one can obtain relationships which make it possible to determine the value of tensile strength occurring in the investigated range of deformation for individual roots in the bundle. As a result, it is possible to obtain a value of tensile strength transmitted by the root system which, after comparing it to the area of the investigated section, can be calculated into the value root cohesion. Schwarz et al. (2013) established that test results on tensile strength and pull-out strength are characterized by substantial variability in the studied property. That is why they proposed to take into account, when calculating the value of tensile strength transmitted by roots, the probability of root breaking described by means of the Weibull survival function, according to the following dependence (*Eq. 11* and *12;* RBM2):

$$F_{tot}(\Delta x) = \sum_{i-1}^{N} F(d, \Delta x) \cdot S(\Delta x_i^*) \quad \text{(Eq. 11)}$$

where: S(Δx*i) – two-parameter Weibull survival function:

$$S(\Delta x^*) = \exp\left[-\left(\frac{\Delta x^*}{\lambda} \right)^{\omega} \right] \quad \text{(Eq. 12)}$$

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where: ω – indicator of the shape of Weibull function, λ – scale parameter, Δx^* – normalized deformations of a root with diameter "d", calculated as the ratio of the value of deformation (obtained from tests) at the moment of breaking to theoretical value of this parameter, F(d, Δx) – value of tensile force of a root with diameter “d” at deformation Δx.

A detailed procedure for determining the value of survival function parameters is described in the paper by Schwarz et al. (2013). To determine the values of Weibull function parameters, results of tests on the root samples tension were used.

Table 1 summarizes similarities and differences of the models described above.

Table 1. *Properties of theoretical models used for calculation of root cohesion*

Properties	Model					
	W-W	**FBM1**	**FBM2**	**FBM3**	**RBM1**	**RBM2**
Tensile force distribution within bundle	Not included	Due to root cross section area	Due to root diameter	Due to number of roots	Due to root cross section area and root’s deformation	Due to root cross section area and root’s deformation
Deformation analysis	Not included	Not included	Not included	Not included	Included	Included
Order of root breakage	Not included	Acc. to tensile strength	Acc. to tensile force	Acc. to tensile force	Acc. to relative deformation	Acc. to relative deformation
Maximum tensile strength of a single root	Constant	Constant	Constant	Constant	Constant	Described by Weibull survival function
Number of model parameters	2	2	2	2	4	6
Difficulty of method	Low	Medium	Medium	Medium	High	High

To determine the significance of differences in values of root cohesion obtained from different methods, averaged values of this parameter in the entire profile were calculated, then ANOVA test was used at a significance level of 0.05.

Results

Root area ratio (Ar/A)

The results of site measurements revealed that total number of roots in the profile in individual test excavations was not very high and ranged from 35 to 92 for black alder and from 34 to 40 in the case of basket willow. In *Table 2* number of roots converted into pieces per square meter is presented. The analysis of root distribution in the profile (*Fig. 1*) showed that values of coefficient B in Gale and Grigal’s equation (1987) are 0.976 for both plants, which indicates that roots are relatively evenly distributed in the soil profile. Similar values of parameter B are given in scientific literature for tropical climate grasses, Oregon ash and some varieties of willow (Pollen-Bankhead and Simon, 2009) or black locust and Manchurian red pine (Zhang et al., 2014).

In general, roots of both species concentrate in two zones of the profile. The first one was at the ground surface, and the second one was at the depth pf 0.55-0.75 m bgl and

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was probably associated with higher moisture content of the soil (*Fig. 2*). During field investigations conducted after rainfall, it was observed that water accumulated at this depth, which means that this zone is less permeable than the other ones. This phenomenon is associated with the presence of a well-compacted layer of silty sand, on whose roof there are proper conditions for water accumulation. Therefore, it can be assumed that the unusual development of the root system observed in both plant species is strongly associated with water availability.

Table 2. *Distribution of roots number and root area ratio (A_r/A) within soil profile*

Depth [m]	Number of roots, roots m^{-2}		Ar/A, %	
	Black alder	Basket willow	Black alder	Basket willow
0.05	173±74	72±28	0.159±0.058	0.023±0.020
0.15	83±36	82±31	0.062±0.035	0.062±0.056
0.25	33±30	42±36	0.018±0.018	0.042±0.037
0.35	30±44	8±12	0.015±0.025	0.001±0.002
0.45	37±47	7±8	0.048±0.082	0.001±0.002
0.55	65±84	40±31	0.060±0.076	0.063±0.072
0.65	85±48	72±31	0.074±0.047	0.041±0.030
0.75	43±60	32±26	0.029±0.044	0.008±0.008
0.85	4.8±66	0±0	0.029±0.041	0.000±0.000
0.95	0±0	-	0.000±0.000	-
Mean	60	39	0.053	0.027

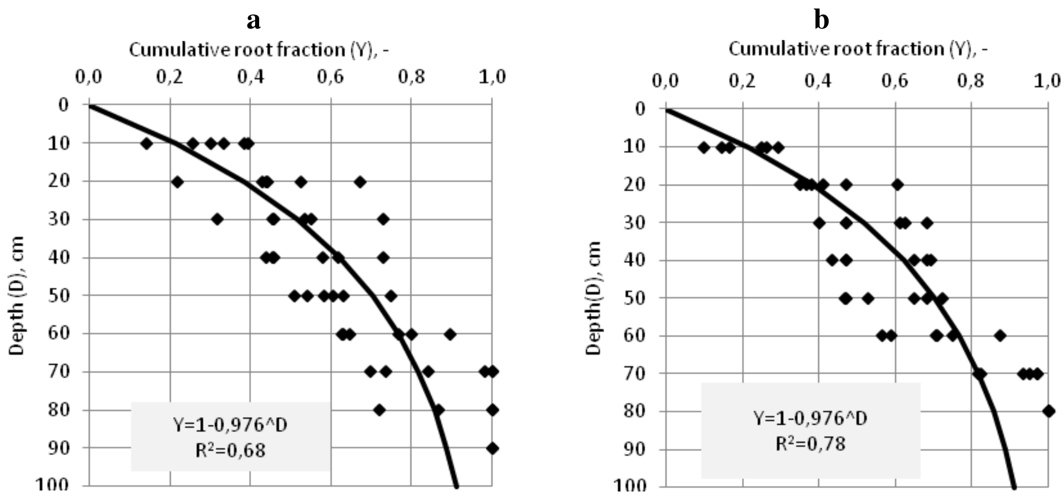

Figure 1. *Cumulative numbers of black alder (a) roots and basket willow (b) roots versus soil depth*

The results of measurements of the root area ratio presented in *Figure 2*, similar to distribution of root number, indicates high diversity in the value of this parameter. The coefficient of variation for the root area ratio in individual layers of the profile was within the range from 24 to 168% (109%, on average), and high values of standard deviation are also indicative of the high variability of the root area ratio. The mean

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value of the black alder root area ratio in the entire profile was 0.053%, and in the case of basket willow – 0.027%. On the other hand, maximum values of this parameter were 0.24% in the case of alder, and 0.17% in the case of willow. Due to lack of normality of the studied property, the results of measurements were analyzed using U Manna-Whitney test. This analysis showed that, at a significance level of α=0.05, root area ratio of the studied species does not differ significantly (p=0.06). A similar relationship was obtained by analyzing the number of roots in the profile (p=0.12).

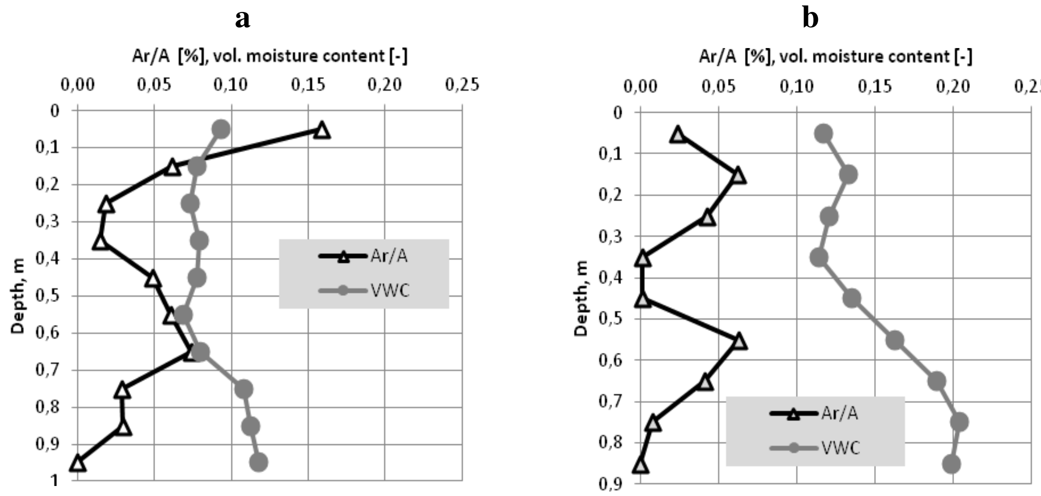

Figure 2. *Root area ratio (Ar/A) and volumetric water content (VWC) of soil in a black alder plantation (a) and basket willow plantation (b)*

Tensile strength

The dependence of tensile force and tensile strength on root diameter is presented in *Figure 3* (for black alder) and in *Figure 4* (for basket willow).

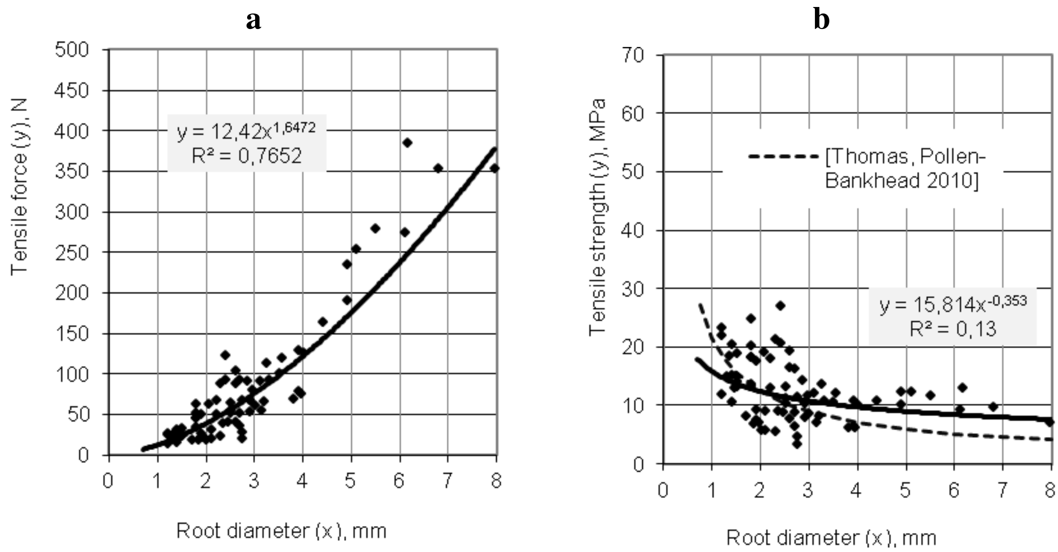

Figure 3. *Tensile force (a) and tensile strength (b) versus root diameter obtained for black alder*

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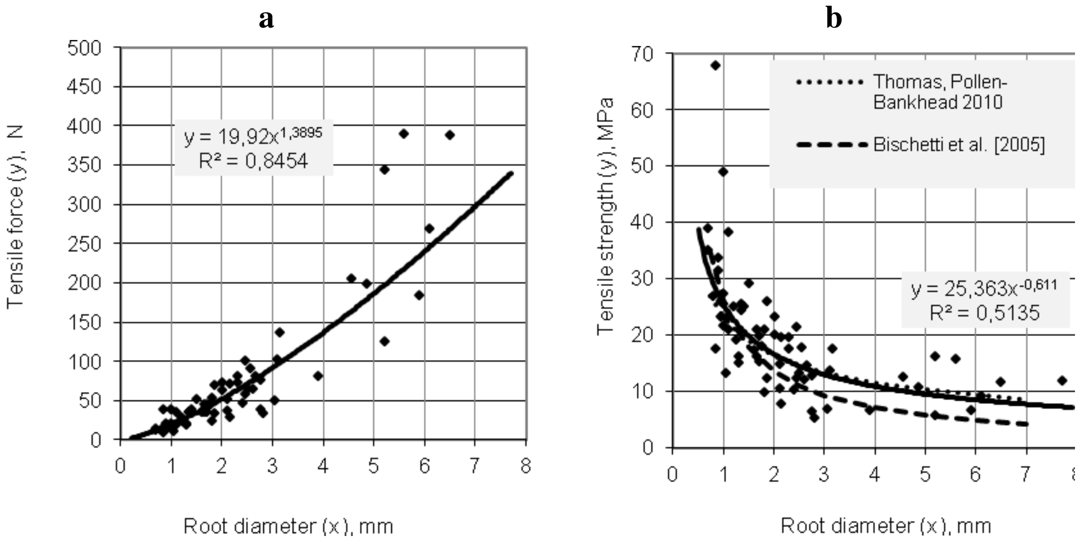

Figure 4. *Tensile force (a) and tensile strength (b) versus root diameter obtained for basket willow*

Generally, it can be said that values of tensile force were much more strongly correlated with root diameter than the dependence of tensile strength on root diameter. According to Vergani et al. (2012), the above relation results from the fact that values of tensile strength are double burdened with the root diameter measurement error. The mean value of tensile strength in the case of grey alder was 12.5 MPa (with mean root diameter of 2.8 mm and standard deviation of 5.2 MPa), and in the case of basket willow – 19.5 MPa (with mean root diameter of 2.3 mm and standard deviation of 10.6 MPa). Statistical analysis at a significance level of 0.01 showed that tensile strength of basket willow roots is higher than that of black alder.

Root cohesion

Results of measurements of the root area ratio in the soil and root tensile strength were used to determine the magnitude of root cohesion. Calculation results presented in *Figure 5* indicate that values of root cohesion change with depth analogically to the changes in the root area ratio. It is noticeable that there is a distinct difference between the values of the analyzed parameter depending on the applied calculation model. Regardless of depth and plant species, the highest values of root cohesion were obtained using the Wu-Waldron model, and the lowest – using the RBM2 model. To present the differences in values of root cohesion obtained by using individual calculation models, the ratio of the value of this parameter obtained from individual fiber bundle models to the value obtained using the Wu-Waldron model was calculated, using the following dependence (*Eq. 13*):

$$k" = \frac{c_{r(FBM1-FBM3,RBM1,RBM2)}}{c_{r(W-W)}} \qquad \text{(Eq. 13)}$$

It was assumed that the k" value was the same as the value of correction factor proposed by Preti (2006) which describes the uncertainty of determining root cohesion

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specified by the Wu-Waldron model. The calculated values of the k" parameter are shown collectively in *Figure 6* in the form of its dependence on the number of roots. It was generally established that, from the fiber bundle models, the highest values of the analyzed parameter were obtained using the FBM1 model, and the lowest – using the RBM2 model. It can also be observed that the more roots in the bundle, the lower the value of the analyzed parameter. This dependence is the least noticeable in the case of the RBM2 model, where values of the k" parameter obtained by this model are relatively stable and are generally within the range from 0.4 to 0.6.

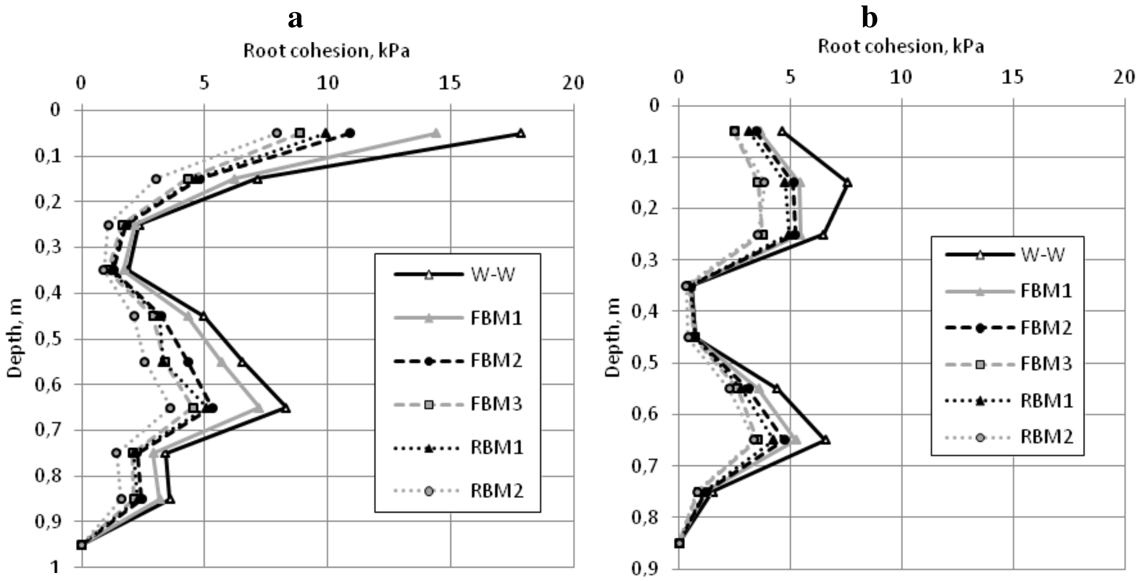

Figure 5. *Mean root cohesion values determined by various models vs. depth for black alder (a) and basket willow (b) obtained using Wu-Waldron model and various fiber bundle models*

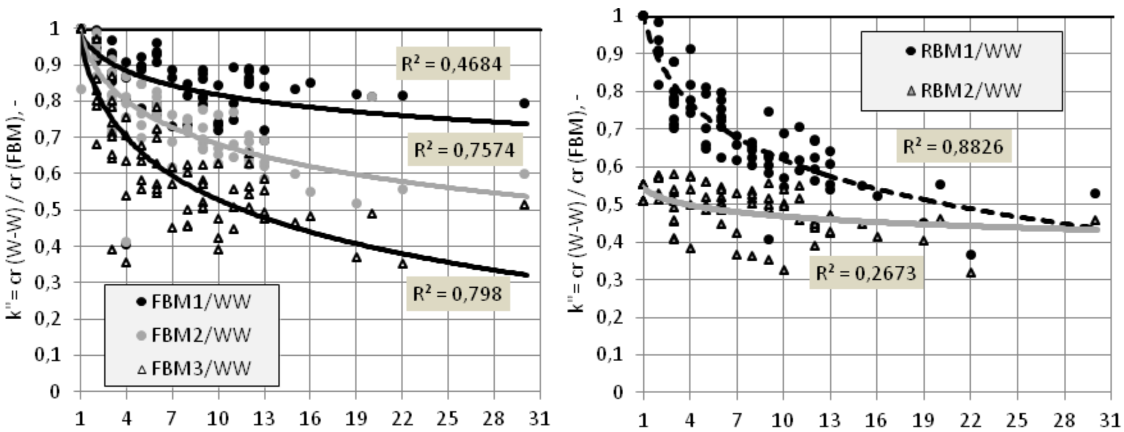

Figure 6. *Values of k" factor vs. number of roots*

Further in the paper, for the clarity of the obtained values of root cohesion, they were averaged within profile. When comparing the studied species of trees, it was established that values of root cohesion averaged in the soil profile (*Fig. 7*) were relatively low,

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namely (on average, according to the W-W model), 6.0 kPa for black alder and 3.6 kPa for basket willow. Values of this parameter obtained from calculations using the RBM2 model are decidedly the lowest – 2.6 and 1.9 kPa for alder and willow, respectively. The statistical analysis carried out for these data (ANOVA) showed that, in case of black alder, values of root cohesion calculated using the Wu-Waldron model differ statistically ($p<0.05$) from the results obtained using the FBM2, FBM3, RBM1 and RBM2 models, and model RBM2 provides statistically lower values of root cohesion in relation to Wu-Waldron, FBM1 and FBM2 models. In case of basket willow, values of root cohesion obtained using FBM3, RBM1 and RBM2 models were significantly lower ($p<0.05$) than those obtained from the Wu/Waldron model. On the other hand, model RBM2 provides statistically lower value of root cohesion in comparison to Wu-Waldron, FBM1 and FBM2 models. It means, that in case of the both studied species root cohesion values determined by RBM2 model are comparable to results obtained using FBM3 and RBM1 models.

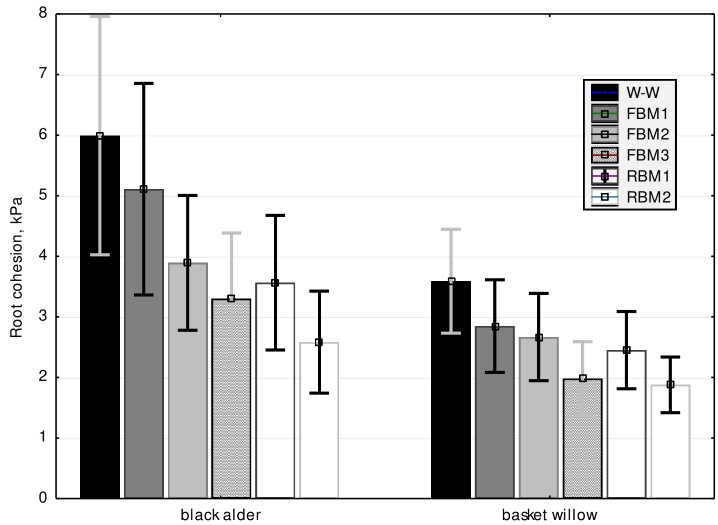

Figure 7. *Site-averaged values of root cohesion*

While comparing the studied plant species, it was shown (ANOVA) that values of root cohesion obtained for black alder were statistically higher ($p<0.05$) than for basket willow in case of W-W, FBM1, FBM2 and FBM3 models, whereas this difference was not observed when analyzing the results of calculations obtained from other models (RBM1 and RBM2).

Discussion

Root area ratio

Generally, it was found that mean values of the root area ratio of the studied plant species reached 0.06% and 0.03% for black alder and basket willow, respectively. The statistical analysis did not show significant differences in the value of the root area ratio of both species. As pointed out by Burylo et al. (2011), lack of statistically significant differences in tests on root systems may result from substantial heterogeneity of the results of determinations of the analyzed parameter.

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Values of the root area ratio of the studied black alder and basket willow are similar to the results obtained for root systems of black poplar and black locust from that region (Zydroń et al., 2016), while measurements of root systems of these species were taken at a slightly different distance from tree trunks (1.0 m). Values of the root area ratio of the studied species are also comparable to the results of measurements of a 17-year-old Chinese thuja, lower than in the case of a 17-year-old black locust (Ji et al., 2012) and higher than root systems of 9- and 10-year-old poplars (Douglas et al., 2010) or young downy oaks and black pines (maximum 0.06%) that are presented in the paper by Burylo et al. (2011). Tests on typical European species occurring in the Alps indicate that mature trees have a much higher root area ratio, on average, by 0.07 to 0.36% (Bischetti et al., 2009), and maximum values of this parameter reach 0.9% (Bischetti et al., 2007; 2009) or even almost 2% (Chiaradia et al., 2012; Mao et al., 2012). So, it can be stated that the area ratio values of the studied plant species are similar to each other and are within the range provided in literature for young trees. It can be expected that the studied species will expand their roots systems.

In most tests on root systems it is characteristic that the largest number of roots, and also the highest Ar/A values, occurs in the surface part of the profile. This is associated with the reducing amount of nutrients and air in soil and an increase in its compaction (Bischetii et al., 2007). On the other hand, in the case of the studied plant species, two zones of root concentration were distinguished: near the surface (0-0.2 m bgl) and in the lower part of the profile (0.5-0.7 m bgl). Test results indicate that the latter zone is associated with the presence of a soil layer with higher moisture content. Tan'a et al. (2006) indicate that water availability is the most important factor determining the development of a root system. On the other hand, the observed root pattern of the studied species corresponds well to the example of root system distribution affected by site conditions (Coppin and Richards, 1990), when presence of well-compacted soil layer force roots to spread laterally.

Tensile strength

The tensile strength test results indicate a relatively good correlation between tensile force values and root diameter. When comparing the tensile strength of the studied plants to properties of other species of the same plant family, a similarity can be observed between the strength of black alder and grey alder (which was studied by Thomas and Pollen-Bankhead (2010)). On the other hand, tensile strength of Geyer's willow (Thomas and Pollen-Bankhead, 2010) growing in North America is similar to that of the studied basket willow, whereas purple willow (Bischetti et al., 2007) has a slightly lower strength. In general, in the light of the results of tests on various plant species, obtained by various research groups and collated in the paper by Stokes et al. (2008), it can be stated that tensile strength of the studied black alder is within the lower range of strength values for deciduous trees, whereas the mean strength value of basket willow is basically typical for shrubs. It should be highlighted, however, that test results presented in the paper by Stokes et al. (2008) were obtained using various methods differing in the way of preparing samples for tests and also in the tensing procedure itself.

Root cohesion

The obtained calculation results indicate a relatively low effect of root systems of the studied plant species on soil stabilization in relation to root systems of young black

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locust trees and Chinese thuja shrub (Ji et al., 2012; Zydroń et al., 2016), Japanese cedar (Genet et al., 2008) or mature tree species occurring in European forests (Bischetii et al., 2009; Vergani et al., 2014). The low effect of the black willow root system on root cohesion was also indicated by Simon and Collins (2002) who obtained the mean value of root cohesion of 2 kPa. However, it should be stressed that the obtained test results are similar to the values of root cohesion of black poplar (Zydroń et al., 2016), of vetiver grass (Mickovski et al., 2009), or higher than some shrub species (Burylo et al., 2011; Adhikari et al., 2013).

When comparing the results of calculations of root cohesion, obtained using the Wu-Waldron model and fiber bundle model, it was found that they were clearly different, which is in accordance with existing knowledge (Pollen and Simon, 2005; Bischetii et al., 2009; Mao et al., 2012). Values of root cohesion obtained using fiber bundle models are, on average, 12-51% lower than those obtained from calculations using the Wu-Waldron model, which means that, in extreme cases, the Wu-Waldron model yields over twice overestimated values of root cohesion.

From the fiber bundle models, the highest values of the analyzed parameter were obtained using the FBM1 model, which assumes that tensile strength distribution is proportional to the root cross-section area, which means that each root is under the same stresses. This assumption is the reason why, when under tension, coarse roots are damaged first, which is contradictory to the results of the paper by Cohen et al. (2011), which indicate that fine roots are usually damaged first. The same relationship was obtained for the other models. Similar relationships concerning the differences in results of calculations of root cohesion and the order of root breaking in the bundle, obtained using FBM1, FBM2 and FBM3 models, are described in papers by Mao et al. (2012) and Ji et al. (2012).

Calculations obtained using deformation models (RBM1 and RBM2) yielded interesting results. These models have the same assumption regarding the way of tensile force distribution as in the FBM1 model, but these models assume that roots have different lengths (roots with small diameter are shorter than coarse roots). The last assumption causes that fine roots, despite their substantial elasticity, are the first to be damaged (broken, pulled out from soil). Values of root cohesion obtained using deformation models RBM1 and RBM2 were, on average, 25 and 51% lower than obtained from the Wu-Waldron model. Values of root cohesion generated by RBM1 model were lower than values of the analyzed parameter obtained using FBM1 and FBM2 models, which is contrary to the results of Ji et al. (2012). In turn, RBM 2 model generated the lowest values of root cohesion among analyzed models. This model takes into account the diversity in root tensile strength, and thereby the value of tensile force transmitted by individual roots is multiplied by the factor of probability of root damage. Contrary to the other models, the ratio between root cohesion determined from this model and the Wu-Waldron model did not depend much on the number of roots (cf. *Fig. 7*) and amounted on average 0.49, which is close to the value of correction factor k” proposed in the paper by Bischetti et al. (2009) (k”=0.5). Therefore, it appears that k”=0.5 may be a parameter that, from the practical point of view, can be used to calculate root cohesion using the classic Wu/Waldron model, thus gaining time and simplification of calculations in relation to calculation procedures used in fiber bundle models.

The authors of the RBM2 model (Schwarz et al., 2013), draw attention to the fact that the results of calculations of the force transmitted by roots are significantly influenced by variability of their strength-related properties, which is expressed by the

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value of the parameter ω. High values of this parameter are indicative of substantial homogeneity of the studied plant material, whereas results of analyses carried out by the authors of this model indicate that this parameter usually amounts to approximately 2, which is similar to the values obtained in the tests herein (for black alder ω=2.6, and for basket willow ω=3.0). Schwarz et al. (2013) also show that results of calculations obtained using the RBM2 model show a relatively good concurrence with results of direct tests. However, as the authors of this model highlight themselves, it requires further work on validation of the values of Weibull function parameters for different combination of factors taking into account the type of test method, method of data elaboration and the number of repetitions.

Conclusion

Based on the obtained results of research on black alder and basket willow root systems from a seven-year plantation, it can be stated that:

1. The studied species are characterized by substantial variability in the root system area ratio, which is typical for this type of material. Moreover, field measurements showed that (in the existing habitat conditions) both plants developed a complex root system in which roots concentrated in the surface part of the profile and in the deeper zone where there are appropriate conditions for drawing water from soil.
2. Tensile strength tests showed a strong connection between tensile force and root diameter. Tensile strength of black alder roots was, in statistical terms, considerably lower than that of basket willow roots.
3. Calculations of root cohesion of both of the studied plant species indicate that they had relatively low values of this parameter, which was associated with their young age.
4. It was shown that evaluation of the effect of the root system on root cohesion is significantly associated with the applied calculation model and calculation assumptions associated with it. It was shown that fiber bundle models yield lower values of root cohesion than the classic Wu-Waldron model. On the other hand, among the fiber bundle models, the lowest values of the investigated parameter were obtained using the Root Bundle Model proposed by Schwarz et al. (2013) that takes into account the function of probability distribution of root tensile strength. Statistical analysis revealed that root cohesion results obtained this model, results from Root Bundle Model described in paper as RBM1 and FBM3 can be considered as comparable.
5. Application of the deformation model which takes into account the Weibull's function of probability distribution leads to obtainment of root cohesion values which are approximately 50% lower than Wu-Waldron model. Therefore, application of the correction factor (proposed by Preti, 2006), at a value of 0.5, in the Wu-Waldron model allows for a relatively easy way to determine realistic values of root cohesion which appear safe from the point of view of engineering applications.
6. Considering application of the obtained test results into practice it seems important to predict development of root system. So, future tests should be concentrated on studies of the root systems growth and its impact on root cohesion and slope stability.

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MATERIAL BALANCE EQUATION OF OVERMATURE ORGANIC-RICH GAS SHALE

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Abstract. Gas shale was treated as a quintuple porosity system. When the source rock matures, a portion of kerogen or bitumen will be transformed into hydrocarbon, and move to displace the water out from inorganic matrix. When the kerogen is recrystallized to graphite, there will be less dissolved gas left in solid kerogen, which was assumed to be substantial in immature source rock. A series of resistivity tests showed the resistivity of shale decreases with higher vitrinite reflectance value, and proved the graphite do exist in overmature shale. With the role of natural fracture in shale analyzed, the water and hydrocarbon stored in overmature shale was summarized, and the overmature organic-rich gas shale should be treated as two porosity system. To find out which kind of pore may accommodate most of the free gas, a generalized material balance equation was presented, and the volume change with pressure drop in organic matrix and inorganic matrix can be calculated simultaneously. The field case herein proved that new material balance equation can be applied to calculate original free gas and adsorbed gas in shale or sandstone, and it was suggested that while in overmature gas shale there is free gas in inorganic pore, free gas in organic pore and adsorbed gas in kerogen, most of the free gas is in the organic pores.
Keywords: *shale gas, kerogen, inorganic matrix, lamination, gas in place*

Introduction

It is well accepted that gas shale is an important unconventional resource which can be viewed as a quintuple porosity system. Because hydrocarbon is stored as free gas in pore and fracture, adsorbed gas in kerogen, and dissolved gas in bitumen or kerogen. a general linear equation was created accordingly (Singh, 2013; Hejazi et al., 2017). When taking into account the organic pore and natural fracture in shale, a material balance equation was proposed for dual porosity gas shale (Zhang et al., 2013; Hashemi, 2017). A similar material balance equation was created, in which the gas stored in natural fracture can be essential (Duarte et al., 2014; Radan et al., 2017). A material balance equation was also constructed for gas condensate instead of dry gas in shale reservoir (Orozco and Aguilera, 2015). However, it is found in many basins that if the kerogen is overmature, the carbon in kerogen is recrystallizing to the mineral graphite, and the organic pore is like the coking observed in retorting of coals. Being unlike to a quintuple porosity system, the gas shale with overmature kerogen has a different distribution of gas and water, and the original material balance equation for quintuple porosity system cannot be applied without change (Ebrahimi et al., 2017). So,

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it is important to improve the conventional material balance equation for overmature shale gas reservoir.

It is also found in many basins that thin laminated layers of sandstone are imbedded in organic-rich shale. These laminated sandstone layers may be of siliceous or carbonaceous mineralogy, and have conventional porosity (Gao et al., 2017). Because the sandstone layers are interconnected with the organic-rich shale adjacent to it, it should be taken as an inseparable part in shale gas reservoirs. Herein the special property of overmature kerogen is analyzed at first, then a general material balance equation for organic-rich shale with thin laminated layers of sandstone is established, therefore original gas in organic matrix and inorganic matrix can be calculated simultaneously (Vazdani et al., 2017).

Materials and methods

The gas storage in overmature shale will be discussed in the following sections. Nomenclature is included in Appendix section.

Carbonification of kerogen in overmature shale

When the source rock is in the overmature stage, the carbon in the kerogen or bitumen was assumed to be in the process of recrystallizing to graphite, which is named as overmature shale. The amount of graphite is hard to measure precisely with X-Ray Diffraction or Scanning Electron Microscope. The electrical resistivity of graphite measured under normal temperature being from $8\times10^{-6}\Omega\cdot m$ to $13\times10^{-6}\Omega\cdot m$, it is possible to measure the amount of graphite indirectly by measuring resistivity. If there is abundant amount of graphite in shale when vitrinite reflectance is greater than 3%, the rock resistivity may be much lower than that with lower thermal maturity (Passey et al., 2010; Xiao et al., 2017).

Resistivity of shale plugs with different vitrinite reflectance, or Ro, have been measured to prove whether the carbon in kerogen has been recrystallized to graphite. Because the electrically conductive material in gas shale consists of clay-bond water, capillary bond water, free water and graphite, it is useful to remove the water before test as much as possible. The shale plugs were dried to constant weight in a humidity oven, and maintained at the 45% humidity and 60 °C temperature until the weight was stable. The resistivity in *Fig. 1* showed that the core plugs with vitrinite reflectance Ro greater than 2% have lower resistivity, which indicated the graphite was available in overmature shale.

Some may argue that the pyrite is also a conductive mineral commonly present in organic-rich shale, and may reduce the resistivity. The amount of pyrite is hard to measure precisely with X-Ray Diffraction or Scanning Electron Microscope. Because the grain density of pyrite is about 5.0g/cc compared with that of 2.6 g/cc for quartz, even if the weight fraction of pyrite scaled to total weight is 5%, the volume ratio of pyrite should be as low as 2.5%, and can be negligible.

Similar to the wet clay or non-connected vug, pyrite framboids can be considered as a separate phase in conductivity model, and the contribution is controlled by their volume fraction (Hassan and Ismail, 2017; Radmanfar et al., 2017; Yang et al., 2017). When analyzing the connectivity equation in porous media, a complex conductivity model was introduced which can be used for the shale with pyrite (Montaron, 2007) (*Eq. 1*).

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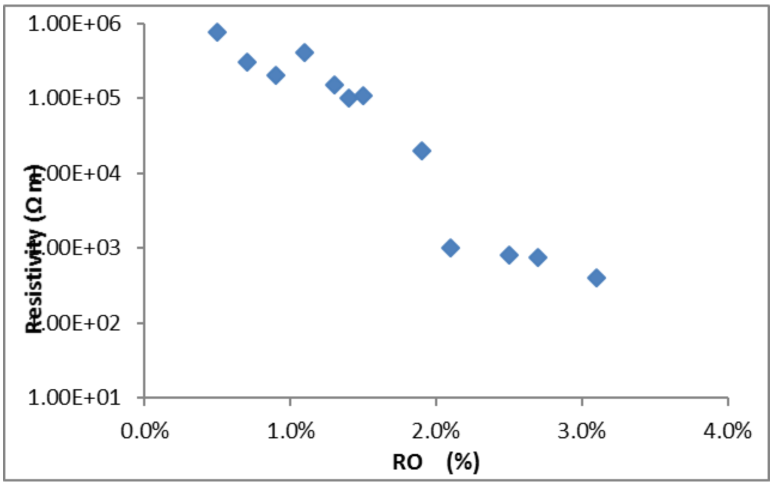

Figure 1. *Resistivity of dry shale samples with different vitrinite reflectance in Xu jiahe shale and Zhenjing shale*

$$\sigma^{1/2} = \sigma_{cw}^{1/2} X_{cw} + \sigma_{w}^{1/2} X_{w} + \sigma_{graphite}^{1/2} X_{graphite} + \sigma_{pyrtie}^{1/2} X_{pyrite} \quad \text{(Eq. 1)}$$

The fact that the volume ratio of pyrite X_{pyrite} is much smaller than other conductive components makes the contribution of pyrite conductivity negligible in shale. It also proved that the lower resistivity of overmature shale indicated the significant amount of graphite, and the kerogen cannot hold any dissolved gas.

Gas storage in shale with different maturity

As shown in *Fig. 2,* all of the pore space in immature shale is full of water initially. A portion of the kerogen will be transformed to hydrocarbon when the kerogen matures, and the hydrocarbon will move to displace some of the water out from the connected inorganic pore (Passey et al., 1990). The displacement process will continue until the capillary pressure balances with the displacement pressure. In overmature shale, there is even less water left in the inorganic pore.

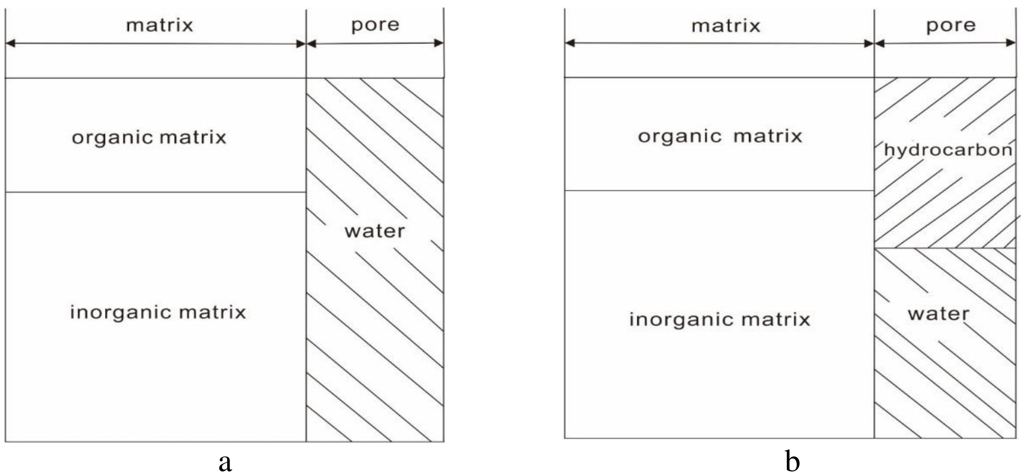

Figure 2. *a) Immature shale rock. b) Mature shale rock*

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It was found that in Antrim shale gas of Michigan Basin, the vitrinite reflectance being 0.4~0.6%, the immature shale is initially water saturated, and the water must be drained to allow gas to desorb from the matrix before production (Jenkins and Boyer, 2008). In Barnett shale gas of Fort Worth Basin, the vitrinite reflectance being 1.0~2.1%, the majority of the gas in pore space is free gas, and there is little initial water production.

The storage mechanisms in shale reservoirs were concluded, and the gas shale was viewed as a quintuple porosity system: free gas in inorganic pore, free gas in organic pore, free gas in fracture, adsorbed gas in kerogen, and dissolved gas in the kerogen or bitumen (Orozco and Aguilera, 2015). Given the similarity between kerogen and bitumen underground, methane dissolved in solid kerogen underground is the same as in bitumen (Swami et al., 2013). However, when the gas shale is in the overmature stage, vitrinite reflectance being greater than 2%, and the carbon in the kerogen or bitumen being recrystallized to graphite, there is little dissolved gas left in kerogen or bitumen, and the dissolved gas in solid kerogen is negligible.

Role of nature fractures in shale

Natural fractures can be sometimes observed in shale reservoirs. After analyzing the testing data of three major shale reservoirs in the US, Wang and Reed (2009) concluded that the pores in kerogen and inorganic minerals contribute the most to the total pore volume and the porosity of natural fractures is less than 0.5%.

The study of Schieber (2011) showed a number of micro-fractures observed in scanning electron microscope (SEM) may be generated due to the poor preservation of shale cores. The fractures in some shale cores may be made from the stress release when they were taken from underground (Ma, 1996). Based on SEM pictures, it was also noted that the natural fractures in Barnett shale reservoir are mostly filled with secondary minerals (Kent, 2007; Gale et al., 2007). It is shown in *Table 1* that the fractures are not applicable to substantial gas storage.

Table 1. *Different porosity in gas shale*

Item	Hayneville shale	Barnett shale	Zhenjing shale
Total organic content (TOC)	3.5wt%	5wt%	>2 wt%
Total porosity of shale	12%	5%	4%
Porosity in natural fractures	<0.5%	<0.5%	<0.5%
Porosity of organics in shale	~0.7%	~1.0%	~0.8%
Porosity of inorganic matrix	>10.6%	>3.3%	>2.7%

It is also shown from the observation of shale cores as shown in *Fig. 3* that visible macro-fractures are not well developed in Zhenjing shale in the Ordos Basin. Because natural fractures in most of gas shales can hardly act as main storage space, the volume of natural fractures in this type of reservoir can be neglected.

Gas in overmature shale

Shale gas reservoirs are structurally more complicated than conventional reservoirs. It was found that there are thin laminated groups of sands imbedded in organic-rich shale in the Lewis shale (Grieser and Bray, 2007). It is also concluded that the Xu 5

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shale in Sichuan Basin is the same class of gas shale as Lewis shale which is shown in *Fig. 4.* At the early stage of reservoir development, primary production is through the thin sands, and black organic bulk shale feeds the sand.

Figure 3. *Core samples (824-827 m) of No. 6 Xinfu well in Zhenjing shale of Ordos Basin*

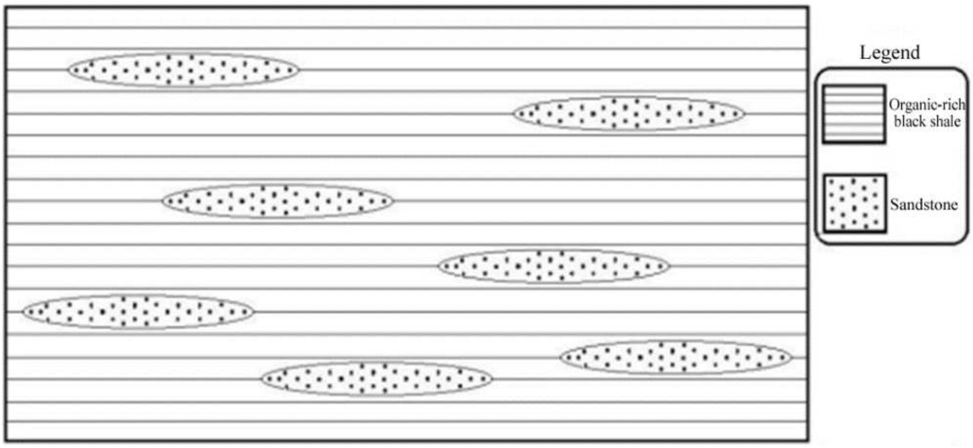

Figure 4. *Laminated sands imbedded in Xu 5 shale of Sichuan Basin*

Table 2 is a summary of the petro-physical properties of this type of shale gas formation, which consists of laminated layers, organic-rich shale and natural fractures. Because the laminated layers are imbedded in the organic-rich black shale, the pores and natural fractures are interconnected, and both of the sand layers and organic-rich shale should be taken as inseparable parts of shale gas reservoir. The kerogen in the organic-rich shale is also called as organic matrix. The inorganic matrix is the laminated sand layer.

The Marcellus shale and Eastern Devonian shale are quite different from Lewis shale, and they are called as black high organic content shale, with the primary production being through gas desorption from kerogen. *Table 3* is a summary of the rock solid, water and hydrocarbon in this type of shale. When comparing *Table 2* with *Table 3,* the role of laminated sandstone layers in gas shale is self evident.

However, both of the gas shales in *Table 2* and *Table 3* share some characteristics in common, and the components can be grouped into inorganic matrix and organic

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matrix. The inorganic matrix can be divided further into solid inorganic rock and fluid. The effective porosity is adopted in material balance calculation. According to the definition of dual water model in wireline log interpretation, clay bond water is not included in the effective porosity. Because inorganic pores are water wet, there is water and free gas in effective inorganic pore. The volume of free gas in effective inorganic pores is defined as G_{nk}.

Table 2. *Components of shale with laminated sand layers*

Rock type	Component	Solid	Fluid
Laminated layer	Inorganic matrix	Non-clay minerals	Free gas
			Mobile water and capillary-bound water
		Clay minerals	Clay-bound water
Organic-rich shale	Organic matrix	Kerogen	Free gas
			Adsorbed gas
Natural fracture			Mobile water
			Free gas

Table 3. *Components of black high organic content shale*

Rock type	Component	Solid	Fluid
Black high organic content shale	Inorganic matrix	Non-clay minerals	Free gas
			Mobile water and capillary-bound water
		Clay minerals	Clay bound water
	Organic matrix	Kerogen	Free gas
			Adsorbed gas
	Natural fracture		Mobile water
			Free gas

The organic matrix consists of solid kerogen and fluid. Because kerogen is non-water wet, there is only free gas and adsorbed gas in the organic pores under the original reservoir conditions. After studying the characteristics of adsorption facies by means of molecular dynamic simulation, it was found that the Langmuir adsorption model can be used for the adsorption process (Ambrose et al., 2010). The free gas in organic pores is defined as G_k, with the adsorbed gas in organic pores being defined as G_A respectively.

If the volume of natural fractures in shale gas reservoir can be neglected, both of the gas shales in *Table 2* and *Table 3* can be simplified to the same volume model in *Table 4.*

Table 4. *Simplified components of overmature gas shale*

Fractional volume of inorganic matrix (V_{pm})	Fractional volume of solid inorganic matrix (V_{sm})
	Fractional volume of effective inorganic pore ($V_{pm}\phi_{pm}$), in which there is capillary bound water and free gas
Fractional volume of organic matrix (V_{pk})	Fractional volume of Solid Kerogen (V_{sk})
	Fractional volume of organic pore ($V_{pk}\phi_{pk}$), in which there is adsorbed gas and free gas

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The relationship between each part is (*Eq. 2*):

$$V_{pm} + V_{pk} = 1 \tag{Eq. 2}$$

where:

$V_{pm} = V_{pm}\phi_{pm} + V_{sm} = V_{pm}\phi_{pm} + V_{pm}(1-\phi_{pm})$

$V_{pk} = V_{pk}\phi_{pk} + V_{sk} = V_{pk}\phi_{pk} + V_{pk}(1-\phi_{pk})$

$V_{pm}\phi_{pm} + V_{pk}\phi_{pk} = \phi_T$

Generalized material balance equation

Supposing the gas is stored in the organic pores, a material balance equation of coalbed methane and shale gas reservoirs was proposed (King, 1990). However, this model can not be applicable to the unconventional gas reservoirs where the pores contained in both organic matrix and inorganic matrix are substantial.

The following assumptions are made for overmature shale on the basis of the geologic characteristics discussed above.

(1) The pores of gas shale consist of inorganic pores and organic pores, which communicate with each other. This is quite true for the organic-rich shale with laminated sandstone layers.
(2) The thickness of adsorbed gas in kerogen is negligible. When the pressure drops below the critical desorption pressure, the adsorbed gas desorbs and leads to deformation of kerogen in the same way as coal.
(3) The gas dissolved in the water and kerogen is neglected.
(4) Inorganic matrix being water-wet, there may be mobile water, capillary bound water and free gas in the effective inorganic pores. In overmature shale gas reservoir, because there is little mobile water in the inorganic pores, the volume of mobile water is negligible, and only the capillary bound water is considered.
(5) Kerogen being non-water wet, there is not any water in the organic pores under the original reservoir conditions.
(6) Natural fractures only act as pathways for flow instead of storage space, so the volume of fractures is neglected.
(7) The difficulty for fracturing fluid to flow back after stimulation makes large amount of slick water remained in gas shale. The fracturing fluid left in gas shale is taken as injected water.
(8) No water influx from aquifer is considered.
(9) The reservoir temperature is constant in the process of reservoir production.

Based on the volume model in *Table 4,* the free gas in the inorganic matrix and the organic matrix are defined as G_{nk} and G_k respectively. *Eq. 3* is the expression for total free gas in place:

$$G = G_{nk} + G_k \tag{Eq. 3}$$

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According to the principle of underground volume conservation, the material balance equation is generally expressed as:
free gas initially in place at Pi = pore volume occupied by free gas at P + change in inorganic pore + change in organic pore + desorbed gas + injected fracturing fluid – produced water.

$$\begin{aligned} &GB_{gi} = (G - G_p)B_g + \Delta V_{nk} + \Delta V_k + \Delta V_d + W_i - W_p B_w \\ &or: G_p B_g + W_p B_w = G(B_g - B_{gi}) + \Delta V_{nk} + \Delta V_k + \Delta V_d + W_i \end{aligned} \tag{Eq. 4}$$

It is shown in *Eq. 4* that the produced gas results from the expansion of free gas and the desorption of adsorbed gas.

The total volume change in the effective pores of inorganic matrix (ΔV_{nk})

When the reservoir pressure drops to *p*, the total volume variation of water and pores in the effective pores of inorganic matrix (ΔV_{nk}) is the sum of the elastic expansion of water (ΔV_{epnkw}) and the elastic variation of effective pores (ΔV_{epnkf}).

(1) The effect of water expansion in inorganic matrix (*Eq. 5*)

$$\Delta V_{epnkw} = \frac{G_{nk} B_{gi}}{1 - S_{nkwi}} S_{nkwi} (e^{\int_p^{p_i} C_w dp} - 1) \tag{Eq. 5}$$

(2) The effect of compressibility in inorganic matrix (*Eq. 6*)

$$\Delta V_{epnkf} = \frac{G_{nk} B_{gi}}{1 - S_{nkwi}} (1 - e^{-\int_p^{p_i} C_{nk} dp}) \tag{Eq. 6}$$

When *Eqs. 5* and *6* are added together, the total effect of formation and the capillary bound water compressibility is (*Eq. 7*)

$$\Delta V_{nk} = \Delta V_{epnkf} + \Delta V_{epnkw} = \frac{G_{nk} B_{gi}}{1 - S_{nkwi}} [(1 - e^{-\int_p^{p_i} C_{nk} dp}) + S_{nkwi} (e^{\int_p^{p_i} C_w dp} - 1)] \tag{Eq. 7}$$

The compressibility of water (C_w) and the compressibility (C_{nk}) can be taken as constant sometimes. If $e^x \approx 1 + x$, *Eq. 7* can be simplified as the common form as that of Moghadam et al. (2011) (*Eq. 8*):

$$\Delta V_{nk} = \Delta V_{epnkw} + \Delta V_{epnkf} = \frac{G_{nk} B_{gi}}{1 - S_{nkwi}} (C_w S_{nkwi} + C_{nk})(p_i - p) \tag{Eq. 8}$$

The volume change in organic matrix (ΔV_k)

Kerogen is non-water wet, so there is no water in the organic pores under the initial reservoir conditions. In the process of production, natural gas is desorbed when the pore

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pressure decreasing. In the overmature kerogen, the volume change of kerogen is similar to that of coal. According to the solid deformation theory, the Palmer and Mansoori (1998) model can describe the effects of compression and matrix shrinkage (*Eq. 9*).

$$\frac{\phi_{pk}^{'}}{\phi_{pk}} = 1 + \frac{C_m}{\phi_{pk}}(p - p_i) + \frac{\varepsilon_l}{\phi_{pk}}(\frac{K}{M} - 1)(\frac{p}{p_L + p} - \frac{p_i}{p_L + p_i}) \quad \text{(Eq. 9)}$$

where (*Eq. 10*):

$$C_m = \frac{1}{M} - (\frac{K}{M} + f - 1)\gamma \quad \text{(Eq. 10)}$$

$\frac{K}{M}$ in *Eqs. 9* and *10* is function of Poisson's ratio (υ), and it is expressed as (*Eq. 11*):

$$\frac{K}{M} = \frac{1}{3}(\frac{1+\upsilon}{1-\upsilon}) \quad \text{(Eq. 11)}$$

Compared with the compressibility of organic pores, the compressibility of solid kerogen (γ) is negligible, so *Eq. 10* is usually simplified as (*Eq. 12*):

$$C_m = \frac{1}{M} \quad \text{(Eq. 12)}$$

Eq. 9 is then simplified as (*Eq. 13*):

$$\phi_{pk}^{'} - \phi_{pk} = \frac{1}{M}(p - p_i) + \varepsilon_l[\frac{1}{3}(\frac{1+\upsilon}{1-\upsilon}) - 1](\frac{p}{p_L + p} - \frac{p_i}{p_L + p_i}) \quad \text{(Eq. 13)}$$

The volume change of organic matrix with decreasing pore pressure is (*Eq. 14*):

$$\Delta V_k = \frac{G_k B_{gi}}{\phi_{pk}}(\phi_{pk} - \phi_{pk}^{'}) = \frac{-G_k B_{gi}}{\phi_{pk}}\left\{\frac{1}{M}(p - p_i) + \varepsilon_l[\frac{1}{3}(\frac{1+\upsilon}{1-\upsilon}) - 1](\frac{p}{p_L + p} - \frac{p_i}{p_L + p_i})\right\} \quad \text{(Eq. 14)}$$

The desorbed volume of adsorbed gas (ΔV_d)

The isothermal adsorption and desorption equation of shale gas is expressed by Langmuir equation (*Eq. 15*):

$$V = \frac{V_L p}{p_L + p} \quad \text{(Eq. 15)}$$

When the reservoir pressure is p, the volume of adsorbed gas is added to the free gas (*Eq. 16*):

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$$\Delta V_d = \frac{G_k B_{gi}}{\phi_{pk}} \rho_{pk} B_g \left(\frac{V_L p_i}{p_L + p_i} - \frac{V_L p}{p_L + p}\right) \tag{Eq. 16}$$

The expression of material balance equation

Eqs. 3, 8, 14 and *16* are substituted into *Eq. 4,* and the material balance equation for the process of reservoir development is as follows (*Eq. 17*):

$$\begin{aligned} G_p B_g + W_p B_w - W_i = G_{nk}[(B_g - B_{gi}) + \frac{B_{gi}}{1 - S_{nkwi}}(C_w S_{nkwi} + C_{nk})(p_i - p)] + G_k\{(B_g - B_{gi}) \\ - \frac{B_{gi}}{\phi_{pk}}[\frac{1}{M}(p - p_i) + \varepsilon_l(\frac{1}{3}\cdot\frac{1+\upsilon}{1-\upsilon} - 1)(\frac{p}{p_L + p} - \frac{p_i}{p_L + p_i})] + \frac{B_{gi}}{\phi_{pk}}\rho_{pk} B_g(\frac{V_L p_i}{p_L + p_i} - \frac{V_L p}{p_L + p})\} \end{aligned} \tag{Eq. 17}$$

Herein new parameters *Y, X* and *E* are defined as (*Eqs. 18, 19* and *20*):

$$Y = G_p B_g + W_p B_w - W_i \tag{Eq. 18}$$

$$X = B_g - B_{gi} - \frac{B_{gi}}{\phi_{pk}}[\frac{1}{M}(p - p_i) + \varepsilon_l(\frac{1}{3}\cdot\frac{1+\upsilon}{1-\upsilon} - 1)(\frac{p}{p_L + p} - \frac{p_i}{p_L + p_i})] + \frac{B_{gi}}{\phi_{pk}}\rho_{pk} B_g(\frac{V_L p_i}{p_L + p_i} - \frac{V_L p}{p_L + p}) \tag{Eq. 19}$$

$$E = (B_g - B_{gi}) + \frac{B_{gi}}{1 - S_{nkwi}}(C_w S_{nkwi} + C_{nk})(p_i - p) \tag{Eq. 20}$$

Eq. 17 is expressed as (*Eq. 21*):

$$\frac{Y}{E} = G_{nk} + G_k \frac{X}{E} \tag{Eq. 21}$$

Eq. 21 is a linear equation with slope of G_k and intercept of G_{nk}. Based on actual production data and gas reservoir pressure data, the relationship between $\frac{Y}{E}$ and $\frac{X}{E}$ is plotted. Then, G_k and G_{nk} are calculated by means of linear fitting.

The calculation equation of adsorbed gas reserves is as follows (*Eq. 22*):

$$G_A = G_k \frac{B_{gi}}{\phi_{pk}} \rho_{pk} \left(\frac{V_L p_i}{p_L + p_i}\right) \tag{Eq. 22}$$

Results

The material balance equation was applied to a pilot production block of a shale gas reservoir in Xu 5 shale in Sichuan Basin of China. During the production, only gas was produced. The remaining fluid of 5100 m^3 did not flow back after stimulation. The gravity of natural gas was 0.65. Other basic reservoir parameters and production data are shown in *Tables 5* and *6* respectively.

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Table 5. *Parameters of shale gas reservoir*

Parameter	Value	Parameter	Value
Initial reservoir pressure (P_i)	27.09 MPa	Langmuir volume (V_L)	42.22 m^3/t
Initial gas volume factor (B_{gi})	3.8×10^{-3} m^3/m^3	Porosity of kerogen (ϕ_{pk})	0.05
Compressibility of inorganic matrix (C_{nk})	0.00125 MPa^{-1}	Langmuir pressure (p_L)	25.5 MPa
Compressibility of water (C_w)	0.000435 MPa^{-1}	Parameters of Langmuir curve (ε_l)	0.0128
Water saturation in inorganic matrix (S_{nkwi})	0.2	Axial constraint modulus (M)	6136 MPa
Density of kerogen (ρ_{pk})	2.0 t/m^3	Poisson's ratio of kerogen (υ)	0.23

Table 6. *Production history*

Pressure (p/MPa)	**Cumulative gas production (G_p/m^3)**	**Gas volume factor ($B_g/m^3/m^3$)**
25.12	4710000	0.004
23.21	6085000	0.0044
20.09	7721000	0.005
15.12	11002000	0.0065
9.04	15530000	0.0115

Parameters Y, X and E are calculated with *Eqs. 18, 19* and *20* are shown in *Table 7.* The relation of $\frac{Y}{E}$ vs. $\frac{X}{E}$ is plotted as *Fig. 5* shown. After regression with linear function, the initial total free gas at standard condition is 0.2901×10^8 m^3, free gas in kerogen being 0.2223×10^8 m^3 and free gas in inorganic matrix being 0.0678×10^8 m^3. Based on the calculation results, the free gas in inorganic matrix is 23.37% of total free gas reserves. Based on *Eq. 22,* the adsorbed gas in kerogen is 0.0774×10^8 m^3.

Table 7. *Calculation of key parameters*

Pressure (p/MPa)	**Total volume change of inorganic matrix ($\Delta V_{nk}/m^3$)**	**Volume change of pores in organic matrix ($\Delta V_k/m^3$)**	**Volume of adsorbed gas ($\Delta V_d/m^3$)**	**Parameter (Y)**	**Parameter (X)**	**Parameter (E)**
25.12	71.46	291.21	9067.44	13740	0.000864	0.000810524
23.21	140.74	559.77	20414.88	21674	0.00134	0.001220704
20.09	253.92	965.60	44717.65	33505	0.002095	0.001837287
15.12	434.20	1510.07	111570.17	66413	0.003995	0.003363581
9.04	654.75	1943.07	350052.54	173495	0.010356	0.008395546

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It is interesting to note that the calculated free gas in kerogen is 76.63%, which agrees with the assumption that most of the free gas may be in the organic pores in the overmature organic-rich shale gas reservoirs (Passey et al., 2010).

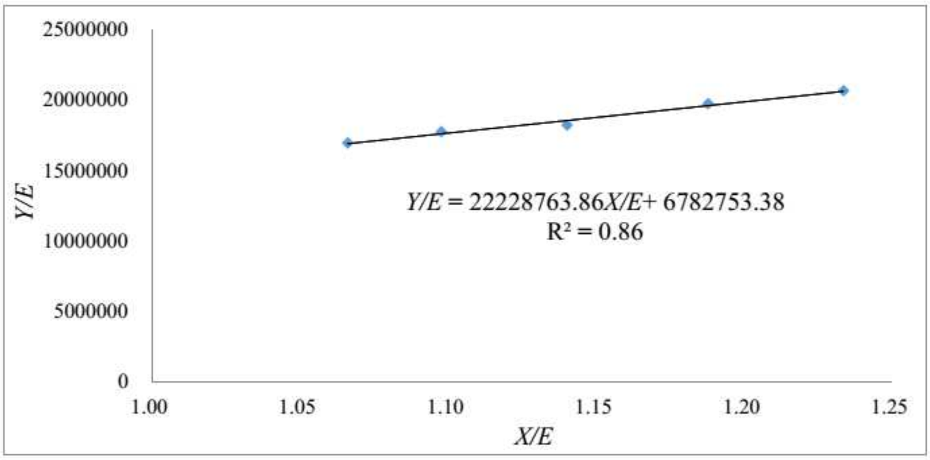

Figure 5. *Y/E versus X/E*

Discussions

The resistivity of dry shale samples with different vitrinite reflectance in Xu jiahe shale and Zhenjing Shale showed that if vitrinite reflectance is greater than 2%, the rock resistivity is much lower than that with lower thermal maturities. It proves that there being abundant amount of solid graphite in overmature shale, and the dissolved gas in the kerogen or bitumen should be negligible.

Natural fractures in most of gas shales can hardly act as a main storage space. Therefore, the volume of natural fractures in this type of reservoir can be neglected.

In some gas shale, thin laminated layers of sandstone are imbedded in organic-rich shale, and free gas in inorganic matrix is also too important to neglect. The distinct feature of inorganic matrix and kerogen should be considered. The inorganic pores are water wet, there being water and free gas in effective inorganic pore, while Kerogen is non-water wet, there is only free gas and adsorbed gas in the organic pores.

For both thin laminated layers of sandstone imbedded in organic-rich shale and high organic content black shale, a generalized material balance equation is developed with the principle of material balance, Langmuir isothermal adsorption model and Palmer and Mansoor (1998) model. Gas in organic matrix and inorganic matrix could be calculated simultaneously, and it is showed that the majority of the free gas is in the organic pores in the overmature organic-rich shale gas reservoirs.

Conclusions

Gas shale was treated as a quintuple porosity system, but the overmature organic-rich gas shale can be simplified as two porosity system.

An advanced gas material balance equation was presented for the overmature organic-rich gas shale, and the corresponding function was also introduced the material balance equation can be plotted as a straight line. A field case showed that in overmature gas shale, most of the free gas is in the organic pores.

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APPENDIX

Nomenclature

σ is the total conductivity of the gas shale;

X_{cw} is the bulk volume fraction of claybound water;

σ_{cw} is the conductivity of claybound water;

X_w is the bulk volume fraction of capillary bound or free water;

σ_w is the conductivity of capillary bound or free water;

$X_{graphite}$ is the bulk volume fraction of graphite;

$\sigma_{graphite}$ is the conductivity of graphite;

X_{pyrite} is the bulk volume fraction of pyrite;

σ_{pyrite} is the conductivity of pyrite;

V_{pm} is the volume ratio of inorganic matrix scaled to the bulk volume of the composite system, dimensionless;

V_{pk} is the volume ratio of organic matrix scaled to the bulk volume of the composite system, dimensionless;

ϕ_{pm} is the effective pores in inorganic matrix, dimensionless;

V_{sm} is the volume ratio of inorganic solid to inorganic matrix, dimensionless;

ϕ_{pk} is the initial porosity in kerogen, dimensionless;

V_{sk} is the volume ratio of solid kerogen to organic matrix, dimensionless;

ϕ_T is the total porosity, dimensionless;

p_i is the initial pressure, MPa;

p is the reservoir pressure, MPa;

G is the total free gas in the reservoirs at standard condition, m^3;

G_{nk} is the free gas in inorganic matrix at standard condition, m^3;

G_k is the free gas reserves in organic matrix at standard condition, m^3;

G_A is the adsorbed gas in organic matrix at standard condition, m^3;

B_{gi} is the gas volume factor at initial reservoir pressure, m^3/m^3;

G_p is the cumulative gas production, m^3;

B_g is the gas volume factor at the reservoir pressure p, m^3/m^3;

W_p is the cumulative water production, m^3;

B_w is the water volume factor at the reservoir pressure p, m^3/m^3;

ΔV_{nk} is the total volume change of water and pores in the effective pores of inorganic matrix, m^3;

ΔV_k is the volume change of pores in organic matrix, m^3;

ΔV_d is the volume of adsorbed gas at the reservoir pressure of p, m^3;

W_i is the cumulative water injection, m^3;

ΔV_{epnkw} is the volume change of water in the effective pores of inorganic matrix, m^3;

ΔV_{epnkf} is the volume change of effective pore in inorganic matrix, m^3;

S_{nkwi} is the water saturation in inorganic matrix, dimensionless;

P_i is the original reservoir pressure, MPa;

p is the current reservoir pressure, MPa;

C_w is the compressibility of water, MPa^{-1};

C_{nk} is the compressibility of inorganic matrix, MPa^{-1};

ϕ'_{pk} is the porosity of kerogen at the pressure of p, dimensionless;

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C_m is the expansion factor of organic matrix, MPa^{-1};

ε_l is parameters of Langmuir curve match to volumetric strain change because of matrix shrink age, dimensionless;

K is bulk modulus, MPa;

M is the axial constraint modulus, MPa;

p_L is the Langmuir pressure, MPa;

f is the factor (0-1), dimensionless;

γ is the compressibility of solid kerogen, MPa^{-1};

υ is the Poisson's ratio of kerogen, dimensionless;

V_L is the Langmuir volume, m^3/t;

ρ_{pk} is the apparent density of kerogen, t/m^3; and

V is the adsorbed gas at the pressure p, m^3/t.

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MULTIFACTORIAL MAPPING OF QTL FOR YIELD AND YIELD COMPONENT IN WHEAT (*TRITICUM AESTIVUM*) IN NORMAL AND DROUGHT CONDITIONS

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Abstract. In order to mapping main and epistatic effects and environmental interactions of QTLs for yield and yield component in normal and water deficit conditions in wheat, A recombinant inbred lines population, comprising 148 lines derived from a cross between two winter wheat cultivars, 'YecoraRojo' and 'No. 49', was evaluated in two location in Iran (Miandoab and Mahabad) during 2014-2016. A linkage map including 177 microsatellite and 51 retrotransposon markers were used in this study. Quantitative trait loci (QTL) were determined for additive effects and additive × additive epistatic interactions using the QTL Cartographer 2.5 and QTL Network 2.0 software based on the CIM and mixed-linear method. Results showed in normal condition 3 QTL (R^2_A = 5.0 to 9.9%), 2 QTL × environments (R^2_{AE} = 5.81%), 10 additive × additive epistatic effects (R^2_{AA} = 1.74 to 7.80%) and 27 QTL × QTL × environmental interactions (R^2_{AAE} = 0.58 to 12.10) were significant. In water deficit conditions, 3 QTLs (R^2_A = 5.0 to 10.94), 1 QTL × environmental interactions (R^2_{AE} = 3.1%), 8 additive × additive inactions (R^2_{AA} = 0.88 to 10.54%) and 20 QTL × QTL × environmental interactions (R^2_{AAE} = 3.46 to 11.26) were identified. In two conditions 4 QTL funded for grain yield with R^2_A value 5.0 to 10.96% which, 3 of them were located on A genome. Also, the largest number of QTLs for yield and yield component located on chromosome 3A which can be used these chromosomes in genetic engineering. Also, most of the repeatedly detected QTL across environments were not significant.
Keywords: *grain yield, epistatic QTL, main-effect QTL, microsatellite marker, wheat*

Introduction

Bread wheat (*Triticum aestivum* L) is one of the world's most important food crops, providing 40% of the world's food. With increasing the number of population in the world, the global appeal for wheat will increase by 40% before 2020. Therefore, a method to solve this problem is to breed more productive varieties of wheat (Dixon,

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2009; Wei et al., 2014). Hexaploid wheat (2n = 6x = 42) has a large genome size of about 17300 Mb (Hussain and Rivandi, 2007). Repetitive DNA elements make for approximately 90% and transposable elements make up 80% of wheat genome (Wanjugi et al., 2009).

Determining the number of controlling genes and them effect on qualitative traits such as yield and yield components are fundamental steps towards molecular breeding of crops (Cooper et al., 2009; Lapitan et al., 2009). Genetic studies under drought stress environments identified quantitative trait loci (QTL) for yield and yield component of wheat (Kirigwi et al., 2007; El-Feki, 2010; McIntyre et al., 2010; Pinto et al., 2010). QTL analysis establishes a link between continuous phenotypic variation and inheritance mechanisms resulting from genetic variation of single gene locations, and QTL identification makes the selection with the help of markers possible (Koroff et al., 2008; Emebiri et al., 2009). The grain yield of wheat and cereals in general, is a polygenic and highly complex trait that is influenced by environmental and genetic interactions at all stages of the plants growth (Slafer, 2003). QTL mapping has been increasingly utilized as a strategy to detect genomic regions important for grain yield and other genetically complex traits in cereal species (Cuthbert et al., 2008; Rebetzke et al., 2012). Therefore the discovery, understanding and eventual incorporation of genes and alleles that beneficially influence yield are major targets for breeding programs worldwide (Simmonds et al., 2014). Many chromosomal regions with minor effects have been detected in controlling yield, but repeatable QTL across environments and different backgrounds are rare.Therefore, concentrating on the exploration and utilization of genomic regions for traits related to drought tolerance may be a more feasible strategy than yield per se approaches. Previous studies have shown that different QTL was detected for grain yield in which are uniformly distributed across the entire genome and all 21 wheat chromosomes have been involved in controlling grain yield in wheat. Most of the QTL were detected on chromosome number of 4A, 3B and 2B (Zhang et al., 2009).

Additive effects and additive × additive epistatic effects are an essential factor affecting the phenotypic expression of complex trait genes and genetic variations in populations (Liao et al., 2001). Commonly, the detected additive × additive interactions contained all three types of epistatic effects, classified on the basis of whether the QTL involved exhibited their own main effects or not. These types were nominated to be epistatic between two additive loci, between an epistatic locus and an additive locus, or between epistatic loci only, which are equivalent to the terms of interactions between QTL, interactions between QTL and background loci, and interactions between complementary loci (Li, 1998). Li et al. (2014) found thirteen significant QTL with additive effects for biomass, grain yield, and straw yield. They also reported of which six exhibited epistatic effects, eleven significant additive × additive interactions were detected, of which seven occurred between QTL showing epistatic effects only, two occurred between QTL showing epistatic effects and additive effects, and two occurred between QTL with additive effects. These QTL explained 1.20 to 10.87% of the total phenotypic variation. In study of Li et al. (2016) a total of 41 QTL with additive effects on different traits were mapped on most wheat chromosomes, excluding 1A, 2A, 3D, 4D, 6D, and 7B. Seven chromosome regions showed either tightly linked QTL or QTL with pleiotropic effects on two to four traits. Ten pairs of QTL showed additive × additive effects (AA), four QTL were involved in additive × environment (AE) effects, and one was involved in AAE effects.

Considering the fact that in previous studies QTL interactions with the environment, QTL × QTL and of QTL × QTL × Environment interaction over different years and different environmental conditions less studied, the present research and its results can be different from other similar studies. The objective of this study was mapping of QTLs for yield and yield component in normal and water deficit stress conditions and there main and epistatic effects and environmental interactions in wheat. The results will be of great significance for helping breeders to enhance the yield of wheat.

Materials and methods

Plant materials

Plant materials used in this experiment, include 148 bread wheat recombinant inbred lines derived from the cross between Yecora Rojo (America originated as a paternal line 149, high Yield, dwarf and early mature) and genotype No. 49 (the origin of Sistan and Baluchestan, Iran as female line, high altitude and late mature). Lines were produced at Riverside University and through of Center of Excellence Molecular Breeding, University of Tabriz was placed at the disposal of this research.

Field evaluation

Studied lines with parents were planted on research farms of Mahabad University and Miyandoab Agricultural Research Center in 2014-2015. The above mentioned regions are grouped into semi-arid areas of Iran. In both experiments used alpha lattice design with two replications under normal and water deficit conditions. Each plot consisted of two rows with 2.5 m long and the inter row and inter plant spacing's were 20 and 5 cm, respectively. Irrigation in stress and non-stress conditions was done after 90 mm evaporation from class A pan, depending on the temperature and evapotranspiration until heading stage. In water deficit stress conditions, irrigation was stopped at heading stage, but in normal irrigation conditions was continued until the heading stage. Crop care was alike for all lines. At physiological maturity number of grains per spike, number of spike, thousand kernel weights, grain yield and harvest index for each plot were measured.

QTL analysis

For QTL analysis the existing linkage map including of 177 microsatellite and 51 retrotransposons markers were used. In this map, 202 markers belonged to 36 linkage groups with a length of 691.36 cm and 26 markers were not associated with any linkage groups (Roder et al., 1998; Roder et al., 1995). According to linkage maps provided for wheat, 34 linkage groups correspond with 19 chromosomes of 21 chromosomes. The average distance between adjacent markers on the map was 3.42 cm. QTL analysis was performed by QTL network 2.0 and QTL Cartographer 2.5 software's through mixed-linear and composite Interval Mapping (CIM) methods. Given that the population of recombinant inbred lines is a permanent population, QTL × environment, QTL × QTL, and QTL × QTL × environment interactions were also examined. It should be noted in cases which QTLs detected by cartographer and not detected by QTL network, there were no interaction between QTL × environment and these effects were not recorded on the tables.

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Results

Phenotypic performance of wheat RILs and parents

The phenotypic variation among wheat RILs and the parents of studied traits, measured in two years and two locations in average of normal and drought conditions are summarized in *Table 1* and *Figures 1* to *4*. Yecora Rojo and No. 49 differed significantly in the measured traits, that phenotypic values of No. 49 for grain yield and grain yield component being much higher than Yecora Rojo. Some RILs had more extreme values than the parents in all conditions, showing substantial transgressive segregation, although the average values of RILs for those traits were intermediate between the parental values. Furthermore, all traits showed considerable phenotypic variation and continuous distributions, indicating their quantitative nature. Based on the results presented in *Table 1*, both the Skewness and Kurtosis of all traits were less than 1.0, implying polygenic inheritance and suitability of the data for QTL analysis (*Table 1*).

Table 1. *Phenotypic summary of yield and grain yield component for Yecora Rojo (P1), No. 49 (P2), and the wheat RILs at two years and two locations in average of normal and drought condition*

Parameters	Spike per m^2	Number of grains per spike	Thousand kernel weight	Grain yield
Yecora Rojo	47.08	20.26	53.65	47.52
No. 49	67.08	23.01	54.03	56.04
RILs mean	59.47	18.82	52.50	46.79
Minimum	45.50	15.05	42.85	29.87
Maximum	77.33	23.06	62.64	61.61
Std. deviation	6.43	1.655	3.76	6.72
Skewness	.378	.183	.040	-.054
Kurtosis	-.362	-.237	-.574	-.774

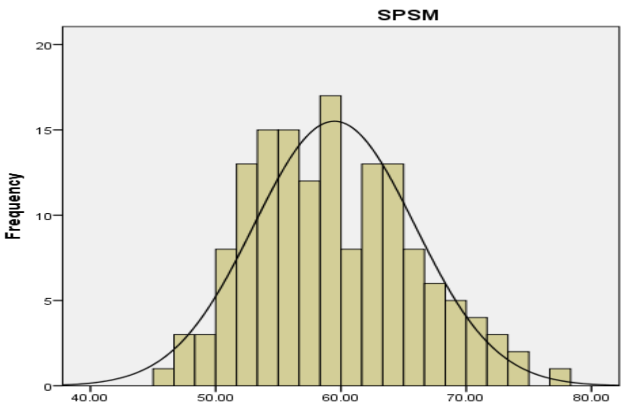

Figure 1. *Frequency distributions of spike per square meter, two years conditions*

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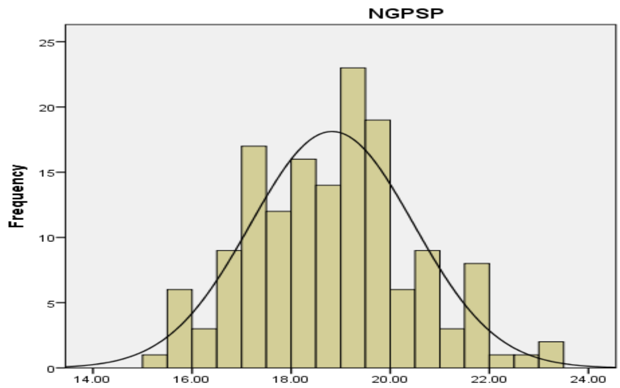

Figure 2. *Frequency distributions of number of grains per two years, two locations and average of two conditions*

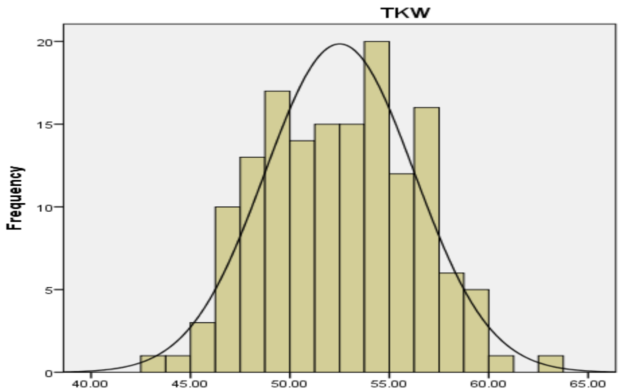

Figure 3. *Frequency distributions of thousand kernel weight in two years, two locations and average of two conditions*

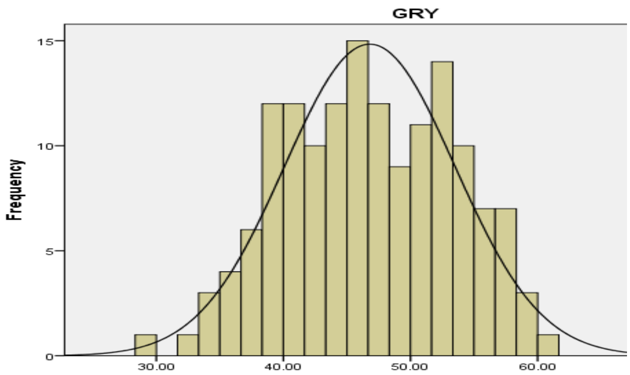

Figure 4. *Frequency distributions of grain yield in two years, two locations and average of two conditions*

QTL mapping

The results of QTL analysis are summarized in *Tables 2, 3,* and *4*. In this research for four traits under study across both years and both locations under normal conditions, 3 QTLs ($R^2_A = 5.0$ to 9.9%), 2 QTL × environments ($R^2_{AE} = 5.81\%$), 10 additive ×

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additive epistatic effects (R^2_{AA} = 1.74 to 7.80%) and 27 QTL × QTL × environment interactions (R^2_{AAE} = 0.58 to 12.10) were significant. Under water deficit conditions, 3 QTLs (R^2_A = 5.0 to 10.94), 1 QTL × environmental interaction (R^2_{AE} = 3.1%), 8 additive × additive inactions (R^2_{AA} = 0.88 to 10.54%) and 20 QTL × QTL × environment interactions (R^2_{AAE} = 3.46 to 11.26) were identified.

Table 2. *Detected QTL and QTL × environment interactions for studied traits in a RIL population of wheat obtained from Yecora Rojo × No. 49 at two years and two locations in normal and water deficit condition*

Trait	Chr.	QTL	Marker interval	Position (cm)	A	R^2_b %	AE_1	AE_2	AE_3	AE_4	R^2_{AE}
Spike per m^2 (normal)	3A	QSPSM3A-N	Wms566- '5LTR.2/Sukkula.380	2.06	-1.72	5.0	-	-	-	-	-
	2A	QSPSM2A-N	Gwm35- 'Gwm296	7.5	-1.93	6.0	-	-	-	-	-
						11					
Grain yield (normal)	7A	QGY7A-N	'Cfa2123- 'Gwm282	53.6	-1.3963	9.9	-	-2.80	2.56	-	5.81
Grain yield (water deficit)	3A	QGY3A-S	5LTR.2/ISSR5.530- 'Gwm66.2	582.2	5.11	10.94	-	-	-	-	0.14
	5A	QGY5A-S	'Barc319- 'Cfa2141	8.0	1.7646	9.82	-	1.34	-	-	3.1
	2D	QGY2D-S	Wms102- 'Wmc18	0	1.64	5	-	-		-	-
						25.76					3.24

A: Additive effect, A positive value indicates that the allele from No. 49 increases the trait value; A negative value indicates that the allele from Yecora Rojo increases the trait value. b: Proportion of the phenotypic variation explained by the QTL. Ae: Additive × environmental effects. R^2_{AE}: Proportion of the phenotypic variation explained by additive × environment effect

Table 3. *Additive × additive epistatic QTL and QTL × QTL × environment interactions for studied traits in two years and two locations at normal condition*

Traits	Chro I	Marker intervals	Position	Chro J	Marker intervals	Position	AA	R%^2_b	AA_{E1}	AA_{E2}	AA_{E3}	AA_{E4}	R^2_{AAE}
Spike per m^2	6B	'Barc178-'Gwm219	109.2	3A	Sukkula/ISSR10.600- 'Sukkula/ISSR7.550	266.9	0.7092	1.8	-3.84	-4.99	5.12	3.80	12.10
	4A	'Wmc468-'Barc170	0.0	7A	'Gwm276-'Cfa2123	35.5	0. 5394	2.2	-3.44	-3.28	3.74	3.14	11.18
								4.0					23.28
Thousand kernel weight	5A	'Barc186-'Barc117	42.1	3A	'Wms566- '5LTR.2/Sukkula.380	56.2	1.12	3.8	1.52	-	-1.58	-	8.42
	5A	'Gwm617-'Wmc327	425.2	31	'Wmc336- 'LTR6150/ISSR3.500	25.2	1.52	4.42	1.95	2.50	-1.98	-2.41	10.18
	3A	'LTR6150/ISSR10.260- '5LTR.2/ISSR5.530	523.2	2A	'Wms122-'Wmc296	17.9	-4.89	1.74	-	-	-	-	0.58
								20.96					19.18
Grain yield	5A	'Gwm129-'Barc1	96.2	6D	'Barc54-'Gwm325	0.0	-2.2463	4.14	-3.08	-3.21	3.69	2.74	9.30
	5A	LTR6149/Nikita.740- 'Barc330	354.2	6A	'Gwm459- 'Sukkula/Nikita.450	5.3	-2.3903	4.86	-	-	-	3.10	6.86
	5A	'Barc330-'Gwm617	403.7	31	'Wmc336- 'LTR6150/ISSR3.500	23.2	5.3206	7.80	5.96	6.41	-5.59	-6.49	10.9
	6B	'Wms88-'Barc24	64.7	1B	Sukkula/ISSR7.230- 'LTR6149/ISSR2.180	3.7	-3.5644	5.94	-2.66	-	-	3.19	4.32
	2A	'Wmc296-'Wms339	19.6	2A	'Gwm35-'Gwm296	1.0	-3.4287	7.0	-2.51	-	-	2.70	5.18
								29.68					36.38

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Table 4. *Additive x additive epistatic QTL and QTL × QTL × environment interactions for studied traits in two years and two locations at water deficit condition*

Traits	Chro I	Marker intervals	Position	Chro J	Marker intervals	Position	AA	$R\%^2_b$	AA_{E1}	AA_{E2}	AA_{E3}	AA_{E4}	R^2_{AAE}
Spike per m^2	2B	'Wms148-'Gwm374	1.0	7B	'Wms297-'Sukkula/Nikita.520	72.8	-0.6881	0.88	-2.211	-2.310	2.474	2.04	11.26
Thousand kernel weight	6B	'Wms88-'Barc24	61.7	31	'LTR6149/ISSR2.260-'Psp2999	5.0	-1.5848	9.1	-	-	-	-	2.84
	3A	'Barc45-'Gwm2	0.0	5B	'Gwm499-'Gwm371	6.1	-0.7424	2.28	-	-	-	-	3.46
	6D	'Barc54-'Gwm325	1.0	2A	'Wms47-'Wmc198	0.0	0.5372	10.54	1.75	2.031	-1.601	-2.18	10.56
								21.92					17.54
Grain yield	5A	'Gwm443-'Wms154	6.0	5A	'Barc180-'Gwm129	84.6	2.8509	6.76	-	-	-2.201	-	5.82
	7B	'Wms400-'Wms46	23.8	7B	'Wms297-'Sukkula/Nikita.520	79.8	20.395	4.0	2.77	3.22	-3.077	-2.970	4.0
								10.76					9.82

AA: Additive x additive effect, a positive value indicates that the effect of the parents' effect is larger than the recombinant effect, and a negative value means that the recombinant effect is larger than the parents' effect. b: Proportion of the phenotypic variation explained by additive x additive QTL. AAe: Additive x additive × environment effects. R^2_{AAE}: Proportion of the phenotypic variation explained by additive x additive × environmental effects

Spike per square meter

In normal condition, two QTLs on chromosomes 3A and 2A were detected for spike per square meter (*Table 2*). These QTLs were linked with markers of Wms566-'5LTR.2/Sukkula.380 and Gwm35- 'Gwm296 and in distance of 2.06 and 7.50 cm. The amounts of additive value of these QTLs were -1.72 and -1.93 and explained 11.0% of total phenotypic variation. Two QTLs (QSPSM3A- N and QSPSM2A- N) were contributed by No.49 parent alleles. Also, in normal condition two pairs of significant additive × additive epistatic effects were observed for spike per square meter (*Table 3*). These interactions were existed between chromosomes of 3A × 6B and 4A × 7A, which showed additive × additive values of 0.7092 and 0.5394, respectively and justified 4.0% of total phenotypic variation. It should be noted that these epistatic (QSSM3A-N × QSSM6B-N and QSSM7A-N × QSSM7A-N) interactions acted by increasing the values of the parents types. In normal conditions, the effects of QTL × QTL were significant in four environments which accounted for 23.28% of the phenotypic variation (R^2_{AAE} = 23.28%).

In water deficit condition, one pair significant epistasis interaction (additive × additive) of QTL between chromosomes 7B × 2B was detected (*Table 4*), which had additive × additive and R^2_{AA} value of 0.6881 and 0.88%, respectively. This QTL acted by increasing the values of the parent's types. In additions, the effects of epistasis interaction between chromosomes (QSSM7B-S × QSSM2B-S) were significant in all environments with R^2_{AAE} of 11.26%. It should be noted there were no common QTL and epistasis effects in both conditions. In normal condition phenotypic variation explained by additive QTL effect was more than QTL × QTL epistasis effect, so can be said additive QTL effect played a more important role in controlling spike per square

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meter. Also, in both conditions the amount of epistatic interactions were small compared to the QTL × QTL × environmental effects, indicating that these epistatic pairs were highly sensitive to environments in the control spike per square meter.

Thousand kernel weight

In normal condition, three significant additive × additive epistasis effects between chromosomes of 3A × 5A, 31 × 5A and 2A × 3A were found for thousand kernel weight (*Table 3*). Amounts of additive × additive values for these effects were 1.12, 1.52 and -4.89, respectively and these epistatic effects controlled 20.96% of phenotypic variation. Two interactions (QTKW3A-N × QTKW5A-N and QTKW31-N × QTKW5A-N) acted to increase the values of the parental types, and the others (QTKW2A-N × QTKW3A-N) acted in the opposite direction, that is, recombinant effects were larger than parental effects. Also, in this condition six QTL × QTL × environmental interactions were significant which 19.18% of phenotypic variations were explained by these interactions.

In water deficit condition, three pairs of additive × additive epistasis were detected between chromosomes 31 × 6B, 5B × 3A and × 6D × 2A, with additive × additive value of -1.5848, -0.7424 and 0.5372 (*Table 4*). The epistasis of QTKW6D-S × QTKW2A-S acted in favor of the parental type and accounting for 10.54% of the phenotypic variance. The other two interactions of QTKW31-S × QTKW6B-S and QTKW5B-S × QTKW3A-S, explained 11.38% of the phenotypic variation together. In this research additive × additive epistasis of QTKW6D-S × QTKW2A was significant in all environments and these interactions justified 10.56% of phenotypic variation.

In our research there were no common epistasis effects observed in both conditions for thousand kernel weight. In addition phenotypic variation explained by additive × additive effect in both conditions exceeded than QTL × QTL × environmental effects, so can be said additive × additive effects are less influenced by the environment.

Grain yield

In normal condition only one QTL was detected on chromosome 7A for grain yield. This QTL (QGY7A-N) was linked with markers of Cfa2123- Gwm282, positioned within a 53.6 cm, by R^2 value of 9.9% and additive effects value of -1.3963 (*Table 1*), Favorable alleles were contributed at this QTL by Yecora Rojo parent. Also, in this condition two significant interaction effects between QTL × environments with R^2_{AE} of 5.81 were detected. Also five pairs of significant additive × additive epistatic effect between chromosomes 5A × 6D, 5A × 6A, 5A × 31, 6B × 1B and 2A × 2A were identified. These epistatic effects evidenced additive × additive value of -2.2463, -2.3903, 5.3206, -3.5644 and -3.4287, respectively and explained 29.68% of the total phenotypic variance. The QTL pair of QGY31-N × QGY5A-N acted in favor of the parental types and effects of QGY6D-N × QGY5A-N, QGY6A- N × QGY5A-N, QGY1B- N × QGY6B-N and QGY2A- N × QGY2A-N acted to increase the values of the recombinant types. It should be noted that 13 significant interactions between QTL × QTL × environments which were detected for grain yield had R^2_{AA} values ranging from 4.32 to 10.9% (*Table 2*).

Under water deficit condition, three QTLs on chromosomes 3A, 5B and 2D were detected for grain yield (*Table 2*). These QTL were linked with markers of 5LTR.2/ISSR5.530- 'Gwm66.2, 'Barc319- 'Cfa2141 and Wms102- 'Wmc18 and in distance of 582.2, 8 and 0 cm. Amounts of additive values of these QTLs were 5.11,

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1.7646 and 1.640 and explained 25.76% of total phenotypic variation. All three QTLs (QGY3A-S, QGY5B-S and QGY2D- S) had positive effects on grain yield` and were contributed by No.49 parent alleles. Out of these, one significant interaction between QTL (QGY5A-S) × environments with R^2_{AE} values of 3.1% was detected for grain yield. Furthermore, two additive × additive epistatic effects were common in water deficit condition for grain yield (*Table 3*). These epistatic effects were located between chromosomes 5A × 5A and 7B × 7B with additive value of 2.8509 and 2.0395, respectively and justified 10.76% of total phenotypic variation. These effects acted by increasing the values of the parental types. It should be noted that in water deficit condition five QTL × QTL × environmental interactions with R^2_{AAE} from 4.0 to 5.82% were identified. In all conditions, additive QTLs effects were more than additive × environmental effects which indicated additive QTLs are less influenced by the environment. In additions, in normal condition phenotypic variation explained by additive × additive epistatic effects were larger than additive QTLs effects. Thus, epistatic QTLs were more important than additive QTLs for grain yields, but in water deficit condition additive QTLs were more important than epistatic QTLs. Furthermore, in normal condition additive × additive epistatic effect was influenced by the environment because of the low R^2_{AA} value compared to R^2_{AAE}. In water deficit condition, these interactions less affected by the environmental effect compared with other conditions.

Discussion

QTLs for Spike per square meter and grain yield were detected on chromosomes 3A, 2A, 7A, 5A and 2D in two years and two locations in normal and water deficit conditions in the present study. Many QTL affecting yield have been reported on all chromosomes, with the exceptions of chromosomes 3D and 5D, in previous studies, and no significant gene by environment interactions were examined (Huang et al., 2006; Cuthbert et al., 2008; McIntyre et al., 2010). The significant QTLs simultaneously identified for spike per square meter and grain yield in the current study were located on chromosomes 3A. In fact, chromosomes 3A are known to carry a number of major genes affecting spike per square meter and grain yield (Huang et al., 2006). Therefore, the QTL on 3A should be considered to increase wheat biomass, grain, and straw in wheat molecular breeding. Furthermore three significant interactions between QTL × environments were detected for grain yield in normal and water deficit condition. In all conditions additive QTL effects were more than additive × environment effects which indicated additive QTL are not influenced by the environmental effects. Marza et al. (2006) and Quarrie et al. (2005) discovered that a number of major genes affecting yield productivity were located on chromosome 5AL. Cuthbert et al. (2008) showed two-grain yield QTL clusters on chromosomes 7A and 7B around the Xwmc273 locus. We detected a QTL (QGY7A- N) close to 'Cfa2123- 'Gwm282 on 7A with significant effects on grain yield. Unfortunately, however, we failed to detect any QTL on chromosome 7B. On chromosome 2D, a QTL that increased grain yield was detected using 402 DH lines from the spring wheat cross Superb (high yielding)/BW278 (low yielding) by Cuthbert et al. (2008). The QTL on 2D affecting GY was also identified in our study. Zhang et al. (2009) and Li et al. (2014) also detected a QTL with significant effects on GY on chromosome 2D. In this study, no QTL for number of grains per spike, thousand kernel weights was observed in both conditions. The reason is probably

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the effect of a large number of QTLs with low effects in control of the mentioned traits (Tanksley, 1993) or influence of environmental effect on quantitative traits (George et al., 2003). Additive effects and additive × additive epistatic effects are an important factor affecting the phenotypic expression of complex trait genes and genetic variations in populations (Liao et al., 2001). Generally, the additive × additive interactions detected included all three types of epistatic effects, classified on the basis of whether the QTL involved exhibited their own main effects or not. In the present study, a total of 16 significant additive × additive interactions were detected for spike per square meter, thousand kernel weight, grain yield in two years and two locations in normal and water deficit conditions using a RIL population. Except additive × additive epistatic effects of QTKW7B-N × QTKW7B-2B-N under normal conditions, QTKW 6B-S × QTKW31-S and QTKW3A-S × QTKW 5B-S under water deficit condition for thousand kernel weight, all of the identified epistatic effects showed a significant interaction with the environment. Furthermore, in normal condition additive × additive epistatic effect was influenced by the environment but in water deficit condition, these interactions less affected by the environmental effect. Overall, the detection of additive and additive x additive effects of a QTL interfered with each other, indicating that the detection of QTL might vary greatly depending on their interactions with other loci in complex traits (Zhuang et al., 2002). Li et al. (2014) found 8 main QTLs, 3 QTL × environments interactions, 3 additive × additive epistatic effects and 2 QTL × QTL × environments interactions for grain yield. Zhang et al. (2014) located 17 QTLs on 14 chromosomal regions) 1A-1, 1B-1, 2B-1, 2B-2, 2D, 3B-1, 3B-2, 4B, 5A-1, 5B-2, 6B-2, 7A-4, 7A-5 and 7B-1 which were associated with grain yield. Furthermore, they identified 13 QTL × environments, five QTL × QTL interactions and nine QTL × QTL × Environment for grain yield. Wu et al. (2012) found 6 main QTLs, 2 QTL × environments interactions, 6 additive × additive epistatic effects and 5 QTL × QTL × environments interactions in wheat DH lines. In the study of Zhang et al. (2016) 10 QTLs were detected on chromosome 1D, 3A, 6B, 6D, 2A, 2B, 2D,5D, 6A-1 and 6A-2 in the five environments which accounting for 5.65–18.62% of the phenotypic variation for Spike per square meter. Wu et al. (2012), reported 13 main QTLs, 10 QTL × environments interactions, 17 additive × additive epistatic effects and 2 QTL × QTL × environmental interactions for thousand kernel weight at multiple rain-fed environments. In normal, water deficit and average of two conditions, additive QTL, QTL × environments, additive × additive epistatic effects by and QTL × QTL × environment interactions justify 68.22, 68.32 and 94 percent of total grain yield phenotypic variation. It can be concluded that interaction effects such as QTL × E, QTL × QTL and QTL × QTL × E play a more significant role in comparison with the main effects of additive QTL in control of grain yield. From total markers, 50 markers showed tight linkage to the QTLs which related to grain yield, and most of them might be useful for marker-assisted selection. In total, chromosome 3A had the largest number of QTLs. In our research most of the repeatedly detected QTLs across environments were not significant.

Conclusions

The goal of crop genomics is to understand the genetic and molecular basis of all biological processes in plants that are relevant to the species (Vassilev et al., 2006). QTL mapping thus represents the foundation for the selection of markers for crop breeding. First, selection should be applied particularly for QTL with main genetic

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effects, which will likely show stable performance across different environments. In one hand, the actions of QTLs involved in epistatic interactions always depend on other loci. On the other hand, these QTLs could produce varieties adapted to specific ecosystems. Thus, it should be more efficient to select superior genetic combinations rather than selection of single genes. Second, selection programs should consider QTLs with interaction effects in different environments (QE), which will also provide confidence for breeders prior to undertaking marker-assisted selection for complex traits in the design and implementation of breeding strategies. However, QE interaction effects might not be well applied across various environments. Third, selection programs should be based on closely linked or pleiotropic QTL. Breeders could possibly simultaneously improve several related traits by selecting such trait-correlated QTL (Liu et al., 2006). In the current study, closely linked or pleiotropic QTLs were not coincident with yield components, suggested that selection for a yield component could not efficiently increase grain yield. These results will make marker-assisted selection not effective for improvement of wheat yield and its compounds. Thus, detailed information on loci involved in related traits and their genetic relationships will not certainly be helpful to improve grain yield potential in our research.

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EFFECT OF DEFICIT IRRIGATION MANAGEMENT ON QUALITATIVE AND QUANTITATIVE YIELD OF SUGAR BEET (*BETA VULGARIS* L.) IN KARAJ, IRAN

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Abstract. Environmental pollution and food security resulting from agricultural input surpluses are of great concern to the world in recent year. Improving use efficiency of agricultural inputs becomes an important way to relieve above issues. Due to the long growing season of sugar beet, its response to deficit irrigation is very important. According to the objective, a field study was conducted to investigate the effects of conventional (control), fixed and alternate furrow irrigation on qualitative and quantitative yields of sugar beet was carried out in Karaj, Iran. The treatments were applied in two consecutive years (2013 and 2014) as randomized complete block design with three replications. The irrigation water applied in the control treatment was 1127 mm, while the fixed and alternate furrow irrigation reduced drainage by 44% and 50%, respectively. The root yield was obtained 79 t/ha under alternate furrow irrigation, and 16% higher compared to fixed furrow irrigation. Average water use efficiency (WUE) for sugar beet root production in conventional, fixed, and alternate furrow irrigation were achieved 7, 11, and 12 kg/m^3, respectively. Moreover, the maximum water use efficiency for of pure sugar production (1.5 kg/m^3 of water used) was obtained in the alternate furrow irrigation treatment. So, it was 23 and 117% higher than fixed and conventional furrow irrigation treatments, respectively. α-amino nitrogen absorption in the alternate furrow irrigation treatment increased by 29% compared to other treatments, which suggest balanced fertilization management is needed in alternate furrow irrigation. Consequently, alternate furrow irrigation management with reduced drainage achieved maximum WUE.
Keywords: *alternate furrow irrigation, fix furrow irrigation, water use efficiency, root yield, drainage*

Introduction

Agricultural input surpluses cause severe environmental pollution and natural resource depletion; thus, the need to improve the utilization efficiency of agricultural inputs has become urgent. However, the high crop production in this region mainly depends on the surplus of agricultural inputs, which has caused serious resource shortage, environmental pollution and soil erosion in recent years (Liang et al., 2010).

Water resource is an important factor influencing crop productivity (Hardin, 2008). Crop production in semi-arid area mainly relies on irrigation. This is true for Iran where annual rainfall is less than 400 mm with negligible amount of rain during the growing season of summer crops (Sepaskhah and Parand, 2006). Drought stress is one of the main problems in the crop production in arid and semi arid area, and it is a serious threat to successful crop production all over the world (Ober, 2001). Akhavan et al.

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(2007), demonstrated that, deficit irrigation, if correctly managed, can be one of the known strategies for making optimal use of water. Increased water use efficiency (WUE) in agriculture plays an important role for maintaining food security and, it is one of the important goals in water use management (Deng et al., 2006). Therefore, to maintain grain supplement stably and safely, it is critical to improve the utilization efficiency of agricultural inputs, save resource consumption and ensure food security (Wang et al., 2016).

Thus, new irrigation strategies must be established to use the limited water resource more efficiently. One of the new irrigation strategies is the deficit irrigation scheduling, which is a valuable and sustainable production strategy for dry region (Greets and Raes, 2009). Under good management practices, deficit irrigation can result in substantial water saving with little impact on the quality and quantity of the harvested yield (Topak et al., 2011). In general, it has been proved that under stress conditions concentrations of dissolved substances increase to correct the conditions, and that this is directly related to the physiological system of the plants and osmotic absorption by the roots (Morillo-Velarde and Ober, 2006). Some field crops including sugar beet can adapt well to deficit irrigation practices (Kirda et al., 2002). Sugar beet is reputed to be a deep rooting crop and relatively insensitive to water stress because of the morphological and physiological characteristics of its root system (Doorenbosand and Kasam, 1979). Sugar beet has the ability to grow in a wide range of salinities and climatic conditions (Tognetti et al., 2003), and Sugar beet is resistant to drought and needs strategies to reduce the effects of drought stress so that it can achieve high yields (Hsiao, 2000). Sepaskhah (1996) reported that, for plants like sugar beet (grown for their leaves or roots) shallow groundwater and alternate furrow irrigation led to high WUE, but deep groundwater might cause substantial reduction in root yield if deficit irrigation was applied. One of the internal responses of sugar beet to water shortage, in addition to reduced growth, is increased sugar concentration in roots. On other hand Uçan and Gençoğlan (2004), reported that the root yield increased as the applied irrigation water increased, and a linear relationship was found between these two parameters. However, drought stress is an important factor in reducing yield which, depending on the climate under which it is grown, varies from 5 to 15 t/ha (Pidgeon et al., 2001).

In the several growth stages of sugar beet, many researchers have investigated deficit irrigation management. Firoozabadi et al. (2003) reported that they applied mild, moderate, and severe stress continuously on sugar beet under normal conditions during the growing season, and achieved 58, 45, and 34 t/ha root yields, respectively. Bazza and Tayaa (1999) have reported that deficit irrigation by 25% in furrow irrigation led to 21% reduction in root yield, but that water use efficiency would increase by 5% compared to the control that received full irrigation. In the study carried out by Rytter (2005), it has been reported that deficit irrigation by 40% reduced sugar beet dry matter by 50% compared to the treatment that received full irrigation. Applying drought stress in the last growing season increased root impurities, especially nitrogen, sodium, potassium, and hence, increased molasses production. Therefore, water stress in the end period of sugar beet season has an effect on sugar concentration of the harvested crop (Clover et al., 2001). Topak et al. (2011) studied the effect of deficit irrigation on sugar beet in semi-arid zone by drip irrigation system, and concluded that 25% and 50% saving in irrigation water caused 6.1 and 45.7% reduction, respectively, in the net income. Albayrak et al. (2010) reported that total sugar yield in alternate furrow irrigation increased compared to conventional furrow irrigation and water use efficiency

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was 29% higher. Mohamoodi et al. (2008) studied the relation to different irrigation regimes in 30, 50, 70 and 90% FC. Irrigation treatment showed that the optimum soil water content for root yield is at 70% field capacity with 78.5 t/ha and maximum quality observed. In a research carried out by Hassanli et al. (2010) it was shown that drip irrigation water management could lead to production of up to 79 t/ha of sugar beet roots, with water use efficiency in root and sugar production being 9 and 1.26 kg/m^3, respectively.

Because of water scarcity in the study area sugar beet cultivation relies extensively on irrigation and is mainly irrigated using conventional furrow systems without soil moisture monitoring or climate based scheduling. Consequently water stress is a common cause of yield loss. Therefore alternative irrigation technologies and more efficient irrigation management should be developed to overcome the problems associated with water stress or over irrigation (Hassanli et al., 2010).

Under these conditions, there is one way for farmers to maximize their profit on sugar beet production. Research results show that the best management of deficit irrigation on sugar beet crop and to choose the most appropriate irrigation scheduling for saving irrigation water is AFI (Topak et al., 2011), therefore deficit irrigation management, the optimum irrigation scheduling for the sugar beet in the arid and semi-arid region.

The objectives of this study were to evaluate the effect of deficit irrigation management on quality and quantity yield of sugar beet. The study examined reduce drainage, yields and IWUE for different irrigation management.

Material and methods

Field experiments of furrow irrigated sugar beet were conducted at the research field of the Kamalabad station of the Sugar Beet Research Institute in Karaj, Iran, at 50°55’E, longitude, 35°55’N latitude and 1313 m altitude during 2013 and 2014 growing season of sugar beet. Climate in this region is semi-arid with total annual precipitation of 265 mm. In the growing season of sugar beet we have no significant rainfall in both years. The soil in this area have no salinity and drainage problems such as water table, some physical properties of the experimental field soil are presents in *Table 1,* and some chemical irrigated water quality properties are shown in *Table 2.*

The experiments were conducted in the same field for the 2-year period. The cultivar, fertilizing, and insect control in all plots were the same for the 2 years. The Pars sugar beet variety was planted on 27 April 2013 and on 2 May 2014, and harvest day were 15 November and 27 October, respectively, for 2013 and 2014. The experimental design was a randomized block with three replications. Each plot consists of 12 row of sugar beet that was 90 m long and 0.5 m wide, the slope of furrow was about 0.00019 m m^{-1}. Sowing density was 3-6 plants per meter.

The design consisted of three irrigation methods. The irrigation methods were alternate furrow irrigation (AFI), fixed furrow irrigation (FFI) and conventional furrow irrigation (CFI). AFI is a deficit irrigation management which one of the two neighboring furrows is alternately irrigated during consecutive watering. The second deficit irrigation is FFI that irrigation is fixed to one of the two neighboring furrows and at last CFI is the conventional method where all furrows irrigated per irrigation, contrast to above mentioned managements.

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Table 1. *Some Physical properties of experimental field soil*

Depth cm	Clay %	Silt %	Sand %	Texture	Bulk density gr/cm^3	Field capacity %	Permanent wilting point %
0-30	31.4	42	26.6	Clay loam	1.47	27.4	14
30-60	34.6	27.4	38	Clay loam	1.42	26.6	14.9

Table 2. *Chemical properties of irrigation water at the study area*

HCO_3 (mg/l)	CO_3 (mg/l)	Cl (mg/l)	K (mg/l)	Na (mg/l)	Mg (mg/l)	Ca (mg/l)	EC (dS/m)	SAR	pH
146	0	0.8	0.8	62	16	23	0.5	2.2	8

Soil water content was measured by gravimetric method in all plots. Irrigation was applied at different intervals according to the soil water content measurement in the root zone. All plot irrigated when the soil water content in the root zone reached 50% of available soil moisture. Doorenbos and Kassam (1979) indicated that the maximum sugar beet yield was usually obtained when the sugar beet plants were irrigated at 50% of available water holding capacity.

The required volume of water was calculated using *Equation 1:*

$$d_n = \Sigma \left(\theta fc_i - \theta_i\right)\Delta z \quad \text{(Eq. 1)}$$

where D_n is the net volume of irrigation water in mm, θ_{fci} plant moisture content at field capacity (in volumetric percentage), and θ_i soil moisture prior to each irrigation (in volumetric percentage), and Δz soil depth in mm. Therefore, the volume of irrigation water was determined beforehand based on soil moisture.

Siphon tubes (25 mm, ID) from an equalizing ditch supplied the water for irrigation treatment. The amount of irrigated water was measured by volumetric methods, and that runoff discharge of the furrows measured by WSC flume (Type II).

The crop evapotranspiration during each irrigation interval (ET, mm) was estimated from *Equation 2* (Heerman, 1985):

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

where ET is the evapotranspiration (mm), I is the depth of irrigation (mm), R is the rainfall (mm), D is the depth of drainage (mm), and ΔW is the change of soil water storage in the measured soil depth. In this study, R was observed at the climatology station in Karaj. The amount of irrigation water applied was checked by inflow and outflow from furrow. ΔW was obtained from the difference between soil water content values to a depth of 0.6 m. The value of D was assumed to be negligible because the amount of irrigation water not increased above field capacity as result of deficit irrigation.

Sugar beet root yield was determined by machine harvesting the five center rows in each plot (each 8 m long). The quality parameters in roots were analyzed in the laboratory of Sugar Beet Research Institute in Karaj, Iran.

Considering the volume of water used and crop yield, water efficiency index was calculated using *Equation 3:*

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$$IWUE = \frac{Y}{W} \quad \text{(Eq. 3)}$$

where IWUE is irrigation water use efficiency (kg produced root/m^3 irrigation water used), Y root or sugar yield (kg/ha), and W the volume of water used (m^3/ha).

Analysis of variance was conducted to evaluate the effects of the treatments on sugar beet root yield (t/ha), white sugar yield (t/ha) and quality parameters, Duncan's multiple range tests was used to compare and rank the treatment means. Differences were declared significant at $P < 0.05$ or $P < 0.01$.

Results

ANOVA of applied water, root yield and sugar yield showed in *Table 3*. ANOVA of the combined 2-year sugar beet root data and Year indicated significant effects for irrigation management. Irrigation management interaction was significant in sugar beet root yield but not significant in white sugar yield (*Table 3*).

Table 3. *ANOVA of irrigation and yield of sugar beet*

		df	Sum of squares	MS	F-value	Sig.
Root yield	Year (Y)	1	98.07	98.07	19.68	0.0022
	Treatment (T)	2	771.59	385.79	77.41	<0.0001
	Y*T	2	138.07	69.03	13.85	0.0025
Sugar yield	Year (Y)	1	54.60	54.60	155.65	<0.0001
	Treatment (T)	2	22.19	11.09	31.63	0.0002
	Y*T	2	0.55	0.27	0.78	0.4901
WUE_r	Year (Y)	1	0.0008	0.0008	0.01	0.9401
	Treatment (T)	2	87.96	43.98	330.59	<0.0001
	Y*T	2	11.29	5.66	42.43	<0.0001
WUE_s	Year (Y)	1	0.69	0.69	94.12	<0.0001
	Treatment (T)	2	2.19	1.09	149.09	<0.0001
	Y*T	2	0.07	0.04	4.76	0.0435

The effect of year on white sugar yield were significant, and the result of Duncan´s multiple range test showed significant differences ($p < 0.01$) among some treatments in white sugar yield for the combined 2 year (*Table 3*). White sugar yield with AFI was always higher than at other full and deficit irrigation management.

The number of irrigation events and amount of applied water, fresh root and white sugar yield values of sugar beet for each irrigation management are shown in *Table 4*. The seasonal amount of applied water was the mean of the two seasonal and amounted to 1127 mm, 599 mm and 625 mm for CFI, FFI and AFI, respectively.

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***Table 4**. Total number of irrigation, amounts of irrigation and yield of sugar beet*

Year	Treatment	Number of irrigation	Irrigation water applied (mm)	Water saving (%)	Sugar beet root yield (t/ha)	Relative root yield (%)	White sugar yield (t/ha)	Relative white sugar (%)
	CFI	13	1197±6.81[a]	0[a]	83.6±1.42[a]	100[a]	10.1±0.76[b]	100[b]
2013	FFI	13	597±8.74[e]	44[b]	74.5±1.58[c]	89[c]	8.8±0.69[c]	87[b]
	AFI	13	667±6.03[c]	56[b]	80.6±0.78[ab]	96[b]	12±0.89[a]	118[a]
	CFI	11	1058±4.51[b]	0[b]	84.7±3.82[a]	100[a]	6.6±0.41[d]	100[b]
2014	FFI	11	602±2.00[d]	43[b]	62.4±1.00[d]	73[b]	5.8±0.18[d]	88[b]
	AFI	11	584±2.00[e]	45[b]	77.8±4.77[bc]	92[c]	8.1±0.68[c]	124[a]
Year (Y)			**		**		**	
Tretment (T)			**		**		**	
Y*T			**		**		Ns	

*significant in 5% level, ** significant in 1% level, ns non-significant

Table 5 shows the WUEr and WUEs for two years and average of the both years. WUE was significant ($p < 0.01$). The WUEr for CFI, FFI and AFI was 7.49, 11.42 and 12.70 kg/m^3 respectively and WUEs for CFI, FFI and AFI was 0.73, 1.21 and 1.59 kg/m^3 respectively.

***Table 5**. Water use efficiency values of root and sugar yield in combined year*

Treatment	WUE_r (Kg/m$^{3)}$			WUE_s (Kg/m$^{3)}$		
	2013	2014	Average of year	2013	2014	Average of year
CFI	6.98±0.08[b]	8.01±0.35[a]	7.49±0.59[a]	0.84±0.06[b]	0.62±0.04[b]	73±0.13[b]
FFI	12.48±0.44[b]	10.36±0.19[b]	11.42±1.27[b]	1.47±0.10[a]	0.96±0.03[a]	1.21±0.30[a]
AFI	12.08±0.19[b]	13.32±0.80[b]	12.70±0.84[b]	1.79±0.14[a]	1.38±0.11[a]	1.59±0.25[a]

WUEr: Water use efficiency of root yield
WUEs: Water use efficiency of sugar yield

ANOVA of the combined 2-year show that, irrigation management were not significant in polarization, white sugar content and molasses. Alkalinity, Sodium, Potassium and amino nitrogen was affected by irrigation management and year. Organic material such as amino nitrogen, potassium and sodium was affected by irrigation management. Molasses was not significant in irrigation management (*Table 6*).

Sugar beet root quality data in relation to different irrigation managements are presented in *Table 7.*

Figure 1 shows the relationships between root yield and applied water under different irrigation managements. Regression analysis showed that there was a polynomial relationship between seasonal water consumption and sugar beet yield, which is a good function and significant (*Figure 2*).

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Table 6. *ANOVA of sugar beet root quality parameters under different irrigation management*

		df	*Sum of squares*	*MS*	*F-value*	*Sig.*
Polarization	Year (Y)	1	31.90	31.90	30.12	0.0006
	Treatment (T)	2	26.74	13.37	12.64	0.0033
	Y*T	2	2.25	1.13	1.07	0.39
Sodium	Year (Y)	1	33.4	33.4	191.80	<0.0001
	Treatment (T)	2	1.87	0.93	5.36	0.03
	Y*T	2	4.43	2.21	12.71	0.003
Potassium	Year (Y)	1	1.12	1.12	10.68	0.0114
	Treatment (T)	2	1.039	0.52	5.02	0.0387
	Y*T	2	1.10	0.55	5.32	0.0340
Amino Nitrogen	Year (Y)	1	2.20	2.019	134.52	<0.0001
	Treatment (T)	2	0.44	0.219	14.63	0.0021
	Y*T	2	0.36	0.181	12.09	0.0038
Alkalinity	Year (Y)	1	234.07	234.07	392.98	<0.0001
	Treatment (T)	2	14.91	7.46	12.52	0.0034
	Y*T	2	5.90	2.95	4.95	0.0398
Molasses	Year (Y)	1	5.42	5.42	77.11	<0.0001
	Treatment (T)	2	0.30	0.15	2.14	0.1800
	Y*T	2	0.19	0.09	1.32	0.3204

Table 7. *Sugar beet root quality parameters under different irrigation management*

Year	**Treatment**	**Polarization %**	**White sugar concentration %**	**Sodium Meq/1000 g**	**Potassium Meq/1000 g**	**Amino Nitrogen Meq/1000 g**	**Alkalinity %**	**Molasses %**
2013	CFI	14.6±0.87[b]	11.8±0.87[bc]	1.7±0.34[d]	4.4±0.12[bc]	1.3±0.03[b]	4.8±0.18[c]	1.9±0.11[b]
	FFI	14.4±1.11[b]	12.1±1.04[b]	2.2±0.39[d]	4.0±0.51[c]	1.3±0.12[b]	4.9±0.56[c]	1.9±0.32[b]
	AFI	17.6±0.95[a]	14.8±0.97[a]	1.7±0.35[d]	5.0±0.17[b]	1.9±0.09[a]	3.5±0.20[c]	2.2±0.14[b]
2014	CFI	11.7±0.63[c]	7.8±0.84[e]	5.6±0.54[a]	4.6±0.19[bc]	0.8±0.05[c]	13.6±1.52[a]	3.3±0.20[a]
	FFI	12.7±0.13[bc]	9.3±0.20[de]	3.7±0.39[c]	5.2±0.26[a]	0.8±0.12[c]	10.9±1.37[b]	2.8±0.09[a]
	AFI	14.2±1.50[b]	10.4±1.01[cd]	4.5±0.35[b]	5.1±0.60[a]	0.8±0.21[c]	10.5±1.69[b]	3.2±0.58[a]
Year (Y)		**	**	**	*	**	**	**
Treatment (T)		**	**	*	*	**	**	Ns
Y*T		Ns	Ns	**	*	**	Ns	Ns

*significant in 5% level, ** significant in 1% level, ns non-significant

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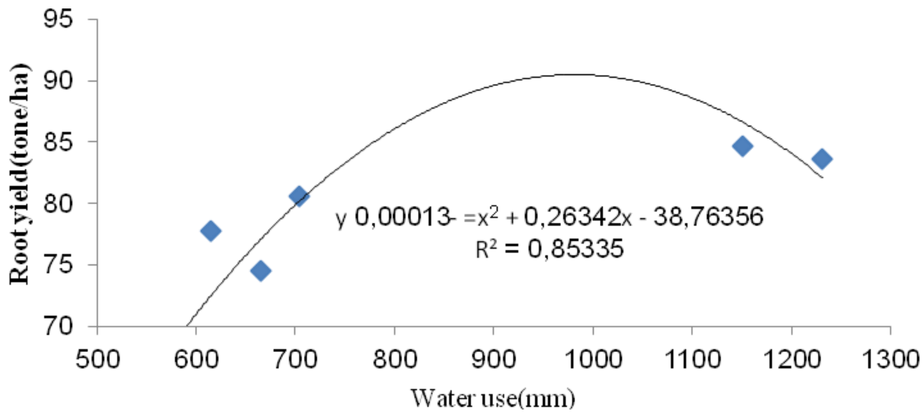

***Figure 1.** Total root yield of sugar beet as a function of water management*

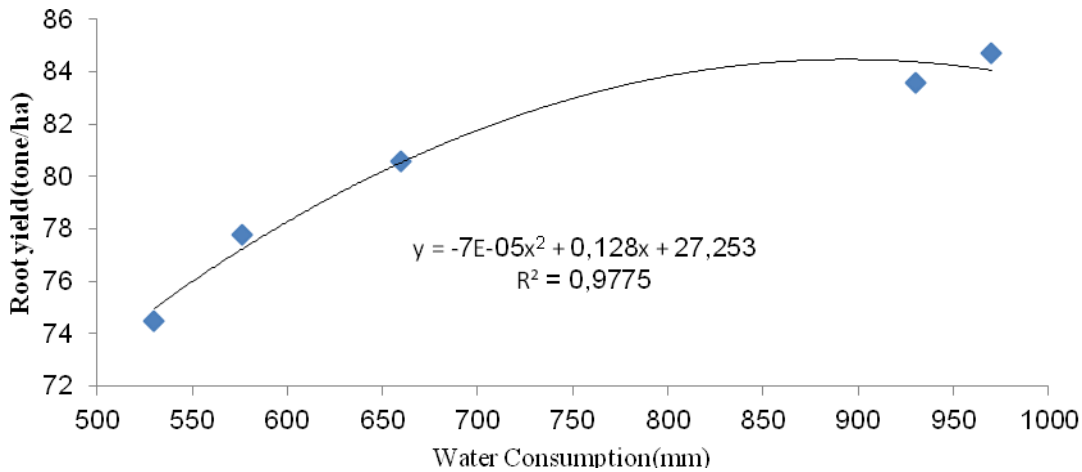

***Figure 2.** Total root yield of sugar beet as a function of water management*

Discussion

In this research, results show that AFI and FFI saved water by approximately 50.5% and 43.5%, respectively, as compared to CFI. The lowest amount of applied water under AFI treatments as compared with CFI might be due to the great reduction of wetted surface in AFI; almost half of the soil surface is wetted in AFI as compared with CFI. The highest Eta occurred in the CFI obviously owing to an adequate soil water supply during the growing season (970 mm). This result supports the outcome obtained by Geraterol et al. (1993), who found that AFI methods can supply water in a way that greatly reduces the amount of wetted surface, with leads to less evapotranspiration and less deep precipitation. Reduced irrigation water due to the alternate furrow management reported by El-Sharkawy et al. (2006), Sepaskhah and Ghasemi (2008), Shayannejad and Moharreri (2009), Ibrahim and Emara (2010) for sugar beet; Nelson and Kaisi (2011).

AFI management, by reducing outlet drainage, can avoid the reduction of groundwater level and deep earth subsidence. AFI management, because of lateral infiltration water in furrow among watering, can cause decreased vertical infiltration. For this reason, nitrate and phosphate that is concentrated in land surface do not move

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into ground water therefore environmental pollution is avoided. AFI management is a good way to reach sustainable agriculture.

Large yield, averaging 84.2 t/ha, was obtained from CFI management plots. Minimum yield was obtained from FFI management plot which averaged 68.5 t/ha. As mentioned by Uçan and Gençoğlan (2004), sugar beet is a crop, which is affected by water deficit. Fluctuation in the yield showed itself to be related to the amount of water given. While the water saving in our study was 44 % (AFI) and 50 % (FFI), the decreases in average beer root yield for 2 years were found to be 6 and 21%, respectively. Therefore, it was observed that the ratio of decreases in beet root yield for each percent deficit rate was not constant. Vamerali et al. (2009) indicated that sugar beet root yields for full and deficit irrigation plots were significantly different. While the rates of decreases in evapotranspiration by Uçan and Gençoğlan (2004), were found as 46.5 and 34%, respectively, the rate of decrease in yield were found as 31.5 and 44%, respectively.

According to these values, it is obvious that there is a parallel relation between the WUEr and WUEs values. Although CFI management that gave the highest root yield and water applied also gave the lowest value of WUEr and WUEs, and the highest value of WUEr and WUEs was obtained in AFI management. This trend supports Febrio et al. (2003), who pointed out that maximum WUE tends to not occur at maximum water applied for sugar beet and usually occurs at an evapotranspiration less than the maximum.

Generally, WUE are influenced by crop yield potential, method of irrigation, method used to estimate or measure water apply and climate characteristics of region.

Root sugar content was generally increased in response to deficit irrigation treatment. Sugar beet roots accumulated more sugar (33%) under AFI management than under CFI management. Sucrose production from sugar beet depends on maximizing storage root growth over along growing season (Topak et al., 2011). It is necessary to apply a suitable irrigation program together with appropriate agricultural measures for taking a high sugar rate in the sugar beet production (Uçan and Gençoğlan, 2004). Dunham (1993) reported that the increase in the sucrose rate to fresh weight root is due to a slower accumulation of water.

The amount of K in sugar beet root generally did not change with the water deficit management during both growing seasons in this study.

The effect of deficit irrigation on Na content of root was significant ($p < 0.05$) in year 2013 and not significant in year 2014. Ober et al. (2005) reported that the effected of water deficit on Na content is less clear and varies from year to year. Na value range from 1.7 Meq/1000 g for CFI to 2.7 Meq/1000 g in year 2013 and 3.6 Meq/1000 g to 4.5 Meq/1000 g in year 2014. Maralian et al. (2008) demonstrated that, deficit irrigation increased Na content of root.

The effect of deficit irrigation management on amino N was not consistent throughout the years. Average amino N value varied from 1.05 Meq/1000 g to 1.35 Meq/1000 g. The most severe effect of water deficit on amino N content was observed in AFI management. However, CFI and FFI management had same effect on amino N content of root in both years. It must be noted that these substances reduce sugar beet quality because they are considered non-compatible dissolved materials contrary to non-toxic or compatible dissolved substances such as some amino acids and non-reducing that can accumulate in large amounts without causing any disturbances in the biological functions of cells (Rontein et al., 2002).

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Finally, the result shows that AFI management with reduced water use and environmental pollution can help achieve sustainable agriculture.

Conclusions

Results showed that deficit irrigation management at sugar beet led to decrease in root and sugar yields. Water use efficiency values increased slightly with increase in water deficit. Water was used more efficiently at the AFI management. Irrigation management AFI could be used for sugar beet grown in arid and semi-arid regions where irrigation water supplies are limited. Under this condition, 44% of water saving was obtained even though there was a 6% yield loss for sugar beet, based on the average of 2 years. The alternate furrow irrigation management had the maximum water use efficiency of root and sugar, 12.70 kg/m^3 and 1.59 kg/m^3, respectively.

In conclusion, this study revealed that if water is limited and deficit irrigation is spread over growth season of the sugar beet, WUE_r and WUE_s may be improved under 44 and 50% deficit irrigation schedule. Alternate furrow irrigation management in sugar beet may be feasible for water saving and reducing drainage. It can be concluded that using deficit irrigation is a good water management technique to protect the environment without reducing the water use efficiency.

The current study has been done in loamy texture soil and it might be different in other soil mixtures. Therefore, it is recommended that further experiments can be implemented in various soil textures and furrow lengths to evaluate deficit irrigation management on qualitative and quantitative yield of sugar beet.

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LAND USE/COVER CHANGE MODELLING IN A MEDITERRANEAN RURAL LANDSCAPE USING MULTI-LAYER PERCEPTRON AND MARKOV CHAIN (MLP-MC)

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Abstract. Mediterranean land use and land cover (LULC) have a very dynamic structure as a result of continuous transformation process due to anthropogenic effects and environmental gradients. LULC dynamics are important indicator of environmental condition in temporal and spatial scales. The aim of this paper was to simulate the future LULC of a Mediterranean type watershed located at the Eastern Mediterranean Region of Turkey by incorporating multi-layer perceptron (MLP), artificial neural network (ANN) and Markov chain (MC) approaches. Landsat TM/OLI images in 1990, 2003 and 2014 over the study area were classified using hybrid classification approach. The Kappa statistics of the hybrid classification that combines K-means, decision tree and object based classification method for these three images were 0.81, 0.85 and 0.87 respectively. The LULC map of 2014 was simulated using LULC maps of 1990 and 2003 for calibration and validation. The simulation results were compared with the actual 2014 LULC map to assess the accuracy of the simulation, and the rate of overlap was found as 89%. LULC map of 2025 was estimated using LULC maps of 2003 and 2014. These results indicated that, the area of bareground will reduce 13.31% whereas the rate of forest and agricultural area will increase 8.70% and 6.51% respectively.
Keywords: *land use/land cover (LULC), hybrid classification, change detection, multi-layer perceptron (MLP), Markov chain, future prediction*

Introduction

Land use refers to man's activities on earth which are directly related to land, while land cover denotes the natural features of the land surface (Singh and Singh, 2011; Ozdogan, 2016). Land use dynamics are important elements for monitoring, evaluating, protecting and planning for earth and ecosystem goods and services (Jakubauskas et al., 1990). LULC which has a dynamic structure results in the change of ecosystem good and service potential due to the transformation. Ecosystem goods and services are the benefits people obtain from ecosystems. During the ecosystem process, there is a flux of energy and material from one pool to another. Terrestrial ecosystems currently store approximate four times more carbon than is found in the atmosphere. Changes in LULC due to timber harvesting, deforestation for agriculture and fire can release substantial amounts of terrestrially stored carbon (Chapin et al., 2011; Conte et al., 2011; Lal, 2004; 2011; Young et al., 2016). Additionally, forests play an iconic role in environmental conservation and are habitats for much of the world's terrestrial biodiversity. Remote

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sensing (RS) technologies are vital to understand the impact of anthropogenic activities and change in natural area. Satellite sensor imageries were used in geospatial investigations in last decades for policy and decision making (Masud et al., 2016). Satellite sensor based data is essential for the study both urban and rural management and planning. In this extent, Geographic Information System (GIS) and RS applications take on a critical role in modern era for natural resource management (Elaalem et al., 2013; Mercant and Narumalani, 2009).

Markov chain (MC) is a very useful approach to forecast urban or rural landscape transition. This approach became more popular due to improvement in GIS and RS technologies. Markov chain methodology intensively utilizes RS data to simulate LULC changes (Dadhich and Hanaoka, 2010a; 2010b; Muller and Middleton, 1994). The aim of this study was to predict the future change in the study area where the rural landscape structure is dominant. The LULC changes were modelled using classified maps derived from 1990, 2003 and 2014 images of Landsat TM/ETM. A hybrid classification approach which combines object-based, K-means and decision tree classification methods was used to increase the accuracy of these classifications. Predicted 2025 LULC map was derived incorporating ANN and MC approaches.

Material and method

Study area

The study area is located in the Eastern Mediterranean Region of Turkey. The region covers an area of approximately 7368.62 km^2 and its altitude ranges between 37 and 3555 m. The forest species richness is substantial and the area has a wide range of heterogeneous topography formed by Taurus Mountain Chain (*Figure 1*).

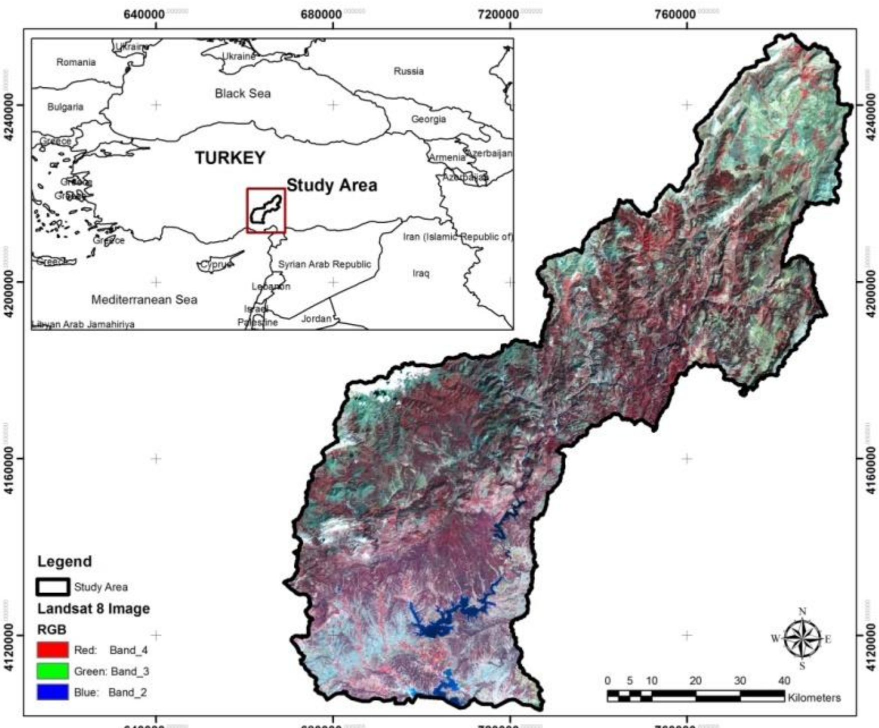

Figure 1. *The geographical location of the study area*

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This area has vast forest land due to the environmental gradients including variation in topography, climate and soils. Forests of southern of Turkey are one of the most important carbon sinks of Turkey. The region covers mostly pure and mixed conifers followed by some deciduous tree species (Donmez, 2016). The climate of the study area is characterized by mild and rainy winters and moist summers with a mean annual precipitation of around 800 mm and distribution of rainfall varies, approximately 75% of rainfall occurs in autumn and winter, the mean annual temperature is 19 °C. Thus, the climate, rich water resources and fertile alluvial plane enabled this area the most productive agriculture region in Turkey.

Characteristics of the dataset

Different dates LULC classification maps were generated with using Landsat images. Additionally Landsat images were used for auxiliary variables that are including vegetation indices to be produced. The remotely sensed data used in this study were given in *Table 1*. The Landsat TM/OLI images were obtained from USGS. Spatial resolution of the dataset was 30 m resolution and georeferenced to UTM, WGS84 projection.

Table 1. *Landsat images used for LULC mapping*

Sensor type	Image date	Patch	Rows	Bant	Radiometric resolution	Spatial resolution (m)
TM	29.08.1990	175	33/34	1,2,3,4,5,7	8	30
TM	17.08.2003	175	33/34	1,2,3,4,5,7	8	30
OLI TIRS	13.09.2014	175	33/34	2,3,4,5,6,7	16	30

The auxiliary dataset was produced for study area LULC classification. Auxiliary variables used in decision tree classification are shown in *Table 2*. It was used decision tree rule with reflectance value on visible bands, vegetation indices and DEM.

Table 2. *Auxiliary variables derived from satellite sensor data*

Auxiliary data	Data date	Metadata
Visible bands	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
NIR	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
SWIR	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
NDVI	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
NDWI	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
Tasseled Cap	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
K-means	1990-2003-2014	Landsat 5 TM – Landsat 8 OLI TIRS
Forest map	2002-2014	Forest General Directorate
DEM	2013	SRTM

Method

This study included six stages; (i) pre-processing of multi-temporal remotely sensed images and forest maps, (ii) production of past (1990-2003) and current (2014) LULC maps using hybrid approach, (iii) accuracy assessment, (iv) modelling of 2014 LULC map, (v) validation of 2014 modelling and (vi) LULC simulation of 2025 (*Figure 2*).

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Land cover change analysis and future land cover modelling studies were carried out within IDRISI TerrSet software.

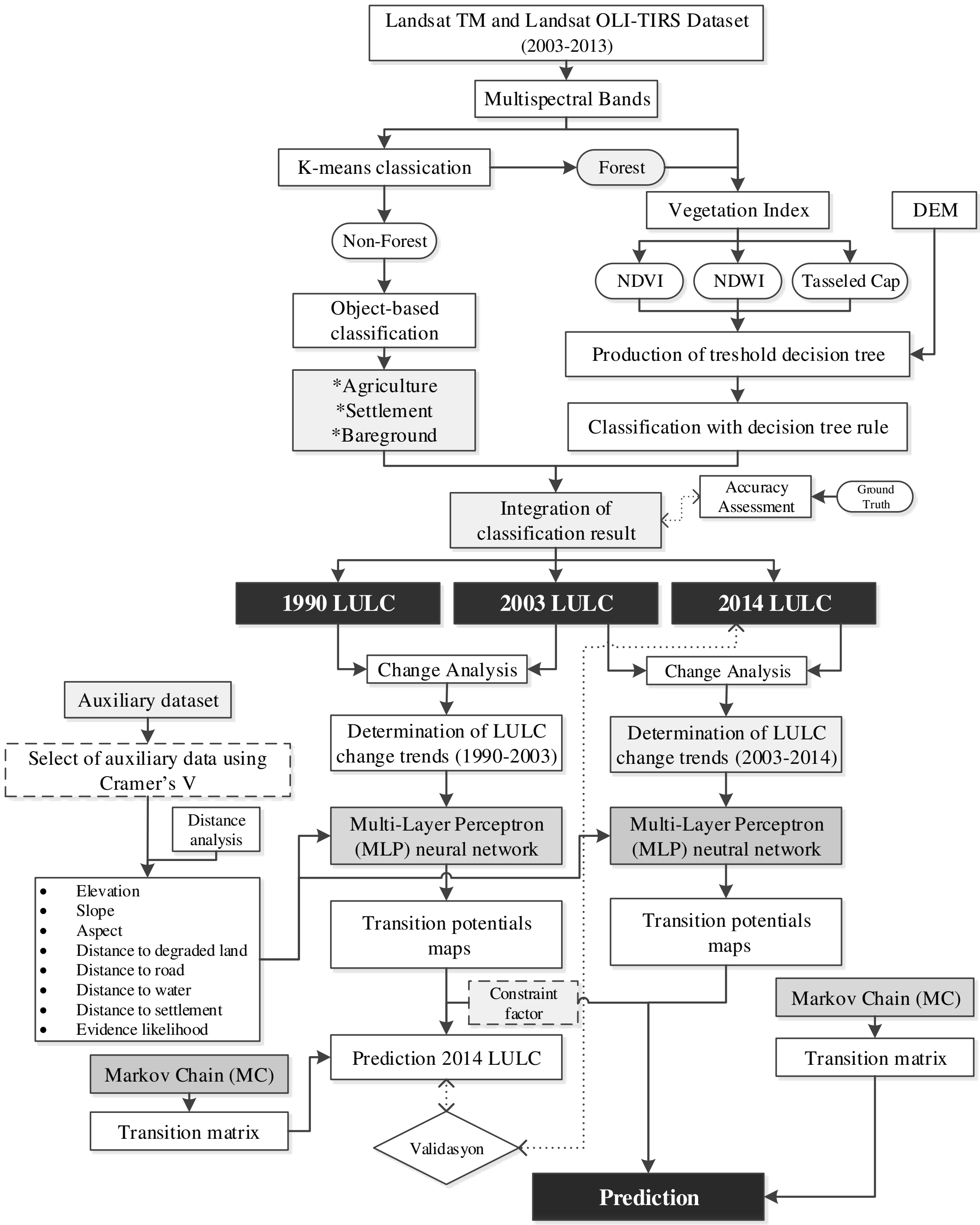

Figure 2. *Flow diagram of the study*

Hybrid classification for land use land cover (LULC)

A hybrid classification was employed in this study by considering the landscape characteristics of the research area. The ground data showed a heterogeneous structure in terms of landscape characteristics largely in dense forest areas. Composition of structural features such as height, percent tree cover, diversity and stand mixture rates,

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make the separation of target objects difficult during the classification of forest trees. Thus, pixel-based and object-based classification approaches were incorporated to extract accurate forest cover information. Combination of these two approaches improved the overall classification accuracy (Caproli, 2003; Satir et al., 2017; Tang and Pannell, 2013). Recent studies showed that combination of various classification methods called hybrid classification were efficiently used to tackle the problem of insufficient reference data. Hybrid classification takes the advantage of both supervised and unsupervised classification techniques (Al-doski et al., 2013; Kamusoko et al., 2011). The unsupervised classification (K-means) method was combined with decision tree and object-based classification methods. Hybrid classification takes advantage of both supervised and unsupervised classification techniques.

The hybrid classification for this study was implemented in five phases including: (i) K-means unsupervised classification approach for determining the forests and non-forest areas, (ii) generation of auxiliary variables from Landsat 5 TM and Landsat 8 OLI-TIRS for decision tree algorithm, (iii) statistical comparison of auxiliary variables, (iv) object-based classification of agriculture, bareground and settlements and (v) accuracy assessment. Post K-means clustering classification provided forest and non-forest covers. The forest cover was determined with using decision tree rule thought vegetation indices while non-forest cover was classified through object-based classification.

Vegetation indices

The auxiliary variables including Normalized Difference Vegetation Index (NDVI), Normalized Difference Water Index (NDWI) and Tasseled Cap were derived as ancillary data from Landsat images (*Table 2*). NDVI was used widely to assess the relationship between spectral variability and the changes in vegetation growth (Bilgili et al., 2014). NDWI is sensitive to changes in water content of vegetation canopies. RS of water in plants is an important application in forestry and agriculture (Satir et al., 2016). Tasseled Cap transformation reduces the Landsat reflectance bands into three orthogonal indices including brightness, greenness and wetness (Ozyavuz et al., 2011).

Unsupervised classification (K-means)

The K-means unsupervised classification is a well-known algorithm uses a simple clustering algorithm based on pixel similarities. The algorithm works in three steps, (i) grouping objects K points into the space represented by the object that are being clustered and these point represent initial group centroids, (ii) assigning each object to the group that has the closest centroid, (iii) as all objects have been assigned, revised the positions of K centroids (Abbas et al., 2016; Giri, 2012; Usman, 2013). The purpose of the K-mean unsupervised classification was applied for determination of forest and non-forest cover type.

Decision tree classification

Decision tree (DT) classification combines both machine learning algorithm and knowledge based data mining technique. It is an efficient tool for LULC classification as a non-parametric approach, particularly in areas facing class mixing problem (Punia et al., 2010). DT is simple, flexible and efficient in classification process noisy and non-linear relation between features both numeric and categorical inputs (Friedman, 1977; Pal and Mather 2003).

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Tree structure in decision tree approach is formed in three basic parts called nodes, branches and leaves. Each feature is represented by a node. The dataset is categorized according to the decision structure defined by the tree, by moving downwards and dividing subparts until reaching a leaf in sequence. The tree is composed of root a node, a set internal nodes an set a terminal nodes (Friedl and Brodley, 1997; Jiang et al., 2011). Each node has only one parent node and more descendant nodes (*Figure 3*). The decision tree creates decision rules by gathering the answers to the questions.

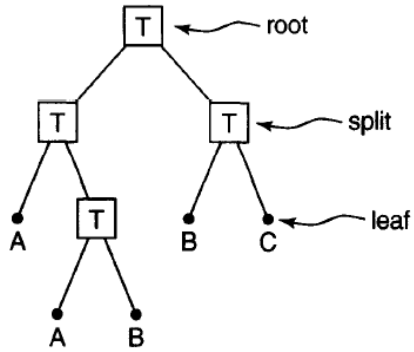

Figure 3. *A decision tree classifier (Friedl and Brodley, 1997)*

The main input of decision tree classification technique were auxiliary variables for this study. The forestlands were derived using K-means unsupervised classification method. The threshold values for DT structure rules collected from auxiliary dataset that generated from satellite sensor images.

Object classification

Object-based classification is useful to analyze groups of contiguous pixels as objects instead of using the conventional pixel-based classification unit. It is being a effective alternative to the traditional pixel based methods (Yu et al., 2006). In contrast to pixel-based classification methods that classify individual pixels directly, object-based classification first aggregates image pixels into spectrally homogenous image objects using an image segmentation algorithm and then classifies the individual objects (Liu and Xia, 2010). Kettig and Landgrebe (1976) proposed this idea and developed the spectral-spatial classifier called extraction and classification of homogeneous objects (ECHO). More recently, some research has adopted this method on land-use or land-cover classification combined with image interpretation knowledge and classification results were significantly improved.

Cukurova plain located in lower part of the study area is a productive agricultural plain and agricultural activities continue throughout the year. Single satellite sensor image is insufficient to map agricultural crop pattern in Cukurova region due to high crop variation and seasonality (Satir and Berberoglu, 2012). Object based classification was used to classify agriculture, settlement and barerock areas accurately. Therefore, multi-temporal images of 1990, 2003 and 2014 were classified using nearest neighborhood algorithm together with multi-resolution segmentation. The heterogeneity of the segments was adjusted experimentally. Agricultural, settlement and barerock areas were classified with object based classification.

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Markov chain for future LULC prediction

Markov chain (MC) analysis has been widely used for LULC modelling which is a stochastic process that takes into account the past state to predict the future changing of variables over time (Brown et al., 2000; Muller and Middleton, 1994). The key factor for a MC is transition probability matrix which defines change trends from past to present and into the future for a certain class type for each grid. The probability matrix is a set of conditional probabilities for the cell to change to a particular new state and takes into consideration several variables that correspond to potential drivers and constrains of change (Berberoglu et al., 2016). MC uses Cellular Automata (CA) approach for the future simulations. CA is a theoretical framework that permits computational experiments in spatial arrangements over time (Clark, 2008). The basic components of the CA approach is identified with the cells, a neighborhood function, transition probabilities, time and space. Markov modelling requires LULC suitability maps for each LULC classes.

Multi-layer perceptron (MLP)

There are six generally used ANN models for pattern recognition; Hopefield network, Hamming network, Carpenter/Grossberg classifier, Kohonen's self-organising feature maps, single layer perceptron and multi-layer perceptron. The multi-layer perceptron described by Rumelhart et al. (1986) is the most commonly encountered ANN model in remote sensing because of its successful generalization capability and only this one is considered in this study. This type of ANN model consist of three or more layers which are generally interconnected to the previous and next layers, but there are no interconnections within a layer, each layer consists of processing elements called unit or nodes. The first layer is called the input layer and serves as a distribution structure for the data being presented to the network. Its holds input values and distributes these values to all units in the next layer and so no processing is done at this layer. The input values can be spectral bands or additional information. The final processing layer is called the output layer and in this case is land cover classes. Layer in between input and output layers are termed hidden layers. The number of hidden layers and units within the network are defined by user.

LULC suitability map of each class as an output of MLP was input to model future LULC map of 2025. This process consisted of three stages (i) classification through decision tree approach, (ii) modelling transformation potential of each class using MLP, and (iii) modelling the future LULC with MC. LULC change map was derived using classified time-series of Landsat TM/OLI images. LULC conversion indicated transition potentials amongst the classes. This change map was linked with explanatory variables including, elevation, slope, aspect, distance to degraded land, distance to road, distance to water, distance to settlements and evidence likelihood, to model transformation potential of each class using MLP. That classifier can be run more efficient with less training data than parametric approaches (Dadhich, 2010a). This process is also called land suitability mapping which is implemented traditionally with multi-criteria approach through user defined settings like weighting and prioritizing land conversion suitability. This subjective process within this study was implemented using MLP with minimum user interference. In the final stage the resulted transformation potential maps for each LULC type utilized within MC approach.

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MC transformation matrices are used to predict the possible conditions of future land cover. However, MC is insufficient to reveal the distributions of spatial changes alone, whereas MLP neural networks allow modelling the general tendency in the trend determined by the change analysis. According to Eastman (2016), MLP-MC combination achieves the most accurate and most obvious results in terms of target transformations. These outcomes are usually assessed under the sub-models major transformations in which urban expansion or human-induced degradation are taken into account. The relation between the grouped transformations in the sub model structure and the driving force is determined by Cramer's V analysis. In this context, it is very important to classify past classification maps with high accuracy so that a reliable model can be performed. In addition, the real and simulated images providing 75% or more of a level of overlap lead to produce the future simulation with an acceptable accuracy. If the overlap between the real and simulated image is less than 75%, the calibration process is applied. It is necessary to check the reliability of the classification maps or re-examine the environmental variables in the MLP-MC process.

Result

The result of this study were presented in three phases (i) multi-temporal LULC mapping and accuracy assessment, (ii) modelling transformation potential of each class using MLP, ROC statistic and (iii) modelling the future LULC with MC.

Multi-temporal LULC mapping and accuracy assessment

The LULC maps for 1990, 2003 and 2014 were derived by hybrid classification of Landsat imagines and auxiliary data (*Table 2*). Nine major LULC were classified as coniferous forest, deciduous forest, agriculture, bareground, water, settlement, barerock, sand dunes and grassland (*Figure 4*). The accuracy of classified images of 1990, 2003 and 2014 were derived using overall accuracy and Kappa statistics (*Table 3*).

Table 3. *Accuracy assessment for LULC classification*

LULC class	1990		2003		2014	
	PA(%)	UA(%)	PA(%)	UA(%)	PA(%)	UA(%)
Coniferous forest	78.0	97.9	90.3	87.9	86.2	90.3
Deciduous forest	88.9	72.7	86.4	98.3	86.1	86.1
Agriculture	70.6	83.0	84.1	84.1	93.5	82.7
Bareground	93.8	88.4	87.2	81.7	83.3	80.6
Water	100	100	96.6	96.6	94.7	94.7
Settlement	96.3	100	93.5	95.6	88.9	88.9
Barerock	82.7	92.9	84.0	85.7	73.3	91.7
Sand dunes	-	-	70.6	85.7	84.6	84.6
Grassland	82.3	83.0	85.3	87.9	85.7	94.7
Overall accuracy	91%		87%		88%	
Kappa statistic(KIA)	0.89		0.85		0.87	

PA: Producer's accuracy, UA: User's accuracy

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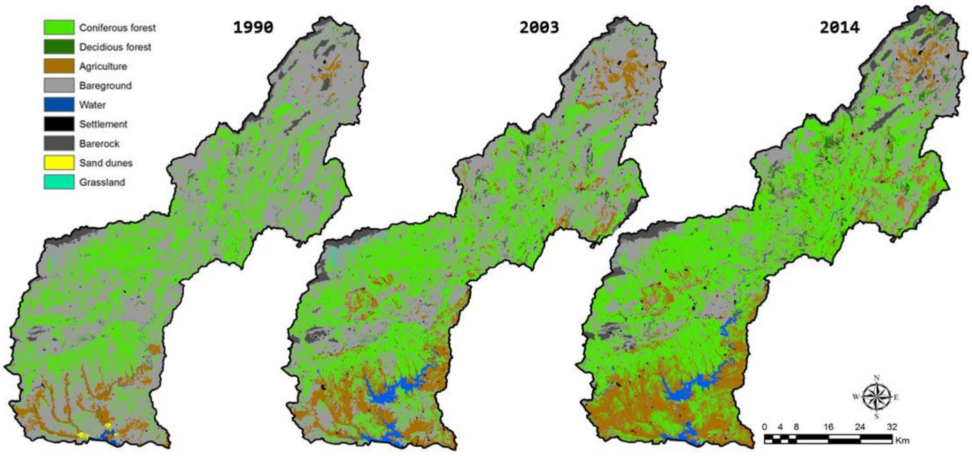

Figure 4. *Hybrid classification results*

Output of three classifications were cross-tabulated to determine from-to change (*Table 4*).

Table 4. *Cross-tabulation of LULC changes (%)*

		1990 LULC								
		Coniferous forest	**Deciduous forest**	**Agri-culture**	**Bare-ground**	**Water**	**Settlement**	**Bare-rock**	**Sand-dunes**	**Grass-land**
2003 LULC	Coniferous forest	82.91	0.87	0.53	15.02	0.31	1.69	0.07	0.32	4.51
	Deciduous forest	0.45	76.0	0.34	0.83	0.11	0.02	0.00	0.01	0.03
	Agriculture	0.73	1.07	78.93	10.15	11.96	4.91	0.04	11.63	0.00
	Bareground	15.73	21.73	4.65	67.20	2.30	0.51	7.24	2.78	13.74
	Water	0.09	0.21	14.67	0.91	85.09	1.06	0.00	84.75	0.00
	Settlement	0.02	0.07	0.85	3.82	0.02	91.78	0.05	0.00	0.00
	Barerock	0.06	0.05	0.00	1.82	0.02	0.00	92.58	0.00	1.34
	Sand dunes	0.00	0.00	0.03	0.01	0.19	0.03	0.00	0.51	0.00
	Grassland	0.01	0.00	0.00	0.24	0.00	0.00	0.02	0.00	80.38
		2003 LULC								
		Coniferous forest	**Deciduous forest**	**Agri-culture**	**Bare-ground**	**Water**	**Settlement**	**Bare-rock**	**Sand-dunes**	**Grass-land**
2014 LULC	Coniferous forest	85.69	21.11	4.74	12.41	0.56	1.74	2.21	1.68	6.46
	Deciduous forest	1.22	54.15	0.36	0.55	0.28	0.08	0.09	0.00	0.19
	Agriculture	1.30	1.46	79.43	9.36	4.91	3.73	0.92	26.51	1.60
	Bareground	11.49	23.03	12.9	73.38	11.22	3.63	3.77	0.73	52.93
	Water	0.01	0.14	1.38	0.19	81.64	0.47	0.01	52.18	0.00
	Settlement	0.07	0.05	0.99	2.13	0.11	90.15	0.06	0.00	0.01
	Barerock	0.19	0.05	0.18	1.41	0.12	0.2	92.91	0.24	1.10
	Sand	0.01	0.01	0.02	0.00	1.10	0.00	0.00	18.66	0.00
	Grassland	0.02	0.00	0.00	0.57	0.06	0.00	0.03	0.00	37.71

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This analysis indicated that agriculture, rural settlement, water surface and deciduous forestlands increased whereas, bareground decreased between 1990 and 2014 period. Forestlands covered 62.52% of the area in 1990 however it decreased to 48.52% in 2003 and further decrease took place in 2014 to 30.91%. Major conversion of forestlands was to agriculture and grasslands over time.

Modelling transformation potential of each class using MLP

LULC changes and their linkage to the explanatory variables were assessed and modelled through MLP. Firstly, the change was grouped and then weighting values of the explanatory variables were determined within the MLP. The major LULC transformations were classified under major groups in the literature (Areendranet al., 2013; Basse et al., 2014; Eastman, 2016; Dadhich et al., 2010a; Megahed et al., 2015; Mishra and Rai, 2016; Ozturk, 2015). These groups are represented by current trends or major changes. For the study, transformation of semi-natural land cover to settlement and agriculture are detected as major changes due to intensive anthropogenic activities during the 1990-2014 (1990, 2003 and 2014) time period. Transformation potential of each LULC type was derived using eight variables including; (i) elevation, slope, aspect, distance to degraded land, distance to road, distance to water, distance to settlements and evidence likelihood and the six major LULC transformations were considered including; (i) from coniferous forest to settlement, (ii) from delicious forest to settlement, (iii) from bareground to settlement, (iv) from barerock to settlement, (v) from bareground to agriculture and (vi) from agriculture to bareground (*Figure 5; Table 5*).

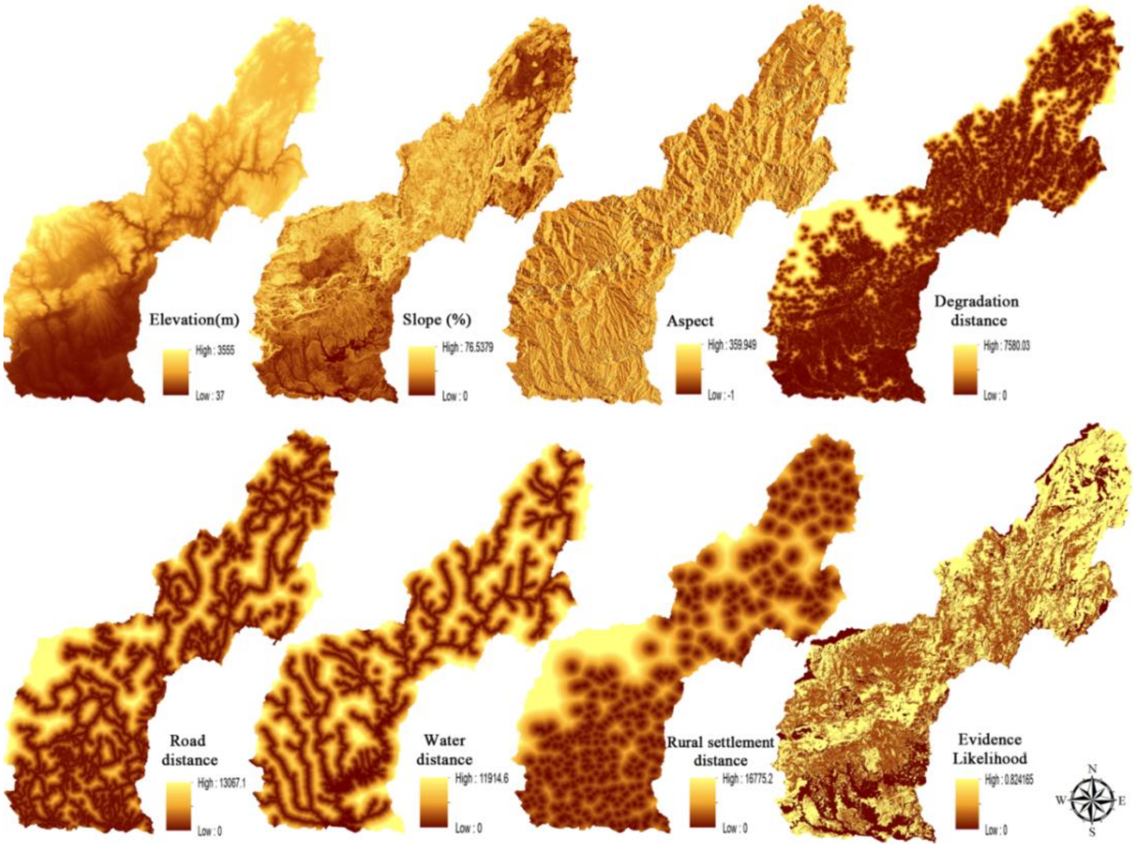

Figure 5. *Input dataset for Multi-Layer Perceptron*

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Table 5. *Driving forces with Cramer's V*

Driving force	Cramer's V
Elevation	0.62
Slope	0.39
Aspect	0.33
Distance to degraded land	0.37
Distance to road	0.49
Distance to water	0.27
Distance to settlement	0.41
Evidence Likelihood	0.45

The current trend of change was indicated that afforestation activities are intense in the region. As a result of afforestation, 1253.55km^2 area was converted to coniferous forest from bareground between the years of 1990 and 2014. On the other hand, adverse effects of anthropogenic impacts such as conversion from bareground to agriculture and settlement took place approximately 986.36 km^2 (*Figure 6*).

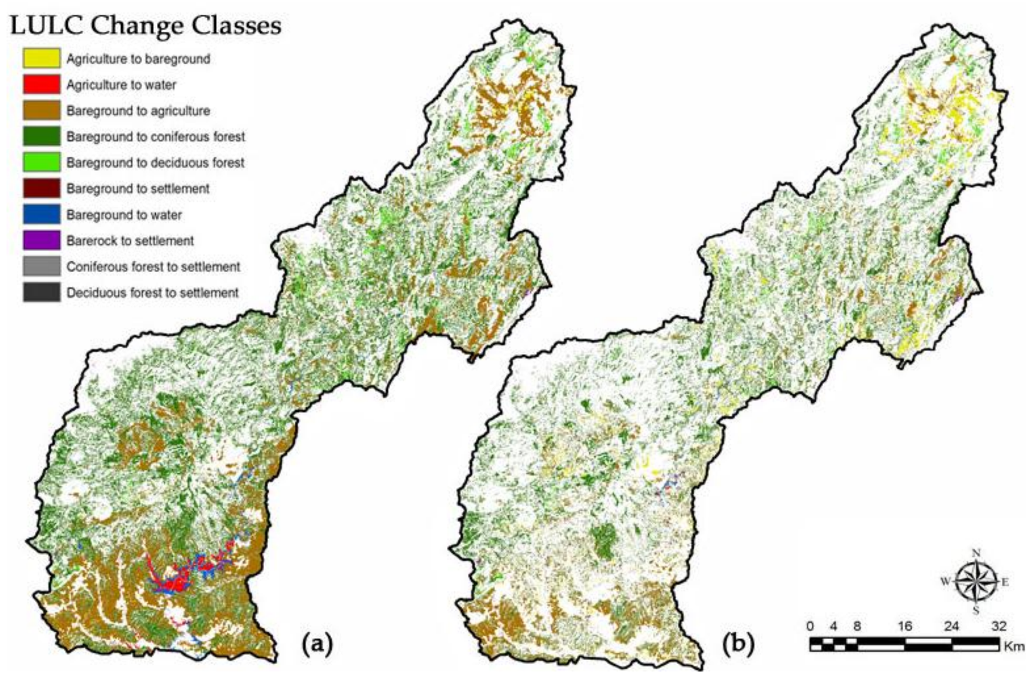

Figure 6. *Change detection result maps (major transformation) (a) from 1990 to 2003 and (b) from 2003 to 2014*

The LULC transitions were linked to explanatory variables in a GIS environment to model transformation potential using MLP. The spatial variables, which affected the land cover change were considered as driving forces. Explanatory variables were determined through literature survey and expert knowledge on land change dynamics of this region. Training set of MLP consisted of six target variables which represent the major LULC transitions and eight driving forces. Training of MLP neural network was evaluated by Cramer's V test. This is the most common method explaining the effects

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of spatial variables in land cover changes. Cramer's V value was calculated with *Equation 1* (Cramer, 1999):

$$V = \sqrt{\frac{x^2}{N(m-1)}} \qquad \text{(Eq. 1)}$$

x^2: Chi-square,
N: Population
m: The numbers of the columns or lines on the table

Static or dynamic properties of spatial variables were identified through Cramer's V. While static variables have a stable characteristic that has not changed over time, dynamic variables can change due to anthropogenic effects over time. In this regard, distance from degradation, distance from road and distance from settlement were identified as dynamic factors and the others were considered as static. The Cramer's V results were considered as weighting values of spatial variables and integrated to MC for future modelling. Categorical maps can be converted into continuous maps using the Evidence Likelihood transformation based on the relative frequency of pixels belonging to the different categories within areas of change (Mas et al., 2014).

The MLP model was trained with 75% of the data and tested with 25%. The ideal MLP training variables were defined experimentally given in *Table 6*. According to result of MLP model process between training and test dataset was observed 76.10% accuracy rate.

Table 6. *MLP model variable value and accuracy rate*

Variables	Results
Hidden layer nodes	17
Start learning rate	0.01
End learning rate	0.001
Momentum factor	0.5
RMS	0.01
Iteration	6000
Training RMS	0.15
Testing RMS	0.17
Accuracy rate (%)	76.10
Skill measure	0.7302

The production of potential change maps by taking advantage of the transformations were input data set required for MC analysis (Mishra, 2016). The number of outputs of the transformation potential maps (*Figure 7*) are equal to the number of major change input groupings. The spatial variables maps were standardized between 0 and 1. The standardization process in between of spatial variable was obtained through MLP transition model results.

Additional testing process was performed for the simulation of 2014. Weights of transition potential images were derived through MLP. This process is also called the model calibration. MC approach was used these transition potential images of 1990 and 2003 to simulate 2014 LULC map. Comparison of the 2014 observed and predicted maps were given in the *Figure 8*.

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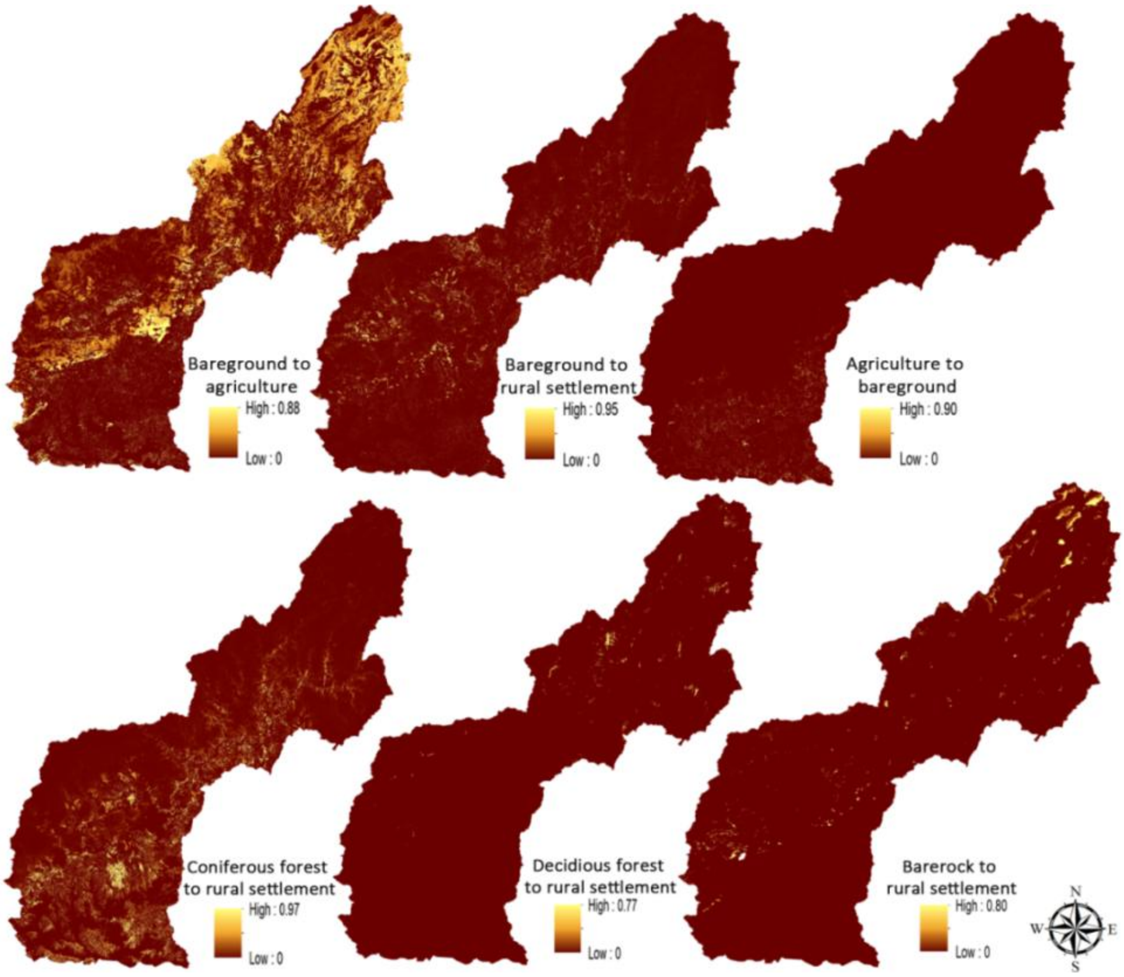

Figure 7. *Transition probability maps of major transition categories*

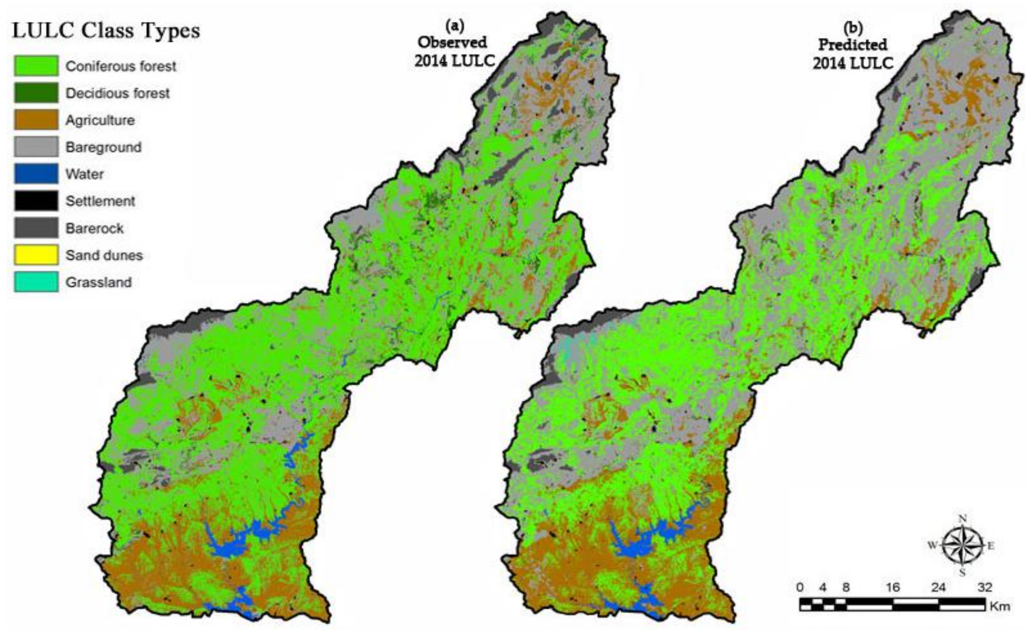

Figure 8. *2014 LULC observed and predicted maps*

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A cross classification analysis was performed for the validation process between the observed and predicted 2014 maps and the Kappa Index of Agreement (KIA) was calculated. KIA indicated the level of overlap between the observed and predicted 2014 LULC types and found as 0.79. Crammer (1999) stated that acceptable accuracy of the model should be ≥ 0.70 in LULC modelling studies. In addition to the KIA, validity of the outputs were evaluated using Receiver Operating Characteristic (ROC) analysis. The main goal of the ROC analysis was to evaluate the performance of the spatial models in selected grid cells that generate possibility in the process of modelling a given phenomenon or situation (Berberoglu et al., 2016; Hamdy et al., 2017). According to testing by correlation method that ROC was obtain 0.89, as for there is high correlation between selected spatial variables and LULC types.

Future prediction of the LULC

LULC of 2025 was estimated after the validation of the model. Major transformations were grouped according to the LULC trends from past to present and future LULC map was produced as an outcome of transition potentials (*Figure 9*).

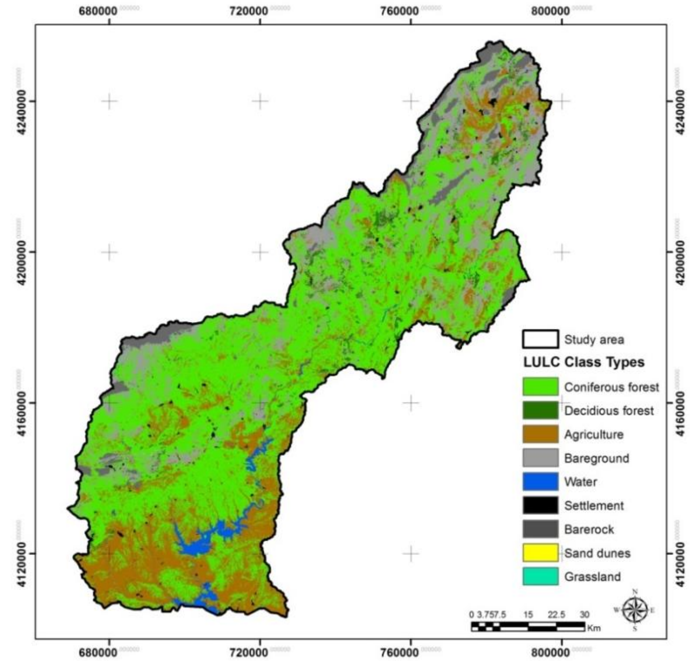

Figure 9. *Predicted LULC map for 2025*

According to modelling results, an increase of 13.43% was observed in forest areas between 1990 and 2014 time periods. As a result of this trend, MLP-MC simulated forestland coverage as 50.83% of the total area (*Table 7*).

It can be stated that the main reason for forest growth was afforestation activities. During the period of past 10-15 years, afforestation were carried out by the Adana Forest General Directorate. However, it was not only the afforestation, but also the microclimatic factors resulted from newly established dams in the study area. Establishments of new dams caused an increase in humidity and triggered the regeneration of the forestlands in the region. This change was also resulted an increase

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in agricultural fields due to irrigation opportunities. Thus, it is clearly seen that agricultural areas are expanding while the dam and water surface are increased. According to the LULC classification and change analysis results the forestlands have been transformed to the larger forest and farmlands whereas, minor changes were observed in dune and pasture areas.

Table 7. *LULC coverage of classified and simulated images*

LULC class types	1990 area (%)	2003 area (%)	2014 area (%)	2025 area (%)
Coniferous forest	28.85	33.42	43.19	50.83
Deciduous forest	0.91	1.31	2.25	2.31
Agriculture	4.09	11.45	16.73	22.24
Bareground	62.52	48.52	30.91	17.60
Rural settlement	0.36	0.59	0.61	0.74
Barerock	2.61	2.88	4.39	4.33
Water	0.49	1.75	1.85	1.88
Grassland	0.05	0.07	0.05	0.06
Sand dunes	0.11	0.01	0.01	0.01

Discussion and conclusion

This study produced past and present LULC by using the hybrid classification approach and 2025 simulation map was also generated based on the change dynamics derived from the different dated LULC maps. The dominant regional landscape characteristics were rural widely shaped by the forestlands.

Major steps of the research can be summarized as; i) LULC mapping with hybrid classification approach and object based classification for more accurate and sensitive results, ii) analyzing the quality and quantity of LULC change with the post-classification technique in order to determine major LULC transformations, iii) preparation of explanatory variables that effect the major LULC transformations and generating of transition probability maps through the MLP and iv) validation of the model calibration and 2025 LULC simulation map with the MC model. The subjected analyses were largely performed with the free of charge Landsat images acquired from the Landsat data archive.

Having altitude difference, fertile soil structure and suitable climatic conditions the study are allocated in the southern east Mediterranean region of Turkey has features that enable the expansion of forest and agricultural areas. According to World Reference Base (WRB) international soil classification system updates in 2014 by Aydin et al. (2016), 67.43% of the study area is in Leptosol-Cambisols soil class. The Leptosol-Cambisols of the area is intersect with limeness brown forest soils and brown forest soils. These soils have a characteristic which are formed on the parent material containing high lime and have high capacity of water-holding and nutrients, natural drainage system and on which shrub and mixed forest can grow. Therefore, the bareground has been transformed into agricultural and especially coniferous forest class due to anthropogenic effects. According to findings of the study, the bareground decreased by 31.61% from 1990 to 2014, while the forest areas increased by 15.68% and agricultural areas increased by 12.64%. In research of Tanriover (2012), the main

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reason for increase in agricultural areas are the population growth of Adana city that in the southern part of the study area, after 1980 and additionally the facilitated irrigation opportunities provided through the dams built on Seyhan River. Furthermore the expansion of forest areas has been determined. It can be stated showing a positive structure for LULC in major changes, afforestation regulations at Adana Forest General Directorate have been successful in the increase of the forest areas. In addition to afforestation, it has been emphasized in a study investigated climate change effect across Turkey by Berberoglu et al. (2014) that forest increase in the northern parts of the eastern Mediterranean region may be an impact of global change. In this direction, the forest increases in the northern part of the research area are parallel to the study of Berberoglu et al. (2014).

Future land cover predictions with the MLP-MC method was experienced in the rural landscape for the study however, it can be also conveniently applied in urban landscapes. The success of the model is mostly depended on the accurate LULC classification and here of presentative LULC transformations. In this frame, MLP analysis was tested for the study area and detected as a useful tool in determining the suitability maps which is one of the most critical steps in the future modelling of MC. The relevance of the auxiliary data was tested using Cramer’s V coefficient during the production of suitability maps. This relationship facilitates the production of more objective transition probability maps. Actually, Multi Criteria Analysis (MCA) is the most common method used in determining the weighting values for preparation of suitability maps in MC modelling (Ahmed and Ahmed, 2012; Akin, 2014; Berberoglu et al., 2016; Jafari, 2017; Kamusoko, 2011; Sayemuzzaman and Iha, 2014). However, this method comes in for criticism due to user intervention during the weighting process. So, this study integrated MC modelling and Multi-Layer Perceptron (MLP) within a neural network interface in order to determine more objective weighting values for the future modelling. One limitation of the MLP-MC analysis was found that the model is unable to generate transition possibilities for all LULC classes. It is necessary to determine major LULC conversion trends and then the model produces transition potential accordingly. This makes the model difficult to use in a wide variety of LULC transitions exist especially in semi-natural areas.

In conclusion, over the past decade remote sensing data enabled the application of hybrid classification approach which is a combination of parametric and non-parametric techniques. This research was demonstrated that MLP-MC integration was beneficial to estimate future LULC of Mediterranean region where LULC change dynamics are complex due to environmental gradients and thousands of years of intensive human activities. Prediction performance of MLP-MC for future LULC was evaluated by ROC coefficient (0.89). Coniferous forest and agricultural areas were statistically distinct as they are expected a large increase in 2025. It should be noted that increase of temperature as a result of climate change will be resulted change in the borders of forest species from south towards to north in the Mediterranean Basin.

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ISOLATION AND CHARACTERIZATION OF A POLLEN-SPECIFIC GENE *ZMSTK2_USP* FROM *ZEA MAYS*

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Abstract. Microsporogenesis in flowering plants is a complex process involving a range of gene expression. In the present study, a pollen-specific gene *ZmSTK2_USP* (serine threonine kinase) was isolated from *Zea mays*. Mutant *ZmSTK2_USP* showed 56.7% pollen viability capacity, 48.92% germination rates comparing with wild-type. Northern Blot results showed that *ZmSTK2_USP* were specifically expressed in mature pollen. The expression level was highest at late pollen development, followed by bicellular stage. And at early pollen stage, no expression was detected. GUS activity was detected in middle stage, late stage of pollens and germinated pollen. These results indicated that *ZmSTK2_USP* expressed at late pollen development and was pollen-specific.
Keywords: *maize, serine/threonine kinase, pollen development, mutant, expression pattern, GUS activity*

Introduction

The floral organ development of plants needs to cooperate with the expression and regulation of various floral organ-specific genes, including a large number of genes related to anther or pollen development.

In plants, a number of anther- or pollen-specific genes have been isolated and identified. Hanson et al. (1989) reported that *Zmc13* expressed in maize pollen and pollen tube. *OSIPA* promoter conferred pollen-specific expression in both rice and *Arabidopsis*systems (Swapna et al., 2011). *TaPSG719* isolated from wheat were specific for mature pollen, and undetectable in other tissues (Jin and Bian, 2004). *TUA1* expressed in stamens and mature pollen in *Arabidopsis thaliana* development (Carpenter et al., 1992). *SBgLR* promoter in potato was identified as critical for gene specific expression in pollen grains (Zhou et al., 2010).

In the present study, we reported a pollen-specific gene *ZmSTK2_USP* (serine threonine kinase) isolated from *Zea mays*. Mutant *ZmSTK2_USP* pollen viability decreased, causing pollen germination disorder and loss of competitiveness, thus affecting the fertility of maize.

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Materials and methods

Plant materials and growth conditions

Table 1 lists all the primers used in this study. *Mu*-induced mutant *ZmSTK2_USP* was donated by Dr. Dooner (Rutgers University, USA) and the insertion information were previously published by Fu et al. (2001). The wild type McC, transgenic lines and mutants were grown in greenhouse with 16 h light /8 h dark cycle.

Table 1. *The list of primers used in the present study*

Gene	Forward primer	Reverse primer
Mu insertion lines *Mu-F* *ZmSTK2_USPA*	TGTGGTTGTCTTGAACCGCT （S0016）	GCCTCYATTTCGTCGAATCC AAGCACAATGGGCAGGTACA （S2011）
Cloning ZmSTK2_USP*B*	ATGGGCAGGTACAGCGACG	GTTGTAGTTGGACCTCCATGGA
Control genes *Actin*	GTTGGGCGTCCTCGTCA	TGGGTCATCTTCTCCCTGTT
Southern Blot Analysis ZmSTK2_USP*E*	AGACGGGCATGCTGGGGGTGAAGT	ATGGCGGCGACGGGCTGGTGT
GUS for transgenic lines	GCAACTGGACAAGGCACT	GAGCGTCGCAGAACATTACA

In vitro pollen viability and germination assays

The pollen of wild type McC and mutant *zmstk2_usp* was collected at 10:00 a.m. every day when the tassel began to loose pollen. After mixing, some of the pollen was quickly brought back to the laboratory within an ice pack.

Proper amount of pollen was put onto clean slides, 2 drops of I2-KI solution were dropped. After treatment with 10 min at 30 constant temperature, the pigmentation of pollen grains were observed under the microscope (ZEISS Axio Scope. A1) and photographed. Mature pollen grains are dark colored, indicating that pollen has strong vitality and shallow coloration indicated that pollen activity is poor or not vigorous. Pollen grains, total no less than 100, were observed under 5 of visual field. Count the number of dark and light colors of pollen grains, and calculate the pollen viability capacity (%). Values are mean with n = 3.

Pollen germination experiments of wild type McC and mutant zmstk2_usp were carried out in vitro referring to the methods described in Schreiber and Dresselhaus (2003). A small amount of pollen were immediately and uniformly scattered to the slides coated with the pollen germination medium [medium formula is followed according to Wang et al. (2017), incubated 2 h in a petri dish, with wet filter paper, at constant temperature illumination and observed under the microscope (ZEISS Axio Scope. A1) within 5 of visual field and photographed. The number of germinated and non-germinated pollen grains was counted and the pollen germination rates (%) were calculated. Values are mean with n = 3.

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Construction of the ZmSTK2_USP expression vector, and maize transformation

The upstream primer STK2B-F and downstream primer STK2B-R were designed based on the sequence of the *ZmSTK2_USP* gene and listed in *Table 1*. STK2B-F with *BamH*I and STK2B-R with *Hind*Ⅲ cleavage site were employed used to amplifiy coding sequences of *ZmSTK2_USP* using McC pollen cDNA as template. *ZmSTK2_USP* target fragments were introduced into the TA clone plasmid, sequenced to gent positive cloning. The plasmids with positive cloning were digested by *BamH*I and *Hind*Ⅲ. *ZmSTK2_USP* target fragments were cloned into pCAMBIA1301 between cleavage sites of *BamH*I and *Hind*Ⅲ, which an expression vector containing a *ZmSTK2_USP*-GUS fusion gene was constructed.

Southern blot analysis

Leaf DNA of T1 transgenic plants with pCAMBIA1301-GUS was isolated and amplified using primers GUS-F and GUS-R listed in *Table 1*. The DNA probe was labeled using DIG. Genome DNA (40 μg) of positive T1 transgenic plants was digested using Hind III, electrophoresised, then transferred to membrane, hybridized and detected according to DIG High Prime DNA Labeling and Detection Starter Kit Ⅰ (Roche). The colored membrane was recorded by digital camera.

Histochemical GUS staining

For GUS activity analysis, we regularly collected various tissues at different developmental stages (organs) of McC and transgenic T_2 generation of *ZmSTK2_USP*-GUS, including immature male flowers, mature male flowers, and mature anthers, three different developmental stages (early stage of pollen development, middle stage of pollen development and late stage of pollen development) and pollen tube.

According to the method described by Jefferson et al. (1987), we put various tissues (organs) into 90% acetone stationary liquid for 20 min in -20 °C refrigerator, PBS buffer rinses 2 times using PBS buffer, each time 10 min, then added GUS staining solution X-Gluc (TritonX-100, 20% methanol), incubated 16 h at 37 °C in dark. After that, the green tissues (organs) was decolorized 2 times using 70%-100%-70% (v/v) ethanol, observed under microscope (ZEISS Axio Scope. A1) and photograph.

Results and discussion

Phenotypic analysis of ZmSTK2_USP mutants

Mu-induced *ZmSTK2_USP*, a non-autonomous 1.4 kb *Mu* element at the beginning of the 5th exon of *ZmSTK2_USP* (*Fig. 1A*), was obtained from Rutgers University, USA (Dooner, unpublished). *ZmSTK2_USP* mutants were detected using the primers listed in *Table 1*.

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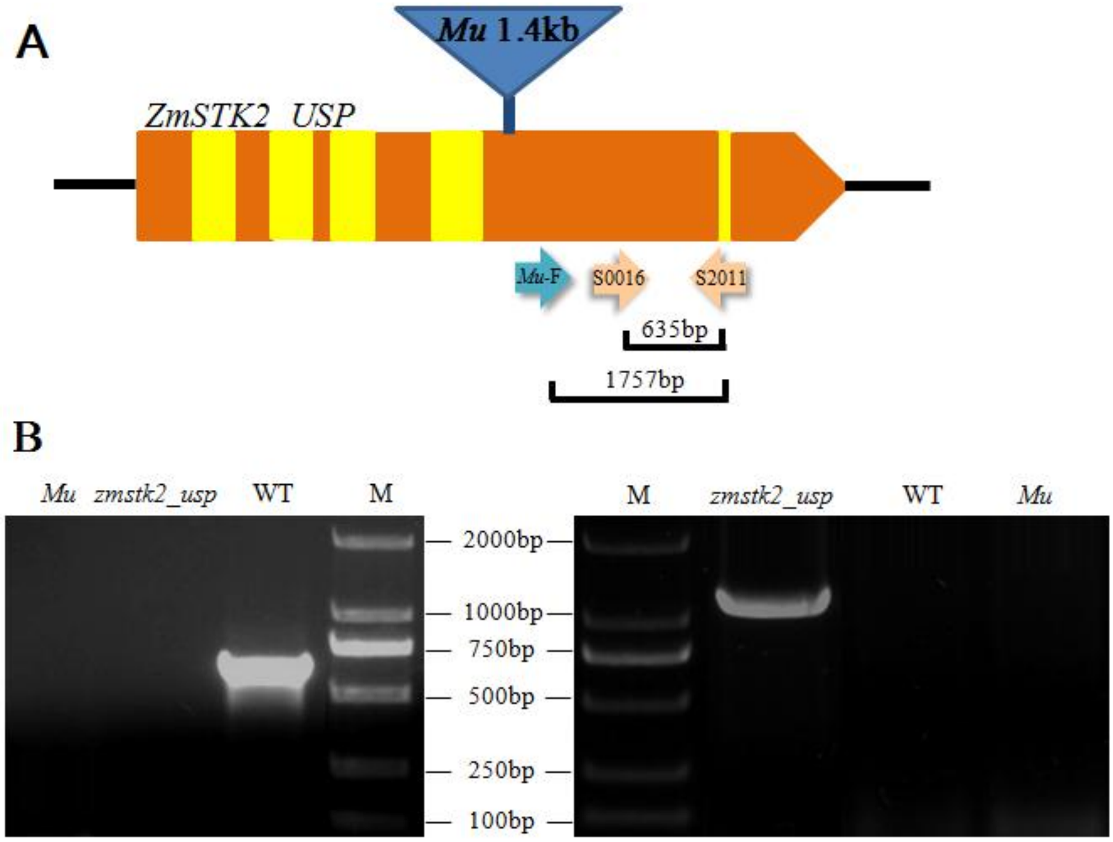

Figure 1. *Mu insertion mutation and detection of homozygous mutant. Note: A. Schematic view of the genomic structure of ZmSTK2_USP in maize and sites of Mu insertions; B. RT-PCR test results. M denotes DL2000. Mu was used as a negative control. The orange solid rectangle is the exon; the yellow solid rectangle is the intron; the inverted blue solid triangle is the Mu transposon and its inserted site (the fifth exon origin); the blue arrow is Mu primers and directions; the pink arrows are a pair of primers and directions on ZmSTK2_USP gene; and the black line segment is the length of the RT-PCR product corresponding to the primer combination*

Fig. 2 shows phenotypic traits of wild-type McC and mutant *ZmSTK2_USP* during the whole period of growth and development. Both wild–type and mutant were semi-compact plant type (*Figs. 2D*, *d* and *E*, *e*) with 12-15 tassel branch (*Figs. 2E* and *e*), purple anther (*Figs. 2F* and *f*) and purple durum grain (*Figs. 2G* and *g*). In our previous study, *ZmSTK2_USP* belonged to receptor-like cytoplasmic kinase (RLCK) and was pollen-specific (Wang et al., 2014; Zhou et al., 2014; Wang et al., 2017). Hence, we observed pollen grains of wild-type plants and mutants. The pollen grains of wild type McC are dyed darkly by I_2-KI , very plump and most of them were germinated, while most of mutant pollen grains are partly wrinkled and hollow, dyed brown and not germinated (*Figs. 2A*, *a* and *B*, *b*). The mutant *ZmSTK2_USP* was artificially pollinated with wild type McC plants, and dyed with aniline blue after 6 h, pollen grain germination and pollen tube elongation of *ZmSTK2_USP* were very slow and half of them did not germinated comparing with wild-type (*Figs. 2C* and *c*).

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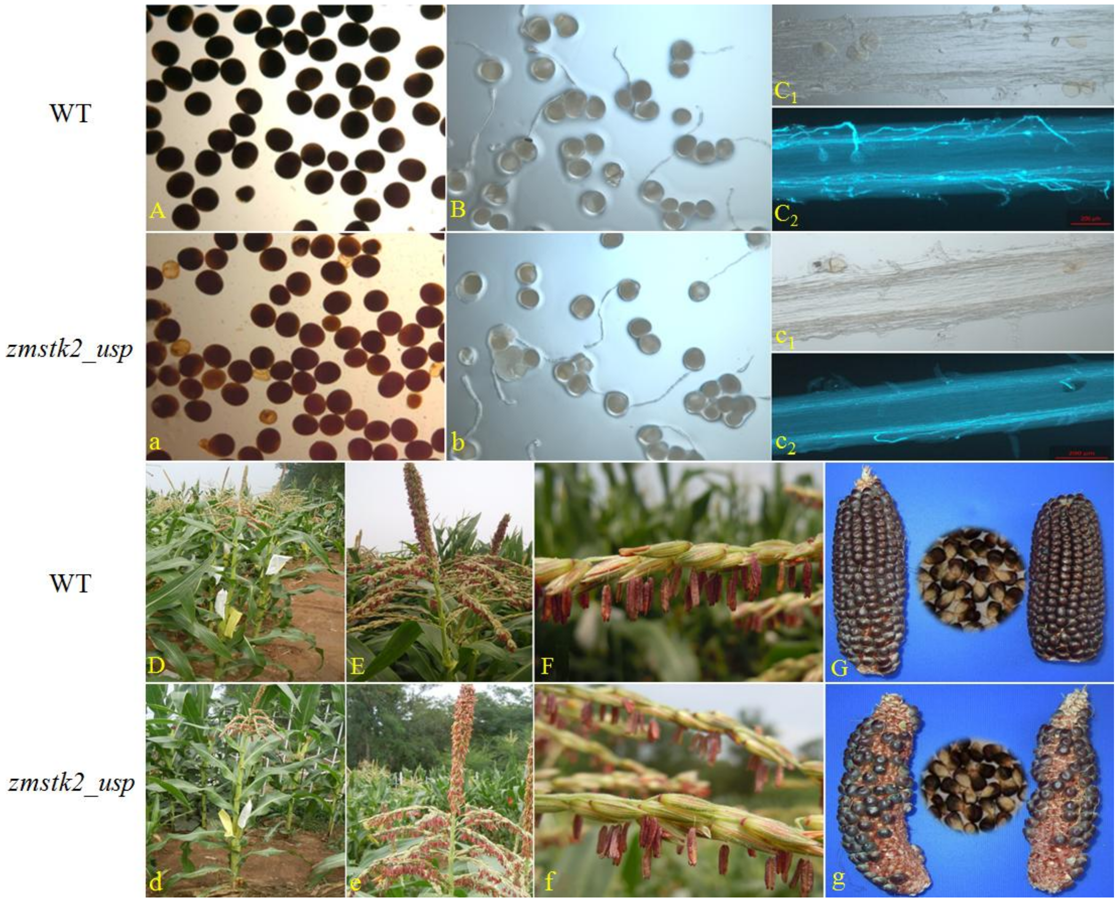

Figure 2. *Phenotypic comparison of wild-type and mutant zmstk2_usp. Note: A and a. Images of pollen viability obtained after staining with I_2-KI; B and b. Pollen germination in vitro after 2 h; C_1, C_2, c_1 and c_2. In vivo of germinated pollen grains in silks after 6 h; C1 and c1 were observed in bright field; C2 and c2 were observed under fluorescence; D and d. Plant type of the wild type and mutant; E and e Tassel of the wild type and mutant; F and f. Anther of the wild type and mutant; G and g. Grain color and grain type of the wild type and mutant*

Fig. 3 shows the statistical analysis results of pollen viability capacity, germination rates, filled grains per spike of mutant *ZmSTK2_USP* and wild-type plants. Comparing with wild-type, mutants showed extremely significant difference with 56.7% pollen viability capacity, 48.92% germination rates. The filled grains per spike of mutant *ZmSTK2_USP* were around 40% of wild-type. In summary, *Mu*-induced *ZmSTK2_USP* cause decreased pollen viability, disordered pollen germination, and lower competitiveness, thus affected the fertility of maize.

ZmSTK2_USP expression pattern

To clarify the expression profiles of *ZmSTK2_USP* gene in Maize, we performed Northern Blot and qRT-PCR analysis (*Fig. 4*). Northern Blot results showed that *ZmSTK2_USP* were specifically expressed in mature pollen and no expression was found in other tissues (organs). The expression level was highest at late pollen development, followed by bicellular stage. And at early pollen stage, no expression was detected. The pattern of *ZmSTK2_USP* expression indicates that *ZmSTK2_USP* expressed at late pollen development and was pollen-specific.

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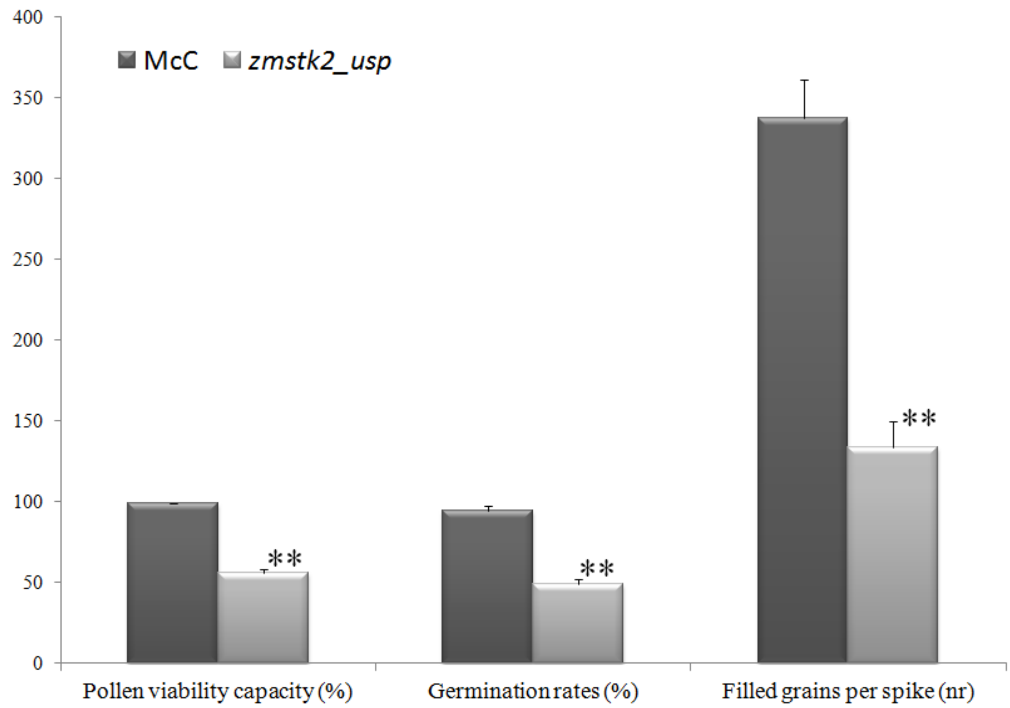

Figure 3. *Analysis of pollen viability capacity (%), germination rates (%), filled grains per spike (nr). Note: ** showed extremely significant difference. The error bars represent standard deviations of the means. Data mean ± SD (n = 3)*

Molecular biology detection of transgenic lines

Using the method of Agrobacterium-mediated transformation of the maize germinating embryo (Wang et al., 2017), four positive plants were conformed by PCR and Southern blot (*Fig. 4*).

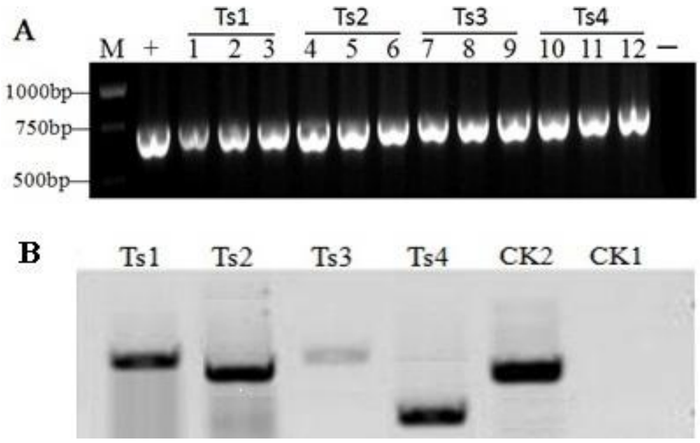

Figure 4. *Molecular biology detection of transgenic lines. Note: A. PCR tests of ZmSTK2_USP-GUS in T_0 transgenic lines; B. Southern blotting analysis of transgenic lines (T_0). M are DL5000 and DL2000, respectively; + denotes transforming plasmids as positive control; — denotes wild-type McC as negative control. Ts1, Ts2, Ts3 and Ts4 are successfully transformed plants with ZmSTK2_USP-GUS. CK1 is the inbred line McC of non-genetically modified maize. CK2 is the GUS gene*

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Among the four positive transgenic lines with *ZmSTK2_USP*-GUS, only T_2 seeds of Ts2 and Ts4 were obtained. To validate the tissue specificity of *ZmSTK2_USP*, using non-genetically modified maize McC as control, T_2 seeds of Ts2 and Ts4 expressing the GUS fusions, and non-genetically modified seeds (McC) were planted in a greenhouse, and the *ZmSTK2_USP* activity was examined by GUS staining at different developmental stages, then observed, photographed, and recorded under stereomicroscope. Ts2 and Ts4 showed no expression of GUS gene in immature male flower and early stage of pollens, while GUS activity was detected in middle stage, late stage of pollens and germinated pollen (*Fig. 5*). These results indicated that *ZmSTK2_USP* expressed at late stage of pollen development.

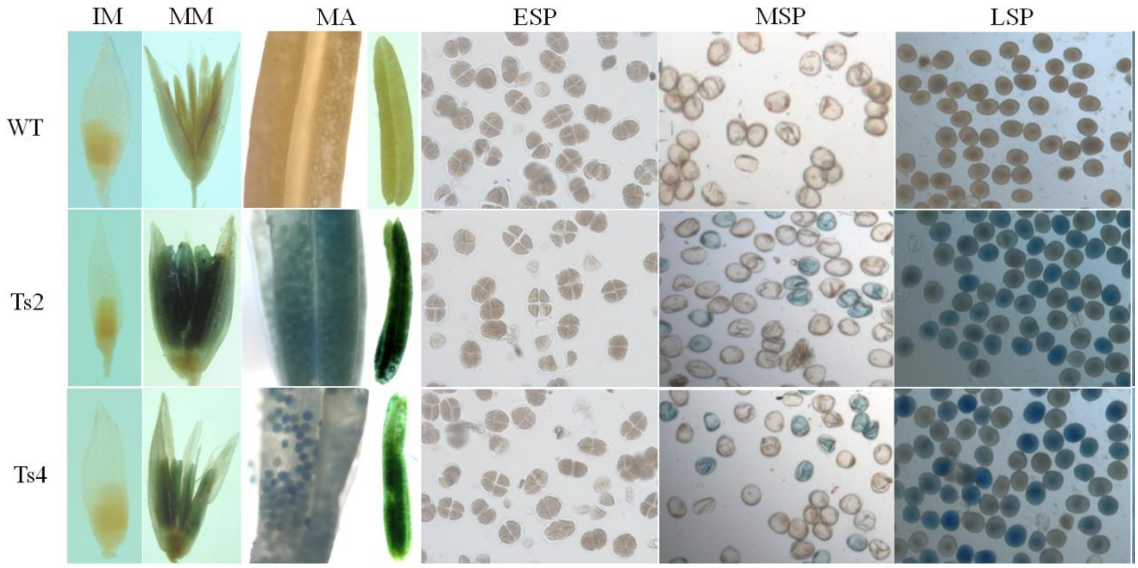

Figure 5. *Histochemical GUS staining of maize transformed with ZmSTK2_USP-GUS. Note: WT is the inbred line McC of non-genetically modified maize; Ts2 and Ts4 are successfully transformed plants with ZmSTK2_USP-GUS; IM: Immature male flower; MM: Mature male flower; MA: Mature anther; ESP: early stage of pollens; MSP: middle stage of pollens; LSP: late stage of pollens*

Conclusion

The molecular mechanisms involved in pollen development and pollen tube elongation are extremely complex. Besides a range of organ differentiation and tightly controlled changes in cellular physiology and biochemistry, a large number of genes are involved in expression and regulation. Most of these genes are also expressed in other tissues of plants, but there are still 10%-20% genes that are specifically expressed in pollen (Willing et al., 1988). At present, there are relatively few reports about pollen development genes in maize.

In our previous study, ZmSTK2_USP belong to the serine/threonine kinase (STK), containing a Usp domain on N-terminal (Wang et al., 2014; Zhou et al., 2014). In the present study, we cloned *ZmSTK2_USP* gene with specific-expressed at late stage of pollen development. The mutant *ZmSTK2_USP*, with wrinkled and withered pollen grains, showed 56.7% pollen viability capacity, 48.92% germination rates comparing with wild-type, which led to the reduction of seed setting rate. To investigate the specific expression of *ZmSTK2_USP*, Northern blot and qRT-PCR analysis results

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revealed that *ZmSTK2_USP* expressed at late stage of pollen development. Further, GUS staining of transgenic plants verified that *ZmSTK2_USP* gene has tissue specific expression at mature pollen. All these results are useful for providing theoretical basis for future study on the molecular mechanism of reproductive development in maize.

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KINETICS AND THERMODYNAMIC PROPERTIES OF PARBOILED BURGOS WHEAT (*TRITICUM DURUM*) IN TURKEY DURING DRYING

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Abstract. The influence of temperature and dryer types on kinetics, thermodynamic properties of parboiled wheat (bulgur) in Turkey was studied during drying. Durum wheat (local name in Turkey, Burgos) was cooked under atmospheric conditions, and then it was dried at 50, 60 and 70 °C. Six semi-theoretical and Fick's diffusivity models with Arrhenius and Eyring–Polanyi approaches were employed to predict drying kinetics and thermodynamic properties. Among all the models, Midilli model was found to have the best fit as suggested average R^2 value of 0.9987, and corresponding RMSE value of 0.010. Effective diffusion coefficient (D_{eff}) increased significantly ($P \leq 0.05$) from 2.38×10^{-11} to 5.84×10^{-11} (59% increase), 4.65×10^{-11} to 8.51×10^{-11} (45% increase), and 8.70×10^{-11} to 14.10×10^{-11} (38% increase) for natural convective air, forced convective air and vacuum drying systems with temperature increase from 50 to 70 °C. The activation energy values of the samples of natural convective air drying (NCAD) (41.46 kJ mol^{-1}) compared with that of forced convective air drying (FCAD) (27.71 kJ mol^{-1}) and vacuum drying (VD) (22.28 kJ mol^{-1}) show a decrease trend which suggests more effective drying. The enthalpy and entropy decreased with increasing temperature for all dryers. The Gibbs free energy increased with increasing temperature. For each dryer, also, general equations to describe the moisture ratio of parboiled wheat as a function of time and temperature were developed. It was concluded that vacuum dryer has provided more effective drying than the other dryers.
Keywords: *model, vacuum, activation energy, bulgur, mass transfer*

Introduction

Wheat is one of the major staple foods in all over world because of ease of storage and ability of its flour to be made into many food materials. Many special food products can be prepared from wheat; one of such special food product is parboiled wheat (bulgur). It is very popular in western countries as frozen food materials and quick cooking foods. Bulgur is a cleaned, washed, parboiled, debranned, crushed and sifted wheat product (Bayram and Öner, 2006). It is an excellent ingredient in pilaf, salad, casseroles, stuffing's, soups, baked goods, and as a meat substitute in vegetarian dishes (Yu and Kies, 1993). It is widely consumed in Turkey, Greece, Cyprus, Middle East, North Africa, and East Europe. From a producer's point of view, the main factors of interest are production costs, processing time, product shelf life and product quality. The selection of one particular drying method above another is dependent on its ability to produce a shelf stable product, as optimizing quality and cost.

Drying of food material depends upon heat and mass transfer characteristics of the product being dried. Knowledge of temperature and moisture distribution in the product is vital for equipment and process design and quality control (Mohapatra and Rao, 2005). Many studies have reported the importance of the kinetics of the drying for various agricultural products such as bean and chickpea (Shafaei et al., 2016), yellow peas (Mercier et al., 2015), pumpkin (Hashim et al., 2014), spearmint (Ayadi et al., 2014) and rice (Correa et al., 2016). These studies identified the effect of temperature

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on the rate and the amount of moisture that is moved between the product and the environment.

The most widely used theoretical model has been Fick's second law of diffusion. Drying of many food products such as rice (Ece and Cihan, 1993), soybean (White et al., 1981), rapeseed (Crisp and Woods, 1994) and sorghum (Suarez et al., 1980) has been successfully predicted using Fick's second law with Arrhenius-type temperature dependent diffusivity (plotting the natural logarithm of effective moisture diffusivity, D_{eff} versus the reciprocal of the absolute temperature).

Semi-theoretical models offer an ease of use that is valid within the temperature, relative humidity, moisture content range for which they were developed. Among the drying semi-theoretical models, the Henderson & Pabis, Lewis, Page's, Wang & Singh, Logaritmic, and Weibull models are used frequently (*Table 1;* Liu et al., 2016).

Table 1. *Mathematical models used for drying curves*

Model name	Number	Model equation	Reference
Lewis	1	$MR = \exp(-kt)$	Bruce (1985)
Page	2	$MR = \exp(-kt^n)$	Page (1949)
Midilli	3	$MR = a * \exp(-kt^n) + bt$	Midilli et al. (2002)
Henderson & Pabis	4	$MR = a * \exp(-kt)$	Henderson and Pabis (1961)
Logarithmic	5	$MR = a * \exp(-kt) + c$	Toğrul and Pehlivan (2002)
Weibull	6	$MR = \exp\left[-(\frac{t}{\alpha})^{\beta}\right]$	Blasco et al. (2006)

k: drying coefficient, t: drying time (min)

Henderson & Pabis model had been used to model drying of corn (Mishra and Brooker, 1980), wheat (Becker, 1959; Watson and Bhargava, 1974), parbolied wheat (Chakraverty and Kaushal, 1982), and rough rice (Wang and Singh, 1978). This drying model was based on the assumption that entire dying process occurred in the falling rate period. The two-term model had been used to describe thin layer drying of parboiled wheat (Mohapatra and Rao, 2005). The Lewis model was used to predict drying of barley (Bruce, 1985). The Page model was the modification of Lewis model and used extensively in determining the drying characteristics of short and medium grain rough rice (Wang and Singh, 1978), soybean (White et al., 1981), shelled corn (Mishra and Brooker, 1980), barley (Bruce, 1985). Empirical model was developed, which suitably predicted drying characteristics of rough rice (Watson and Bhargava, 1974).

Some researchers have reported on thermodynamic properties such as enthalpy, entropy, Gibbs free energy in different products such as rice (Correa et al., 2016), chia (Velasquez et al., 2015), potato flakes (Lago et al., 2013), pineapple (Bispo et al., 2015), and bulgur (Bayram et al., 2004b).

Although extensive work has been done on drying of wheat and parboiled wheat, limited literature is available on vacuum drying characteristics of parboiled wheat of Burgos variety comparison with natural convective air and forced convective air drying systems. Whereby drying is an important process to produce parboiled wheat, present study has given importance on drying behaviour of parboiled wheat.

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The aims of the present study were to demermine the influence of the time, temperature and dryer type on parbolied wheat (Local name, Burgos (*Triticum durum*)) during drying, to examine the capabilities of six semi-theoretical and Fick's Diffusion models, to find the best drying kinetics model, to calculate the thermodynamic parameters (Activation energy, Entalpy, Entropy and Gibbs free energy), and to derive the new equations related to moisture ratio, time and temperature of parboiled wheat for each dryer.

Material and methods

Material

Wheat sample (Burgos, *Triticum durum*), which is one of the main cultivars used for pasta and bulgur production, was obtained from Şanlıurfa Commodity Exchange, Turkey. The moisture content was determined by standard oven method (AOAC, 2002). The average dimensions (L: length, W: width and T: thickness in mm) of wheat kernels were measured with Mutitoyo No. 505-633, Japan, digital micrometer. The average equivalent diameter ($D_e = (LWT)^{1/3}$) and sphericity ($\phi = (LWT)^{1/3} L^{-1}$) of grains were also calculated (Mohsenin, 1980).

Wheat cooking

Wheat kernels, cleaned from extraneous matter, were combined with water at a ratio of 1:6 (weight basis) and cooked by a heater (IKA Model HP 30, Staufen, Germany) at 97 °C until the starch was entirely gelatinized (about 80 to 95 min). The average initial moisture content of parboiled wheat on a dry basis (d.b) was 126.98%.

Drying systems

The drying process was carried out at 50, 60 and 70 °C for 480 min. Parboiled wheat samples were laid on each pan of dryers (1600 g m^{-2}). Natural convective air dryer (NCAD) (Elektromag, M7040-R, Turkey), forced convective air dryer (FCAD) (Elektromag, M7040-R, Turkey) with air velocity of 1.2 m s^{-1} and vacuum dryer (VD) (WiseVen, WOV-70, Witeg, Germany) with a pressure of 33.33 kPa were used for dehydration of parboiled wheat.

Experimental procedure

Fifty grams of representative parboiled wheat samples was uniformly spread in single layer over the drying pans of dryers. The moisture loss from the parboiled wheat, during drying was determined every 1 h, by lifting the drying pan and quickly weighing the sample with an electronic balance. The drying experiments were carried out till 8 h. The experiments were conducted in triplicate. Average values of moisture loss were taken for final calculations. The moisture content of samples in dry basis at any drying time was calculated by *Equation 1:*

$$M_t = \left[\frac{(M_o + 1) * W_t}{W_o} - 1 \right] * 100 \qquad \text{(Eq. 1)}$$

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where W_o is initial weight (g), W_t is weight of sample (g) at any drying time (t). M_o and M_t are the moisture contents of wheat samples in dry basis initially and at different drying time, respectively.

Theory

Modeling of drying curves

For studying the drying characteristics of parboiled wheat, it is very important to model the drying behavior effectively. The data obtained at different temperatures of drying were fitted into six commonly used drying models, listed in *Table 1*.

In the drying experiments, the moisture ratio (MR) of parboiled wheat was calculated using the following equation (*Eq. 2*):

$$MR = \frac{M_t - M_e}{M_o - M_e} \qquad \text{(Eq. 2)}$$

where M_t is the moisture content at any time (%, d.b.); M_e is the equilibrium moisture content (%, d.b.); and M_o is the initial moisture content (%, d.b.) of the samples. In the present study, this equation was simplified to *Equation 3,* considering that M_e is negligible compared to M_t or M_o (Rayaguru and Routray, 2012).

$$MR = \frac{M_t}{M_o} \qquad \text{(Eq. 3)}$$

The drying rate (DR) of parboiled wheat samples was calculated using *Equation 4* (Kavak Akpinar, 2002)

$$DR = \frac{MR_{t+dt} - MR_t}{dt} \qquad \text{(Eq. 4)}$$

where MR_{t+dt} and MR_t are moisture ratio at the time t + dt and t (dimensionless). t is the drying time in min.

Effective diffusivities calculation

Fick's diffusion model is generally used to describe the drying characteristics of the biological products. The solution of Fick's diffusion model in spherical coordinates, with the assumptions of moisture migration being by diffusion, negligible shrinkage, constant diffusion coefficients and temperature, is given by (*Eq. 5;* Crank, 1975):

$$MR = \sum_{n=1}^{\infty} \frac{6}{\pi^2 n^2} \exp\left(-\frac{D_{eff} * n^2 * \pi^2 * t}{R_e^2} \right) \qquad \text{(Eq. 5)}$$

where n is the positive integer, D_{eff} is the effective moisture diffusion coefficient ($m^2\ s^{-1}$), t is drying time (s) and R_e is the average radius of wheat (m). Simplifying this by taking the first term of the series solution, gives (*Eq. 6*)

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$$MR = \frac{6}{\pi^2} \exp\left(- \frac{D_{eff} * \pi^2 * t}{R_e^2} \right) \tag{Eq. 6}$$

Thermodynamic properties

Effect of temperature on effective diffusivity is generally expressed using an Arrhenius-type relationship, since temperature has the significant effect over the drying process rather initial moisture content of the product (*Eq. 7*).

$$\ln(D_{eff}) = \ln(D_{ref}) - (\frac{E_a}{R}) * (\frac{1}{T}) \tag{Eq.7}$$

where D_{eff}, T, E_a and R are effective diffusion coefficient of the Fick's model, soaking temperature (K), activation energy for the drying process in kJ mol^{-1} and ideal gas constant in 8.314×10^{-3} kJ mol^{-1} K^{-1}, respectively. D_{ref} is reference diffusion coefficient for the Fick's model.

The thermodynamic properties of mass transfer process in parboiled wheat were determined by Eyring–Polanyi model (*Eqs. 8, 9* and *10*; Correa et al., 2012):

$$\Delta H = E_a - RT \tag{Eq. 8}$$

$$\Delta S = R[\ln(A_o) - \ln(\frac{k_B}{h_p}) - \ln(T)] \tag{Eq. 9}$$

$$\Delta G = \Delta H - T\Delta S \tag{Eq. 10}$$

where ΔH is the enthalpy, J mol^{-1}; ΔS is the entropy, J mol^{-1} K^{-1}; ΔG is the Gibbs free energy, J mol^{-1}; k_B is Boltzmann's constant, 1.38×10^{-23} J K^{-1}; and h_p is Planck's constant, 6.626×10^{-34} J s^{-1}.

Statistical analysis

The drying data were fitted to different models using Sigma plot 10 (Jandel Scientific, San Francisco, USA) software. Nonlinear regression analysis was performed on all runs to estimate the parameters associated with considered models from the experimental data. Data were compared using the Duncan's test at $p \leq 0.05$ (SPSS Inc., version 16, USA). Correlation coefficient squared (R^2) and root mean square error (RMSE) (*Eq. 11*) was used as the criteria for the accuracy of the fit.

$$RMSE = \sqrt{\frac{1}{n} \sum_{1}^{n} \left[(MR_{\exp} - MR_{pre}) \right]^2} \tag{Eq. 11}$$

where n, MR_{exp} and MR_{pre} are the numbers of observations, the experimental moisture ratio and predicted moisture ratio, respectively.

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Results and discussion

Moisture ratio and drying rate change

Initial moisture content of the wheat grain was about 9.99 (%, d.b.). The average L, W, T, D_e and R_e (equivalent radius) values of raw wheat grains were found to be 7.81±0.12, 3.21±0.09, 3.10±0.07, 4.22±0.15 and 2.11±0.18 mm, respectively. The sphericity of it was calculated as to be 0.54±0.01.

By recording the weight change over time in the different dryers, moisture contents (%, d.b.), moisture ratios and drying rates of samples were calculated by *Equations 1, 3* and *4,* respectively (*Figs. 1* and *2*). The initial moisture content of parboiled wheat was found to be 126.98 (%, d.b.). Moisture contents after 8 h drying of parboiled wheat at 50 °C were found to be decreasing as 16.39, 15.63 and 9.15 (%, d.b.) for the natural convective air, forced convective air and vacuum dryers, respectively. Similarly, increasing of temperature to 60 °C decreased the final moisture contents to 11.26, 10.96 and 6.83 (%, d.b.). Also, drying at 70 °C temperature showed that the final moisture contents were found to be 6.44, 6.11 and 5.63 (%, d.b.) that were in the decreasing trend.

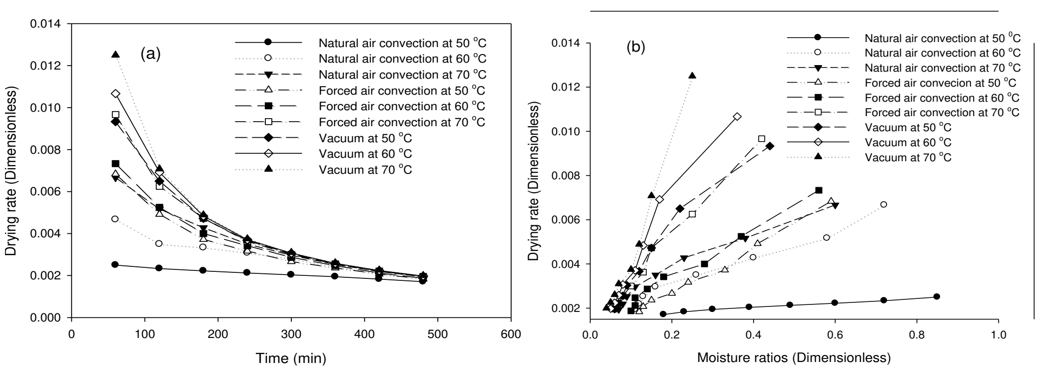

***Figure 1.** Drying rate curves of parboiled wheat at different temperatures for different dryers (a: drying rate vs. time(min), b: drying rate vs. moisture ratio)*

Change of drying rate is shown in *Fig. 1*. It can be seen from *Fig. 1* that significant ($P \leq 0.05$) differences in drying rate were found between the three drying methods, i.e. natural convective air drying, forced convective air drying and vacuum drying. At the beginning when moisture ratio was high, the drying rate under all drying conditions increased with time. The constant drying period is rarely observed in food drying studies and several authors reported that no constant rate period occurred during drying of different agricultural products, such as potato (McMinn and Magee, 1996), carrot (Toğrul, 2006), parboiled wheat (Mohapatra and Rao, 2005), bell pepper, pumpkin, tomato and several others (Krokida et al., 2003). The entire drying process was found to be taken place in falling rate period only, which indicated that moisture diffusion was the governing factor (Singh and Sodhi, 2000) deciding drying behaviour of parboiled wheat (*Fig. 2*). It was observed that the drying of the parboiled wheat is accompanied by the falling drying rate stage (*Figs. 1* and *2*). Lack of a constant drying rate period was also observed in other studies of vacuum drying of porous materials (Sander, 2007).

As can be seen from these values, the temperature increase for the three dryers caused a decrease in the final moisture values. In other words, in order to reach the final

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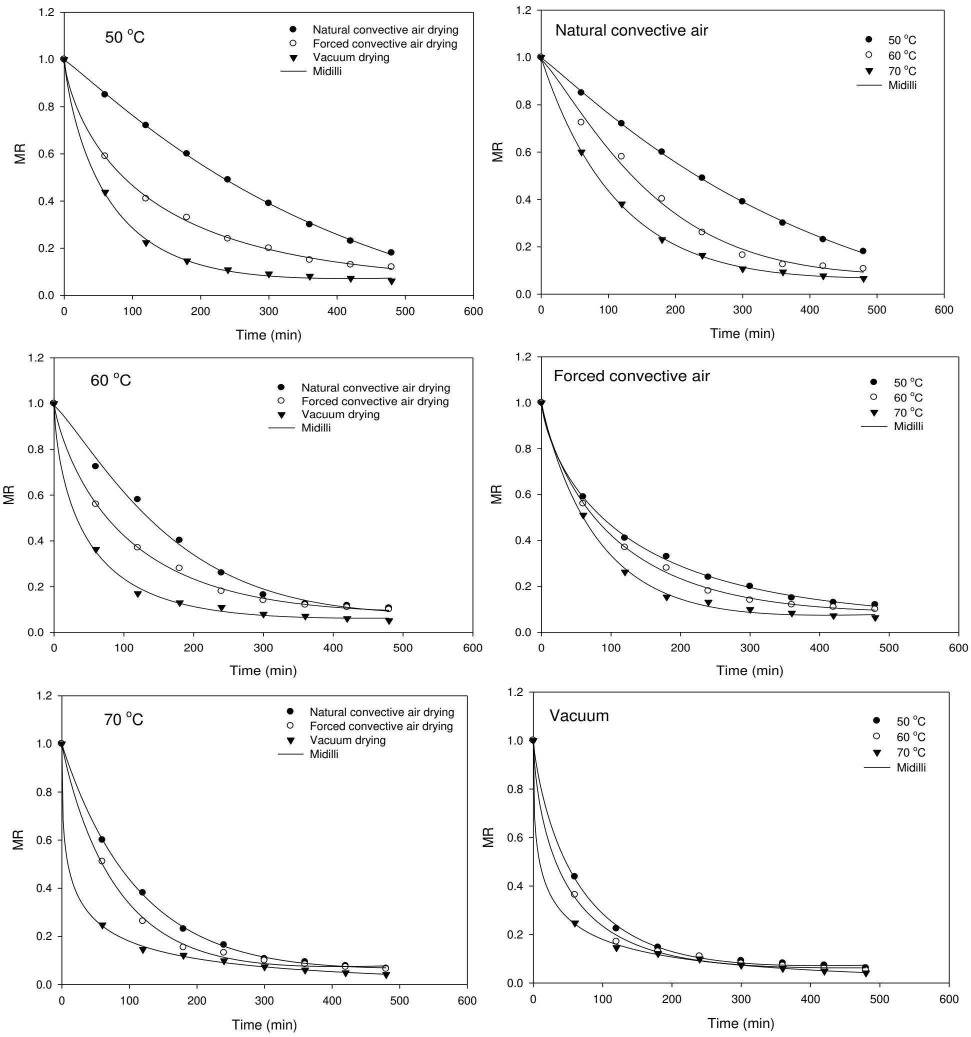
moisture content, the vacuum dryer required a shorter time. Likewise, the increase in temperature also shortened the drying time. As the drying temperature increased, the drying rate increased during drying of parboiled wheat (*Fig. 1*). In all dryers and temperatures during the first hour of drying, the initial drying rate is high due to easy separation of free water.



a **b**

Figure 2. *Simulated moisture ratio during drying of parboiled wheat at different temperatures (a) for different dryers (b) from Midilli model*

For NCAD system, during 60 min drying period, increase in temperature from 50 to 60 °C resulted 46.43% increase in drying rate, and 62.50% increase from 50 to 70 °C. Similarly, FCAD increased the drying rate by 6.82% and 29.31% at the same temperature increments for the same drying period. We see similar temperature effect during vacuum drying system that the drying rate increased by 12.50% and 29.69%. During 60 min drying periods at 50, 60 and 70 °C temperatures, 63.41% and 73.21%, 36.36% and 56.25%, 31.03% and 46.67% increments were found respectively when we compared drying rates of NCAD with FCAD and vacuum dryers. Results are confirmed by previous studies that vacuum drying is more effective for similarly forced convective

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air drying for mints (85-90% reduction) (Giri and Prasad, 2007) and mushrooms (70-90% reduction) (Therdthai and Zhou, 2009). Mohapatra, and Rao (2005), Ghaitaranpour et al. (2013) investigated that the drying temperature of parboiled wheat was affected the rate of drying. The results of this study are also confirmed by studies of Özdemir and Derves (1999) for hazelnut, Mohapatra and Rao (2005) for bulgur and Tulek (2011) for mushroom. Drying temperature, forced convective air and vacuum drying systems have greatly increased the drying rates.

Fitting of the drying curves of parboiled wheat

The drying kinetics is often used to describe the combined macroscopic and microscopic mechanisms of mass transfer during drying, and it is affected by drying conditions, types of dryer and characteristics of materials to be dried. The drying kinetics models are essential for equipment design, process optimization and product quality improvement.

Parboiled wheat samples were dried in different dryers, and at different temperatures to obtain experimental data for the change in moisture ratio (MR) over time (*Fig. 2*). Data obtained from drying experiment; basically the moisture content was converted to moisture ratio (MR) and was fitted to the six models listed in *Table 1*. As root mean square error (RMSE) values approach zero, the closer the prediction is to experimental data. Different drying models were compared on the basis of their R^2 and RMSE so as to evaluate their respective goodness of fit. The statistical results of different model including their model coefficients are listed in *Table 2*. In all models and all cases, R^2 values were higher than 0.9526, and RMSE values were lower than 0.063. The average R^2 value of Midilli model at all temperatures and dryers were 0.9987, and corresponding RMSE value was 0.010. Among the used models, Midilli model was exhibited the best fit for drying kinetics of parboiled wheat.

It can be seen from *Table 2* that the drying rate constant 'k' for Midilli model increases in absolute values with increasing temperatures. This fact is expected because higher temperatures lead to a higher drying rate, reaching equilibrium water content faster. Also, effect of dryer on k value can be seen from *Table 2*.

Table 2. *Statistical constants of the six drying models ($p \leq 0.05$)*

Dryer*	Model	T^a (°C)	k**	a	b	c	n	α	β	R^2	RMSE
NCAD	1	50	5.27×10^{-5}							0.9898	0.027
		60	8.78×10^{-5}							0.9923	0.026
		70	12.9×10^{-5}							0.9928	0.025
	2	50	8.45×10^{-6}				1.19			0.9989	0.009
		60	4.86×10^{-5}				1.06			0.9932	0.025
		70	4.73×10^{-4}				0.86			0.9974	0.015
	3	50	1.50×10^{-5}	1.00	-2.90×10^{-6}		1.09			0.9997	0.005
		60	1.85×10^{-5}	0.99	1.75×10^{-6}		1.20			0.9948	0.022
		70	1.70×10^{-4}	1.00	1.91×10^{-6}		0.98			0.9997	0.005
	4	50	5.47×10^{-5}	1.03						0.9921	0.024
		60	8.87×10^{-5}	1.01						0.9924	0.026
		70	1.27×10^{-4}	0.98						0.9932	0.025
	5	50	3.33×10^{-5}	1.35		-3.48×10^{-1}				0.9995	0.006
		60	8.73×10^{-5}	1.01		-6.57×10^{-3}				0.9924	0.026

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		70	1.51×10^{-4}	0.95		5.39×10^{-2}				0.9997	0.005
	6	50						1.86×10^{4}	1.19	0.9989	0.009
		60						1.15×10^{4}	1.06	0.9932	0.025
		70						0.75×10^{4}	0.86	0.9974	0.015
FCAD	1	50	1.03×10^{-4}							0.9606	0.054
		60	1.22×10^{-4}							0.9696	0.049
		70	1.68×10^{-4}							0.9780	0.043
	2	50	1.98×10^{-3}				0.68			0.9991	0.008
		60	1.89×10^{-3}				0.70			0.9973	0.014
		70	2.02×10^{-3}				0.72			0.9928	0.025
	3	50	3.76×10^{-4}	1.00	8.04×10^{-7}		0.72			0.9992	0.007
		60	7.51×10^{-4}	1.00	1.91×10^{-6}		0.81			0.9991	0.009
		70	1.45×10^{-3}	1.00	2.41×10^{-6}		0.92			0.9984	0.012
	4	50	9.55×10^{-5}	0.94						0.9683	0.048
		60	11.60×10^{-5}	0.95						0.9734	0.046
		70	16.40×10^{-5}	0.98						0.9787	0.043
	5	50	1.49×10^{-4}	0.86		1.23×10^{-1}				0.9941	0.021
		60	1.67×10^{-4}	0.89		9.93×10^{-2}				0.9970	0.015
		70	2.13×10^{-4}	0.93		7.19×10^{-2}				0.9991	0.009
	6	50						8.89×10^{3}	0.69	0.9991	0.008
		60						7.44×10^{3}	0.70	0.9973	0.014
		70						5.43×10^{3}	0.72	0.9928	0.025
VD	1	50	1.94×10^{-4}							0.9698	0.050
		60	2.37×10^{-4}							0.9632	0.055
		70	3.17×10^{-4}							0.9526	0.063
	2	50	5.45×10^{-3}				0.62			0.9951	0.020
		60	14.20×10^{-3}				0.53			0.9962	0.018
		70	61.40×10^{-3}				0.38			0.9996	0.006
	3	50	1.18×10^{-3}	1.00	2.20×10^{-6}		0.81			0.9993	0.008
		60	4.37×10^{-3}	1.00	1.69×10^{-6}		0.67			0.9982	0.012
		70	65.00×10^{-3}	1.00	-9.69×10^{-8}		0.38			0.9996	0.006
	4	50	1.89×10^{-4}	0.97						0.9707	0.049
		60	2.32×10^{-4}	0.98						0.9638	0.055
		70	3.12×10^{-4}	0.99						0.9529	0.062
	5	50	2.54×10^{-4}	0.92		7.63×10^{-2}				0.9993	0.008
		60	3.14×10^{-4}	0.92		7.50×10^{-2}				0.9973	0.015
		70	4.37×10^{-4}	0.92		7.48×10^{-2}				0.9929	0.024
	6	50						4.32×10^{3}	0.62	0.9951	0.020
		60						3.09×10^{3}	0.53	0.9962	0.018
		70						1.46×10^{3}	0.38	0.9996	0.006

*NCAD: Natural convective air dryer, FCAD: forced convective air dryer, VD: vacuum dryer. T^a: Drying temperature. ** drying rate constant

Comparing k value of natural convective air drying with forced convective air drying and vacuum drying systems, k values increased from 1.50×10^{-5} to 3.76×10^{-4} and 1.18×10^{-3} at 50 °C, respectively. Similar effect was obtained at 60 and 70 °C drying temperatures. As a result, vacuum drying provides faster drying than the natural convective and forced convective air drying systems.

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Determination of effective diffusivities of parboiled wheat during drying

The statistical data of the Fick's model used to explain the drying phenomenon that occurred in the falling rate stage of parboiled wheat were investigated and the experimental data obtained at different temperatures and different dryers were applied to the Fick's model by nonlinear regression analysis. As shown in *Table 3,* the regression coefficients (R^2) and the root mean standard error (RMSE) values ranged from 0.9534 to 0.9923 and 0.06 to 0.03. The theoretical moisture ratios were calculated using the results of the model and compared with the experimental data in *Fig. 2*. As shown in the *Fig. 2,* it was observed that the experimental data were compatible with the theoretical data.

Table 3. *Thermodynamic properties (E_a: activation energy, ΔH: enthalpy; ΔS: entropy; ΔG: Gibbs free energy) and effective moisture diffusivity of dried parboiled wheat for different drying systems*

Dryer	T^a (°C)	D_{eff} x 10^{11} ($m^2 s^{-1}$)	R^2	RMSE	E_a kJ mol^{-1}	ΔH J mol^{-1}	ΔS J $mol^{-1}K^{-1}$	ΔG J mol^{-1}
NCAD*	50	2.38 ±$0.10^{a,x}$	0.9898	0.03	41.46 ±3.24^{z}	38774.75 ±$9.21^{a,x}$	-320.49 ±$1.25^{a,x}$	142291.46 ±$22.45^{a,x}$
	60	3.97 ±$0.13^{b,x}$	0.9918	0.03		38691.61 ±$6.37^{b,x}$	-320.74 ±$1.69^{a,x}$	145497.59 ±$18.42^{b,x}$
	70	5.84 ±$2.72^{c,x}$	0.9923	0.03		38608.47 ±$7.12^{c,x}$	-320.98 ±$0.87^{a,x}$	148706.21 ±$15.67^{c,x}$
FCAD*	50	4.65 ±$1.45^{a,y}$	0.9606	0.05	27.71 ±2.68^{y}	25028.13 ±$5.25^{a,y}$	-357.98 ±$2.38^{a,x}$	140656.10 ±$9.28^{a,y}$
	60	5.52 ±$1.21^{b,y}$	0.9696	0.05		24944.99 ±$8.78^{b,y}$	-358.23 ±$1.45^{a,x}$	144237.19 ±$18.45^{b,y}$
	70	8.51 ±$1.46^{c,y}$	0.9628	0.05		24861.85 ±$5.44^{c,y}$	-358.48 ±$1.65^{a,x}$	147820.77 ±$12.27^{c,y}$
VD*	50	8.70 ±$1.28^{a,z}$	0.9693	0.05	22.28 ±1.29^{x}	19591.03 ±$2.36^{a,z}$	-369.29 ±$2.66^{a,x}$	138871.17 ±$23.56^{a,z}$
	60	10.80 ±$1.29^{b,z}$	0.9634	0.06		19507.89 ±$4.92^{b,z}$	-369.54 ±$0.98^{a,x}$	142565.32 ±$19.31^{b,z}$
	70	14.10 ±$0.35^{c,z}$	0.9534	0.06		19424.75 ±$8.98^{c,z}$	-369.79 ±$1.55^{a,x}$	146261.98 ±$32.21^{c,z}$

*NCAD: natural convective air dryer, FCAD: forced convective air dryer, VD: vacuum dryer. T^a: Drying temperature. Means in the same column with different superscript letters are significantly different, a-c (temperature), x-z (dryer), and $P \leq 0.05$. Second values are standard deviations

When we look at the calculated D_{eff} values for the parboiled wheat samples from the Fick's model in *Table 3,* we see that increase in temperature for three drying systems increased D_{eff} values. Increase of temperature from 50 to 70 °C increased in D_{eff} values from 2.38×10^{-11} to 5.84×10^{-11} (59% increase), 4.65×10^{-11} to 8.51×10^{-11} (45% increase), and 8.70 $x10^{-11}$ to 14.10×10^{-11} (38% increase) for natural air convection, forced air convection and vacuum drying systems, respectively. Mohapatra and Rao (2005) reported that the effective diffusion coefficient of parboiled wheat during drying at a temperature range of 40-60 °C varies between 1.20×10^{-10} and 2.90×10^{-10} m^2s^{-1}. The D_{eff} values lie within in general range of 10^{-11}–10^{-9} m^2 s^{-1} for food materials (Rizvi, 1986).

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These results show that the Fick model can represent drying behavior of parboiled wheat in all dryers at all drying temperatures. When the drying kinetics of different foods such as sour yeast produced with Amaranth flour (Rozylo et al., 2014), popcorn (Doymaz and Pala, 2003), saffron (Acar et al., 2015) and Izmir Crispy snack are examined (Turkut et al., 2015), it has been seen that the Fick's diffusion model is a suitable model.

Compared to NCAD, FCAD and vacuum drying systems at the same temperatures, diffusion coefficients increased. This can be explained by easy evaporation of the moisture in the product at higher temperatures and an increase in drying rate in different dryers. The higher the diffusion coefficient, the higher the mass transfer. Salehi et al. (2017) and Walde et al. (2006) reported that D_{eff} was higher in vacuum drying compared to forced convection in the drying of the mushroom.

The temperature effect was the highest in natural air convection, and the least in vacuum drying. This shows that the effect of vacuum is greater than temperature effect due to more mass transfer of water in vacuum system. Similarly, compared to natural air convection, the movement of hot air by forced air convection drying reduced the temperature effect. In other words, mass transfer is affected by heat transfer. Mass transfer (moisture) in the vacuum dryer is less affected by temperature. Also, at the constant temperatures, D_{eff} values of the forced air convection dryer were higher than that of the natural air convection, while that of the vacuum drying was faster than both the forced air convection and the natural air convection dryers (*Table 3*). The increase in D_{eff} value indicates that drying is faster. The increase in drying speed means that it will dry up in a shorter time. As seen in *Fig. 2,* both the temperature and the type of dryer affected the drying rate. As a result, vacuum drying provides faster drying than the other two drying systems.

Thermodynamic properties

Thermodynamically, the activation energy is expressed as water molecules passing through the energy barrier when moisture transfers from the inside to the outside of the product. The lower values of the activation energy give higher values of moisture diffusion in the drying process. The reduction in the activation energy of a process is due to the increase in the average energy of the water molecules (Devahastin, 2000). Activation energy can be interpreted as the energy barrier that must be overcome in order to activate moisture diffusion (Babalis and Belessiotis, 2004).

The temperature dependence of D_{eff} can be described by an equation of the Arrhenius type as given in *Equation 7*. The moisture diffusivity was plotted against reciprocal of absolute temperature in *Fig. 3*. The slope of the curve gives the E_a/R, while the intercept gives the D_{ref} value. The R^2 values, which indicate regression compliance, were found between 0.9329 and 0.9961. The linear regressed equations from the Arrhenius approach for NCAD, FCAD and vacuum dryer were found as to be $\ln(D_{eff}) = -9.01 - \frac{4986.76}{T}$, $\ln(D_{eff}) = -13.52 - \frac{3333.36}{T}$ and $\ln(D_{eff}) = -14.88 - \frac{2679.39}{T}$, respectively.

The activation energy values for natural convective air, forced convective air and vacuum drying were found to be 41.46, 27.71 and 22.28 kJ mol^{-1}, respectively. These values are close to the value of the dried boiled wheat (37.013 kJ mol^{-1}) found by Mohapatra and Rao (2005). In addition, the activation energy values we found were found to be less than the value found in the drying of raw wheat (51.00 kJ mol^{-1})

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(Becker, 1959). Turkut et al. (2015) also found the activation energy of Izmir Crispy snack to be 32.78 kJ mol^{-1}. In another study (Ah-Hen et al., 2013), the activation energy values of blueberry was found to be 59.27 and 34.30 kJ mol^{-1} for natural air convection and vacuum drying, respectively. The smaller the activation energy is, the more easily a particular process occurs, in other words, the lower the energy that is required for the physical processing. Thus, vacuum drying of parboiled wheat showed the lower activation energy that indicates less temperature effect. However, it can be concluded that the drying rate is faster than other dryers.

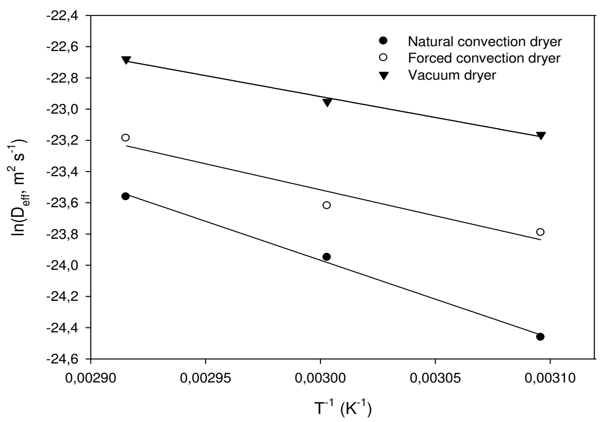

Figure 3. *Arrhenius plot of Fick's law model of D_{eff}, of parboiled wheat over the drying temperature range of 50-70 °C*

Table 3 shows the values that were obtained from the thermodynamic properties relating to the drying process of parboiled wheat. The differential enthalpy decreased significantly ($P \leq 0.05$) with increasing temperature for natural convective air dryer, forced convective air dryer and vacuum dryer. The differential enthalpy values were positive as this is a process with heat absorption, that is, endothermic (Shafaei et al., 2016). Similar results are reported in previous studies (Jideani and Mpotokwana, 2009; Correa et al., 2016; Shafaei et al., 2016). Lower values of enthalpy difference indicate that a lower energy is required for the process to occur. Differential entropy is a thermodynamic quantity that is associated with the degree of disorder, as it is a state function where the values increase during a natural process in an isolated system. Analyzing the behavior of the entropy, it is concluded that this thermodynamic property showed similar behavior to the enthalpy in which the values decreased with increasing temperature (*Table 3*). This trend indicates that there is an increase in the system order, as it is entropically unfavorable (Jideani and Mpotokwana, 2009). This fact can be explained by the theory of activated complex in which a substance in an activation condition may acquire negative entropy if the degrees of freedom of translation or rotation are lost during the formation of the activated complex (Dannenberg and Kessler, 1988).

The Gibbs free energy increased with increasing temperature, and their values were positive, indicating that the drying in this research conditions were not spontaneous. The positive value of the Gibbs free energy is characteristic of an endergonic reaction that requires the addition of energy from the environment in which the product is involved for the reaction to occur (Reusch, 2007). The similar results were reported previously by Jideani and Mpotokwana (2009), Correa et al. (2016) and Shafaei et al. (2016).

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General model as a function of drying time and temperature

When the equations found from the regression of Arrhenius relationship (*Eq. 7*) in *Table 3* were combined with *Equation 6* for three dryers, time and temperature dependence of moisture ratio, the following general models were derived to describe the drying kinetics of parboiled wheat:

Natural convective air dryer (*Eq. 12*),

$$MR = \frac{6}{\pi^2}\exp\left(-\frac{\pi^2}{R_e^2}1.22x10^{-4}\exp(\frac{-4986.76}{T})t\right) \quad \text{(Eq.12)}$$

Forced convective air dryer (*Eq. 13*),

$$MR = \frac{6}{\pi^2}\exp\left(-\frac{\pi^2}{R_e^2}1.34x10^{-6}\exp(\frac{-3333.36}{T})t\right) \quad \text{(Eq.13)}$$

Vacuum dryer (*Eq. 14*),

$$MR = \frac{6}{\pi^2}\exp\left(-\frac{\pi^2}{R_e^2}3.45x10^{-7}\exp(\frac{-2679.39}{T})t\right) \quad \text{(Eq.14)}$$

Equations 12-14 can be used to find the moisture ratio of parboiled wheat during drying at any time (seconds) and temperature (K) providing that R_e is known. Also, when the moisture ratio and drying temperature of parboiled wheat are known, the drying time can be found for the dryers. Using these equations can be beneficial to each dryer during drying processing of bulgur.

Conclusions

The changes of moisture ratio have been described by six semi-theoretical and Fick's second diffusion models. The model yielded the best description. The effective moisture diffusivity was significantly ($P \leq 0.05$) increased when forced air convection and vacuum drying was applied, compared with natural air convection drying. When the temperature of drying changed from 50 to 70 °C, 59, 45 and 38% changes in D_{eff} values were found for natural convective air, forced convective air and vacuum drying systems, respectively. The effective moisture diffusivity during drying of parboiled wheat by natural air convection, forced air convection and vacuum dryers varied from 2.38×10^{-11} to 14.10×10^{-11} m^2 s^{-1}, in the range from 10^{-11} to 10^{-9} m^2 s^{-1} reported for other food stuffs. Drying temperature, forced convective air and vacuum drying systems have greatly increased the drying rates. Results confirmed that the dehydration rates of the vacuum dried parboiled wheat were higher than those of the forced convective and natural convective air dried ones. The activation energy values for natural convective air, forced convective air and vacuum drying systems were found to be 41.46, 27.71 and 22.28 kJ mol^{-1}, respectively. In the processes of parboiled wheat drying, both differential enthalpy and entropy decreased with increasing temperature. The

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differential enthalpy values are positive, indicating endothermic drying process. The Gibbs free energy increased with increasing temperature and with positive values, indicating that drying does not occur spontaneously in the working conditions; thus, this process requires the addition of energy from an external source. In this study, vacuum drying of parboiled wheat showed the lower activation energy, higher effective diffusivity and rate constant (k) that indicates less temperature effect while the drying rate is faster than other dryers. New equations related to the moisture ratio at any time and temperatures for different dryers were derived. Using these equations can be beneficial to each dryer during drying processing of bulgur. Further investigation about the effect of natural convective air dryer, forced convective air dryer and vacuum dryer on quality characteristics of parboiled wheat is needed during bulgur production.

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CHARACTERISTICS AND LABORATORY FREEZE–THAW TEST OF SALINE SOIL IN WEST JILIN, CHINA

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Abstract. For the purpose of investigating the characteristic and material composition of saline soil in western Jilin, China the saline soil distribution area of Da'an in west Jilin Province was sampled during a site investigation, after which the fundamental properties of the soil were investigated in the laboratory. The soluble salt ranged from 0.28% to 0.60%, decreasing with depth from 0 to 40 cm blow the surface. Conversely, the moisture content increased with increasing depth, but showed little change beneath 40 cm. An indoor freeze–thaw simulation test revealed that the moisture migrated under the temperature gradient, while the salinity migrated upwards under the combined action of the temperature gradient and concentration gradient, with the optimum degree of compaction for salinity migration being 90%. Following freezing, the inside structure became looser, and this phenomenon was more obvious when the temperature was lower. The research provided the basis for prevention of the secondary salinization and frost heaving the disease of saline soil in western Jilin province.
Keywords: *saline soil, freeze-thaw test, water migration, salt migration, microstructure*

Introduction

Saline soil is a kind of special soil whose salt content is more than 0.3% (Chai, 1983). Saline soil is widely distributed on the Earth's surface, resulting in expansion, dissolution or corrosion characteristics that can cause serious damage to agricultural and architectural environment (Wang et al., 2011; Hejazi et al., 2017). Some damage to roads in saline soil areas are related to the particular characteristics of saline soil. The formation of saline soil occurs in response to evaporation caused by underground water migration and the hot-dry climate (Kovda, 1958; Blaser and Schere, 1973; Kovda and Szabolcs, 1979). Upward migration of salinity with water migration is the main form of salinity accumulation in soil (Lv et al., 1999). Moreover, changes in temperature affect the migration of salinity in saline soil (Bear and Gilma, 1995), with saline soil being distributed in cold regions, especially those subjected to seasonal freezing, showing salt expansion damage due to

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variations in water, salinity, temperature and stress (Deng and Zhou, 2009; Zhang et al. 2010). Because salt in the soil has a different impact on the freezing point (Ma et al., 2016; Bing et al., 2016), the experiment of salt centent and freeze-thaw characteristic is necessary.

Western Jilin Province is a typical seasonal frozen soil distribution area of northeast China that has large amounts of saline soil. This region has severe cold winters and torrid summers, with low annual precipitation. The rainy season is from June to August, while spring and autumn are dry and windy. Due to the strong evaporation effect of the earth's surface, salinity in soil easily gathers on the surface and forms saline soil (Zhang, 2010; Hashemi, 2017). Furthermore, water migration during the freezing process in winter brings salinity to the freezing front, and salinity again accumulates during spring due to surface evaporation, aggravating the degree of soil salinity. Soil salinization not only brings serious harm to agricultural production, but also leads to damage to engineering projects such as frost heave and differential settlement of highways. Therefore, it is essential to investigate the characteristics of saline soil in western Jilin (Radan et al., 2017).

In this study, soil samples were collected from Da'an in Jilin Province and subjected to a series of laboratory and freeze–thaw simulation tests to investigate their properties. Scanning electron microscopy (SEM) was also used to examine the microstructure of the different samples to evaluate the effects of the freezing process.

Materials and Methods

Soil materials

Soil samples were collected from the Da'an area of Jilin Province during spring. A total of six samples were collected, one each from depths of 20cm, 30cm, 40cm, 50cm, 70cm and 100cm. Soil samples were digged from the nature soil layers below the ground. The surface of the field was dry and gray-white because of the massive crystals of soluble salt, while the underground soils were wet and black-brown or gray.

Soils were analyzed (Wang et al., 2013; Talitha et al., 2013) for particle composition, moisture content and salinity content (*Table 1*).

Table 1. *Basic characteristics of soil samples*

Depth of soil samples/ cm	Density/ g/cm^3	Particles mass fraction /%			Moisture content/ %	Liquid limit moisture content/%	Plastic limit moisture content/%	Ip	Soluble salt content /%
		Sand 2-0.075mm	Silt 0.075-0.005mm	Clay <0.005					
20	1.69	9.6	59.2	31.2	12.51	32.00	21.40	10.60	0.6
30	1.79	5.5	52.8	41.7	19.79	35.10	20.00	15.10	0.55
40	1.66	9.5	53.6	36.9	20.68	36.00	22.10	13.90	0.49
50	1.79	4.6	50.6	44.8	20.55	35.80	20.40	15.40	0.35
70	1.81	4.1	52.0	43.9	20.77	34.50	21.60	12.90	0.28
100	1.82	4.6	52.7	42.7	18.14	34.10	21.00	13.10	0.29

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Additionally, the mineral composition of samples from 20 cm and 100 cm were analyzed by X-ray diffraction (XRD) (*Table 2*). The results revealed that the clay content of the soil samples ranged from 31.2% to 44.8%. The natural moisture content of samples collected from 0 to 40 cm increased with depth, but little change in moisture was observed below 40 cm. The soluble salt content decreased with increasing depth. Based on a 0.3% soluble salt content indicating saline soils, the soil was saline soil at above 50 cm in the research area. The soil was primary mineral based, and included quartz, alkali-feldspar and plagioclase (Vazdani et al., 2017).

Table 2. *Mineral composition of soil samples*

Depth of soil samples/cm	Percentage composition of minerals /%							
	fs	Q	Pl	Cc	I/S	K	I	Am
20	20	39	21	6	6	4	4	
100	11	41	21	11	7	4	4	1

fs: alkali feldspar, Q: quartz, Pl: plagioclase, Cc: calcite, I/S: illite-smectite mixed layer, K: kaolinite, I: illite, Am: hornblende

Methods

A temperature-controlled capillary water tester was designed for this study to simulate freeze–thaw conditions in the field. The equipment was composed of three temperature-controlled, compartments that could be controlled separately. Each compartment had a capacity of 50cm of soil, and there was a hole between the adjacent compartments so the soil samples could be compacted into a tube stand in the device that ran through the three compartments. The photo and diagram of the tester are shown in *Fig. 1.* and *Fig. 2*.

Figure 1. *Temperature-controlled capillary water tester*

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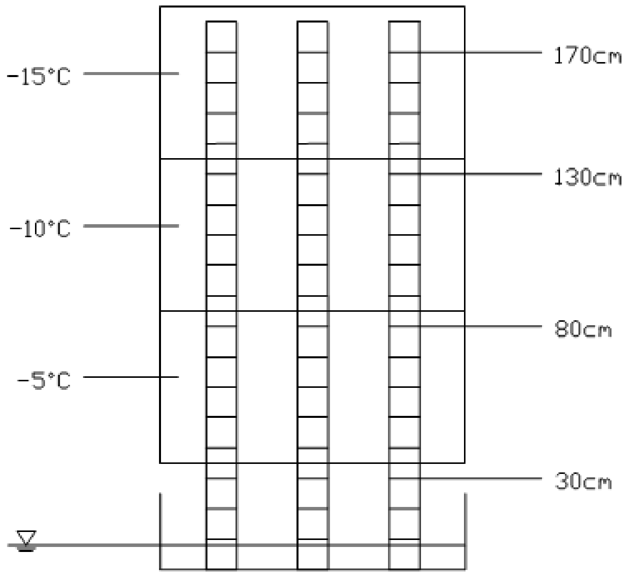

Figure 2. *Diagram of the temperature-controlled capillary water tester*

After field monitoring of the winter ground temperature in western Jilin, the temperature of the three compartments was set as −5°C, −10°C, and −15°C. Soil collected from 20 to 30 cm was selected for the freeze–thaw simulation test. Briefly, the initial moisture content was adjusted to 18% to simulate the native state, after which the soil was compacted into three 180 cm tubes with a compaction degree of 85%, 90% and 95%. The bottom 30 cm of the tubes were then placed in a water channel under room temperature.

During the first 2 days, the temperature was gradually reduced in the compartments until the pretest temperature was reached. The soil beneath the compartments was constantly maintained at an indoor temperature (approximately 18°C). Normally winter lasts 4 months in study area, and the soil is frozen for 3 months in nature. Therefore, the freezing process lasted for 3 months, after which the soil was immediately sampled before thawing. Soil samples were collected from the tubes in 10 cm intervals, after which the moisture content was immediately measured. In addition, other soil samples were collected from each room and analyzed for salt content and microstructure.

Results and Discussion

Change in moisture after freezing

The moisture contents at different heights after freezing are shown in *Fig. 3*. There was little change in moisture observed with increasing height for all compactions, indicating little variation in the initial moisture content. However, the moisture contents increased slightly near the temperature dividing line (Xiao et al., 2017). As a result, a portion of the observed water migration was in response to the temperature gradient. This was mainly due to the temperature gradient causing film water to migrate in the soil, while capillary water did not migrate. Due to the temperature gradient, the original soil water potential balance was broken at a different position, resulting in migration of the water from unfrozen soil into frozen soil. This led to a redistribution of the moisture

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distribution. In this experiment, the moisture contents of unfrozen soil were lower than the initial content at 150 cm, but the moisture contents of frozen soil were higher than the initial content, and the water migrated slightly at that position. Moreover, different degrees of water migration were observed at 50 cm and 100 cm due to the temperature gradient. However, the film water of soil in the same temperature-controlled compartment did not migrate because the temperatures were the same.

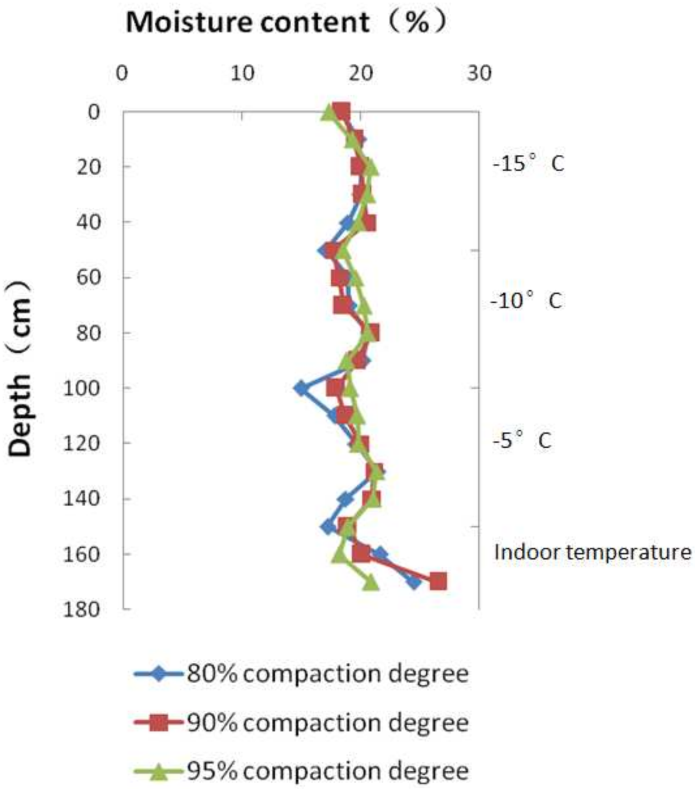

***Figure 3.** Relationship between moisture content and height*

Change in salinity after freezing

The soluble salt contents of soil samples after freezing were tested by an oven drying method, and the variations in soluble salt contents with depth are shown in *Fig. 4*. The variations in salt content were similar between soil samples at compaction degrees of 85% and 90%, with an increase occurring from 50 to 150 cm, but little change from 10 to 50 cm. The variation in salt content at 90% compaction differed, showing an increase, followed by a decrease and then another increase with depth. At this level of compaction, the changes in salt content occurred around 50 cm and 100 cm, while the salinity migrated between 50 cm and 100 cm. These findings indicate that the degree of soil compaction influenced the migration of salt with water, with the salt carried by water in the soil migrating effectively under high compaction, but being restricted above a threshold. In this study, 90% was found to be the optimum degree of compaction.

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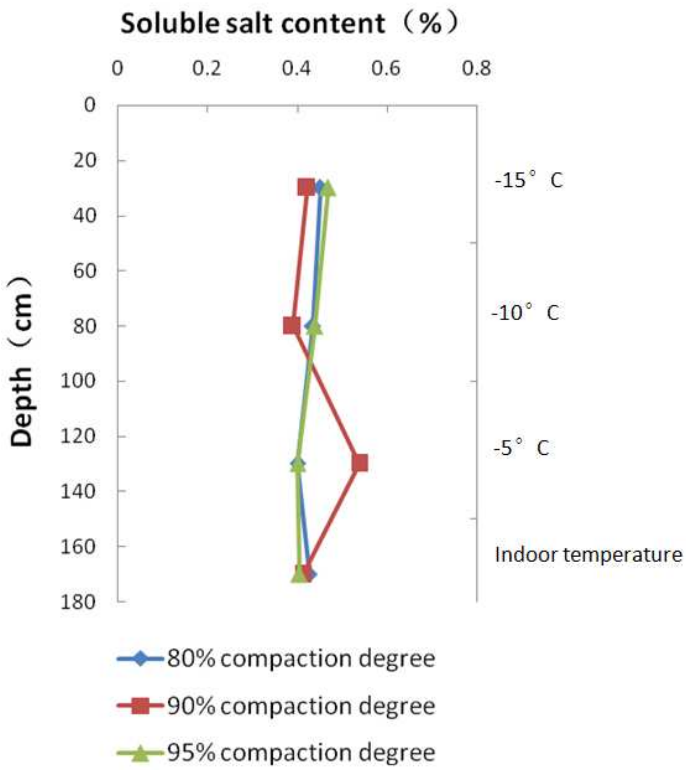

***Figure 4.** Relation curves of soluble salt content and height after freezing*

The soluble salt content in the native state is shown in *Fig. 5*. The salt content obviously increased with depth, especially at 20–50 cm. The salt migration trend was similar to that of the laboratory test, but more obvious. This is because salt migrates into soil via the surface over a long period when soil is in its native state.

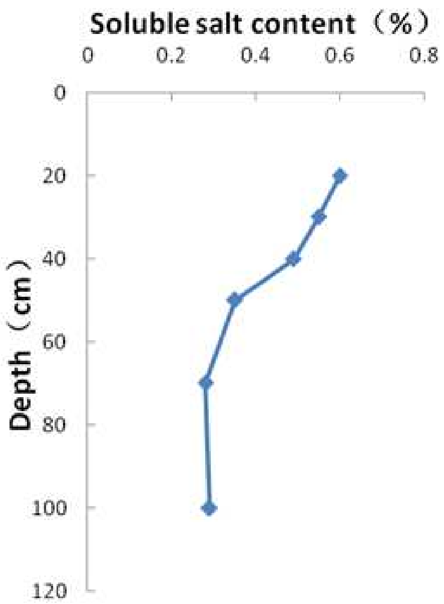

***Figure 5.** Relation curves of soluble salt content and height at native state*

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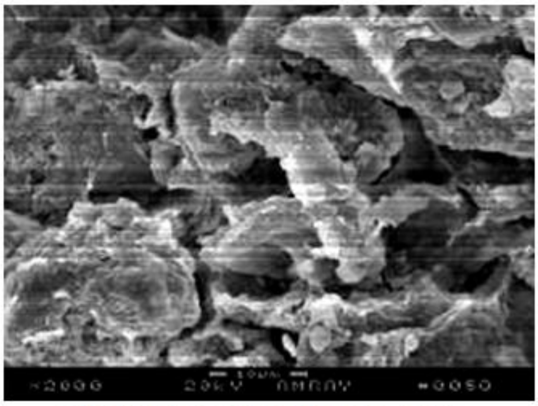
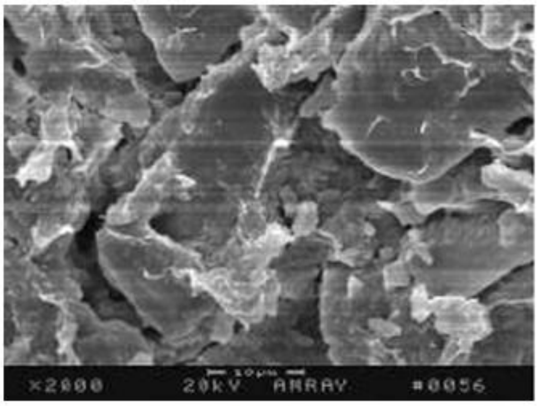
Changes in microstructure after freezing

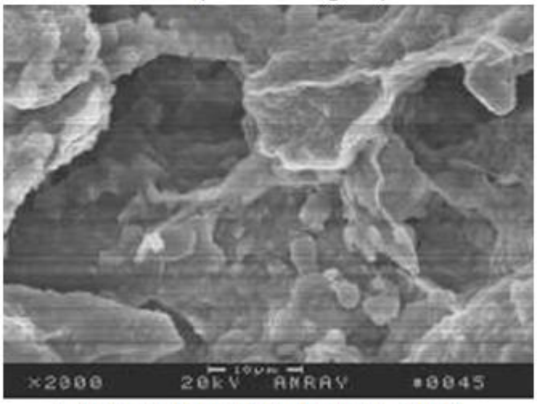
SEM was used to investigate the soil microstructure before and after freezing. During the test, liquid nitrogen was used to freeze the soil samples quickly to ensure that the water did not turn into amorphous ice and break the original microstructure. The ice in the soil was then sublimated in a low temperature vacuum apparatus and pumped out with a vacuum pump to ensure that the pores in the soil did not become deformed. *Fig. 6* shows the SEM images of soil samples with an 85%, 90% and 95% compaction degree at different freezing temperatures, as well as the soil sample with a 95% compaction degree and normal temperature.



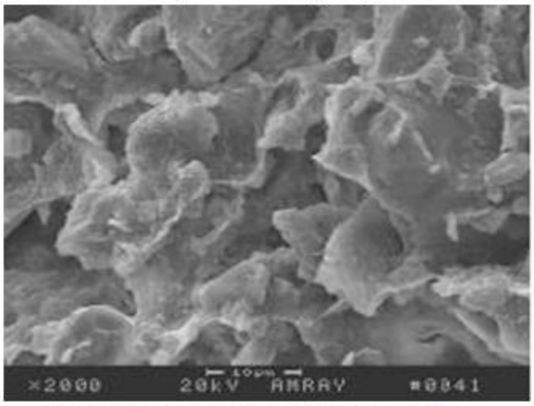
a. 85% compaction degree, -5 °C



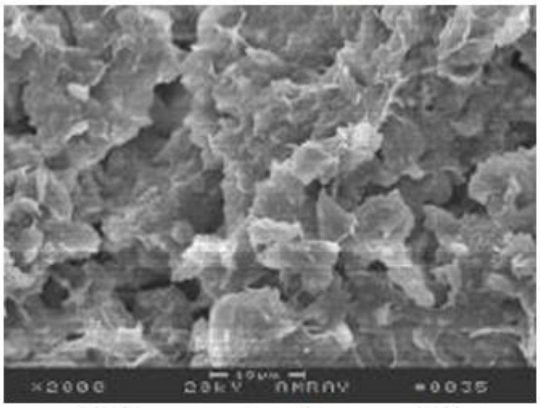
b. 85% compaction degree, -10°C



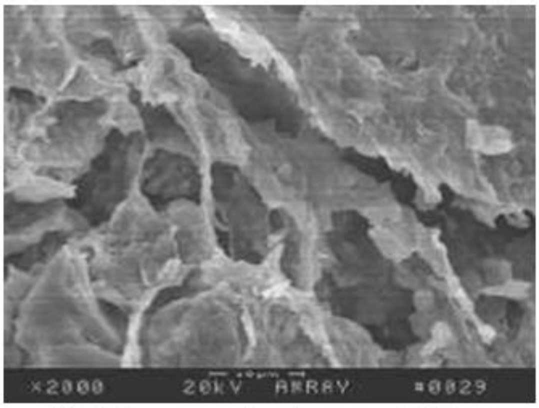
c. 85% compaction degree, -15°C



d. 90% compaction degree, -5°C



e. 90% compaction degree, -10°C



f. 90% compaction degree, -15°C

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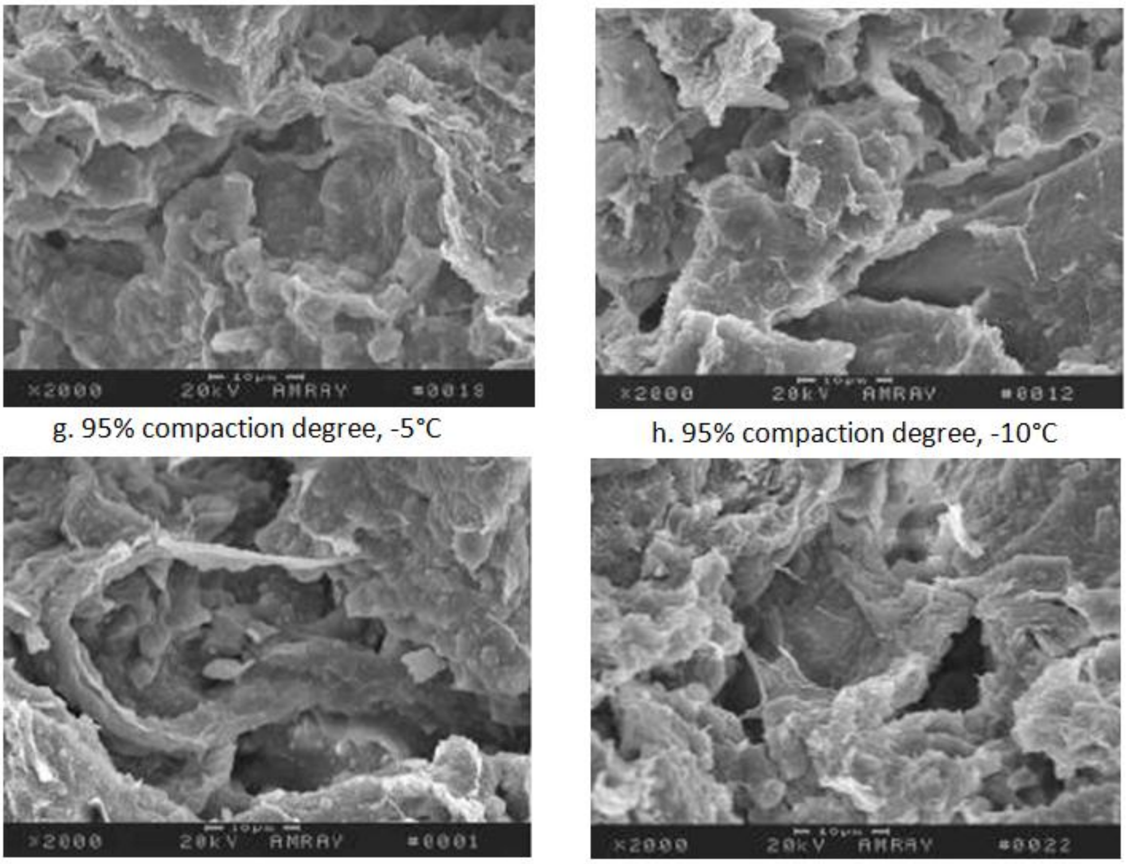

g. 95% compaction degree, -5°C

h. 95% compaction degree, -10°C

i. 95% compaction degree, -15°C

j. 95% compaction degree, normal temperature

Figure 6. *SEM micrograph of soil samples indifferent freezing temperatures*

As shown in *Fig. 6.*, soil particles had a flocculence granular structure, with clay minerals primarily composed of illite and an illite-smectite mixed layer. In addition, there was a high volume of xenomorphic minerals with a poor degree of crystallinity. As a result, the clay contents and xenomorphic analysis indicate different regulation of the mineral contents (*Table 1* and *Table 2*). The structures are primarily formed via cementation inosculation, the structure is loose and cracked, and salinity crystals are present in the soil pores.

Comparison of the SEM images at different temperatures revealed that changes in temperature not only affect the size of soil structural units and pores, but also influence the arrangement of soil structural units and pores (Zhang et al. 2017). Lower freezing temperatures are associated with looser soil structural units, while higher pore volume is associated with more obvious formation of seepage channels. This phenomenon is due to lowering of the freezing temperature (Wan et al, 2013; Yang et al., 2017), which results in water in the soil continually turning to ice, after which the ice crystals break the original structure and enlarge the pore volume. Moreover, the unfrozen water content decreases, and saline ions increase in solution. When the solution concentration exceeds the solubility of sodium bicarbonate, sodium bicarbonate crystal precipitates. As the temperature decreases, ice and salt crystals increase continually, causing the soil particles to be stretched and squeezed. This causes the distance between soil particles to

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increase and pore volumes to expand, leading to the soil structure becoming looser, large particles breaking into small particles, and the large soil particle content decreasing.

Comparison of the SEM images at different compaction degrees reveals that the particle content decreased most obviously at 90% compaction as the temperature decreased. As the compaction degree increased, the deformation of soil particles and pores were depressed to a certain extent. Additionally, increasing ice crystals and salt crystals can enlarge the pore diameters when the soil sample is loose; however, if the soil sample becomes excessively loose, the increasing ice crystals and salt crystals primarily fill the original pores. As the SEM results show, the deformation of the soil was greater at 90% compaction.

Conclusion

Investigation of saline soils by an indoor freezing–thaw simulation test revealed the following:

1. The soil in the research area is low liquid-limit clay. The moisture content of this soil decreases with increasing depth due to evaporation in shallow earth, but does not change greatly deeper down. Soil more than 50 cm underground is saline, which can cause damage to industry and agriculture.
2. The moisture and salt all shows migration during freezing progress. The water content only increased slightly with depth near the temperature dividing line. This phenomenon was caused by film water migration, not capillary movement of water. The salinity migrates upward with film water under the combined action of the temperature gradient and concentration gradient. This is one of the main reasons for the high soil salinity in western Jilin.
3. Salt migration is more obvious when the degree of compaction is larger; however, if soil is compacted too much, it will cause the soil particles to be too close together, limiting the migration of water and salt. The optimum degree of compaction for salt migration was 90% in the research area.
4. Due to the limitations of experiment, this paper only discussed the soil collected in spring, the following research will take more experiments of summer and autumn soil and investigate more characteristic of saline soil in western Jilin.

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USING STRUCTURAL EQUATION MODELING APPROACH TO INVESTIGATE FARMERS' PERCEPTION CONSEQUENCES OF DROUGHT (CASE STUDY: BIRJAND TOWNSHIP, IRAN)

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Abstract. As a natural disaster and inevitable phenomenon, droughts are frequently seen in vast areas of different countries, particularly in areas with hot and dry climates. It has resulted in heavy economic, social, and environmental losses. Undoubtedly, the first step to cope with droughts and their consequences is to have an accurate understanding of droughts and their effects on different aspects by specifying the related strategies and approaches in this regard. Given the importance of this issue, the present study was conducted to investigate how to model farmers' perception of consequences of drought through structural equation modeling in Birjand Township, Iran. All farmer households living in rural areas of Birjand (a city in the Iranian province of Southern Khorasan) composed the statistical population of this study (N=6057). As many as 200 households were chosen for the study by using Bartlett's table. In total, 20 villages were considered as the target districts using stratified random sampling. The results showed that the most important consequences of droughts understood by the farmers are their economic (0.341), environmental (0.261), and social (0.138) consequences. The highest indirect effects on the farmers' perception of consequences of drought in Birjand were: reduced income (0.286), increased bank debts (0.272), reduced purchasing power (0.265), and the wells, aqueducts and springs that have dried (0.224) respectively. In this regard, measures such as supporting the investment and entrepreneurship, granting banking facilities and credits, governmental oversight on water withdrawals from groundwater sources, development of infrastructures and irrigation and water supply networks, reuse of wastewater, identification and promotion of products compatible with saline and unconventional waters, development of support services and social security for rural communities and development and promotion of drought insurance are proposed in order to reduce the consequences of drought.
Keywords: *drought, consequences of drought, structural equation modeling, farmers' perception, Birjand Township*

Introduction

Most recently, the phenomenon of drought, its intensity, frequency and duration have drawn many concerns around the world (Sivakumar et al, 2014; Peterson et al., 2013). This phenomenon is constantly paid attention rather than a natural phenomenon due to reasons like a constantly growing population, its intangible and long-term impacts, and the duration and severity of damages (Rezaei et al., 2010). Droughts are natural hazards threatening human life and the natural ecosystems (Jamshidi, 2014). Droughts lead to dwindling surface and underground water sources and thereby lead to multiple negative effects in all aspects of rural life–especially in economic and agricultural structures.

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Droughts and the resultant shortage of water leading to declining agricultural production have become major global concerns these days (Liu et al., 2008). Although this crisis is not considered as a new phenomenon for farmers, the complexity of the contributing factors and the interweaving of the negative impact of droughts make these a main concern of farmers living in areas with critical situations (Campbell et al., 2011). In addition, frequent droughts have made rural communities more vulnerable (Speranza et al., 2008) in such a way that many rural communities try to survive in the face of a drought (Campbell et al., 2011). Iran has experienced the problem of water shortage and droughts in the past two decades, with its regions and provinces being more or less affected. According to a report of the National Drought Warning and Monitoring Center, the Southern Khorasan ranks third when it comes to facing the onslaught of droughts (National Drought Warning and Monitoring Center, 2016). Owing to recent droughts, 905 villages in this province have been suffering from scarcity of water. The extent of damages to the agricultural sector due to such droughts was over $375m during the years 2014–2015 (Agricultural Jihad Organization of Southern Khorasan, 2016). These are just a few instances of damage and consequences of drought in Southern Khorasan. The comprehensive identification of the effects of drought can play a very effective role in the formulation of policies and strategies to deal with such a natural disaster. Besides, without any knowledge of farmers' attitude to drought, the probability of the success of formulated plans for drought management and dealing with its consequences will be very poor. Therefore, the main question of this study is: What are farmers' perspectives on consequences of drought in Birjand Township?

Review of the literature

Droughts imply deviations from normal or medium conditions of precipitation; they are seen when the precipitation is less than 75% of the average precipitation in a region over a period (usually 25–30 years) (Wilhite, 1993). In general, a drought could be defined as a situation of shortage of rainfall and increased temperature that may occur at any climatic conditions (Alizadeh, 2002). This phenomenon disturbs vital water systems and thus creates high risks for the region (Kaviani and Alijani, 1999); it is one of the most persistent and pernicious economic disasters that is believed to stem from a complex mechanism, and its nature is less well-known than other natural disasters (Javanmard et al., 2000). This phenomenon forms a group of effects that occur in various aspects and are visible gradually over a long period following the occurrence. Diverse perspectives are discussed in this regard–some of these classify the effects of drought into direct and indirect or primary and secondary categories (Wilhite, 1993; Kardavani, 2001). Direct effects of drought are often associated with climate and ecological characteristics, while the indirect effects are subtler and related to economic and social damages that can be barely distinguished due to their nature and features (Wilhite, 1996).

Droughts are counted among major Iranian climate characteristics, and these can be seen in both wet and dry conditions. The characteristics of a drought in Iran show that any region of the country has suffered this event and its destructive effects (Nasaji Zavareh, 2001). Undoubtedly, drought involves a mental picture of barren lands, destroyed crops, and survival efforts for most people. Therefore, a change in the weather pattern is expected, along with a disruption of daily activities, in the event of a drought. The effects of drought can be divided into economic, social, and environmental

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impacts (Eskandari and Mosayebi, 2008). Reduction of the area for cultivation of crops, reduction of the fertility of pastures and forests, increases in fire incidents, dwindling underground water levels, increasing mortality rate of livestock, and increasing damage to wildlife and habitats of fish are argued to be some of the direct effects of this phenomenon. The decline in production and area under cultivation of crops could reduce the revenue of farmers and workers in this sector, raise food prices, increase unemployment, decrease tax revenues, and increase crimes and lawsuits relating to legal problems due to delay in repayment of bank loans and facilities are also known as indirect effects of drought (Saleh and Mokhtari, 2007; Jamshidi, 2014). Several studies have been conducted on droughts and their effects on human communities in domestic and foreign territories of Iran. Some of these works are discussed here.

Nasaji Zavareh (2001) recognized the most important economic consequences of drought in the form of declining income for farmers and workers, increasing input prices, rising unemployment and immigration, decreasing agricultural land values, and increasing food prices. Mohammadi Yeganeh and Hakimdoust (2009) concluded that droughts in multi-year time scales negatively impacted agricultural and rural economy, altered the function of rural land, and reduced the income and employment opportunities for villagers. Moreover, Alimoradi (2009) argued that the drought of Ilam Province had increased from the southwestern part to the northeastern in 2007–2008 and had resulted in reduction of the discharge capacity of rivers and underground water level of alluvial aquifers. Beikmohammadi et al., (2005) showed that Sistan had experienced a profoundly negative impact on the rural economy in both agriculture and animal husbandry sections because of the drought of 1998–2004. Furthermore, Sharafi and Zarafshani (2011), in a comparative study, elucidated that wheat farmers in Rawansar Township were the most vulnerable against droughts, while wheat farmers in Kermanshah Township dealt with the lowest socio-economic vulnerability.

Holden and Shiferaw (2004) indicated that the indirect effect of drought on family welfare through its effects on prices of livestock and crops was greater than those of direct impact. Sivakumar et al. (2005) believed that agricultural productivity in Asian tropical regions not only increased the temperature, but also was sensitive to the altered nature and characteristics of monsoon showers. The simulation of climate change showed that the changes increased the frequency of droughts, the greatest risk for farmers.

Kenny (2008) considered physical and mental stress, anxiety, depression, family conflicts, reduced quality of life, increased immigration, and increasing poverty as the social consequences of droughts. Gentle and Maraseni (2012) suggested that climate changes could influence such components as livelihoods, food security, and increasing poverty and social inequality in Nepal. Antwi-Agyei et al. (2012) found that climate change and droughts in Ghana had the strongest effect on the agriculture section in terms of the vulnerability of crops. Ayinde et al. (2011) studied the effect of climate change on agricultural productivity in Nigeria; they revealed that changes in climate, particularly in the pattern of rainfall, had a significant impact on the agricultural productivity. Lilleor and van den Broeck (2011) believed that environmental changes, along with economic and social factors, provided the conditions for immigration. Baba et al. (2011) demonstrated large-scale immigration of the global population and thereby portrayed political and economic crises, poverty, and starvation caused by the reduction in of agricultural land resources, the intense decline in biodiversity due to the incompatibility of some species to adapt to new climatic conditions, and increased morbidity and mortality as results of climate change. Schilling et al. (2012) had shown

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that the Moroccan economy and poor people were strongly dependent on the agricultural sector, while climate change and droughts probably had the strongest effect on this sector.

Materials and methods

Introduction of the studied area

Birjand Township is the capital of Southern Khorasan province, which is the third biggest province of Iran in terms of area (150800 km^2, equal to 9.15% of the entire area of the country) and is located in 59° 13' E and 32° 53' N. (*Fig. 1*). Since it is situated on a dry and semidry climatic belt, far from sea, adjacent to desert regions, and exposed to the 120-day peripheral winds of Sistan, this township has always been subject to drought (Khosravi and Akbari, 2009).

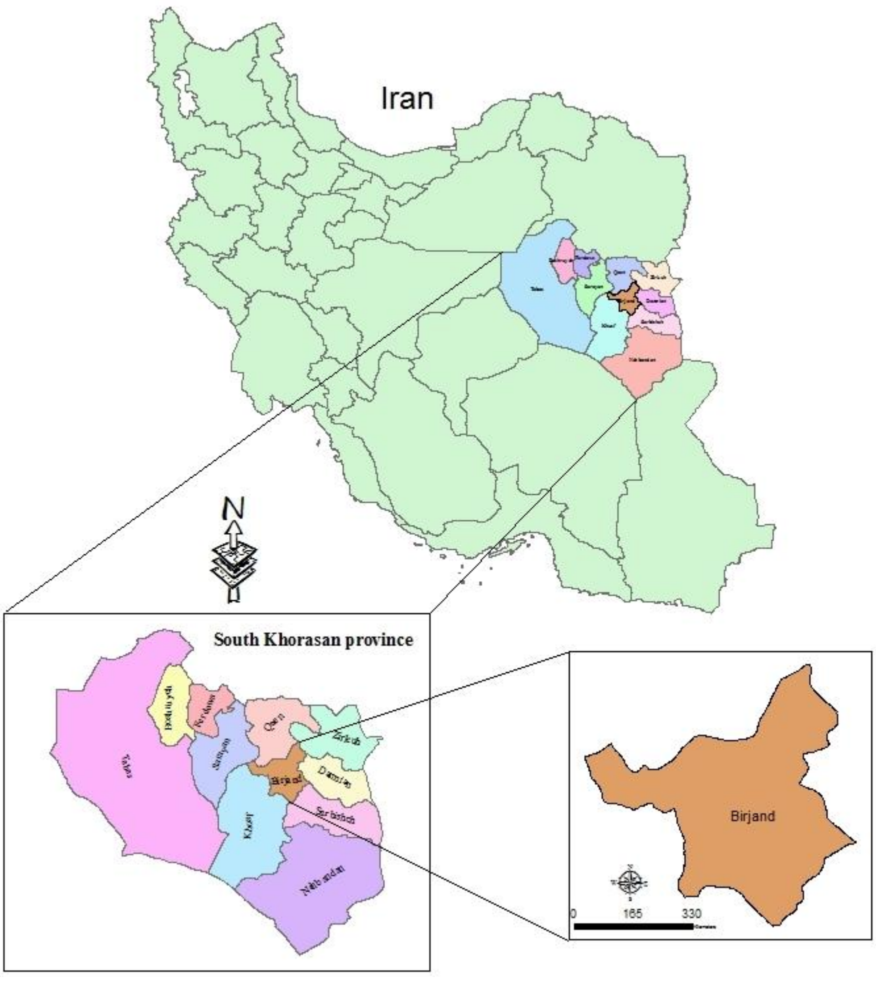

Figure 1. *The location of study area in Birjand, Southern Khorasan province, Iran*

The average precipitation of the Birjand Township is equal to 151.6 mm, i.e. around half of the average precipitation of the country and one-fifth of the global average. Ombrothermic diagram, designing monthly mean of temperatures and precipitation

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data, as well as time series analysis of changes in precipitation within a 30 year period indicates a descending trend with a relatively sharp slope, clearly indicating the critical status of precipitation and provision of water resources of the Birjand Township throughout this period (*Fig. 2, 3*). This has caused incidence of frequent droughts across the township: in a 30 year period, it has experienced 15 years of drought (National Drought Warning and Monitoring Center, 2016).

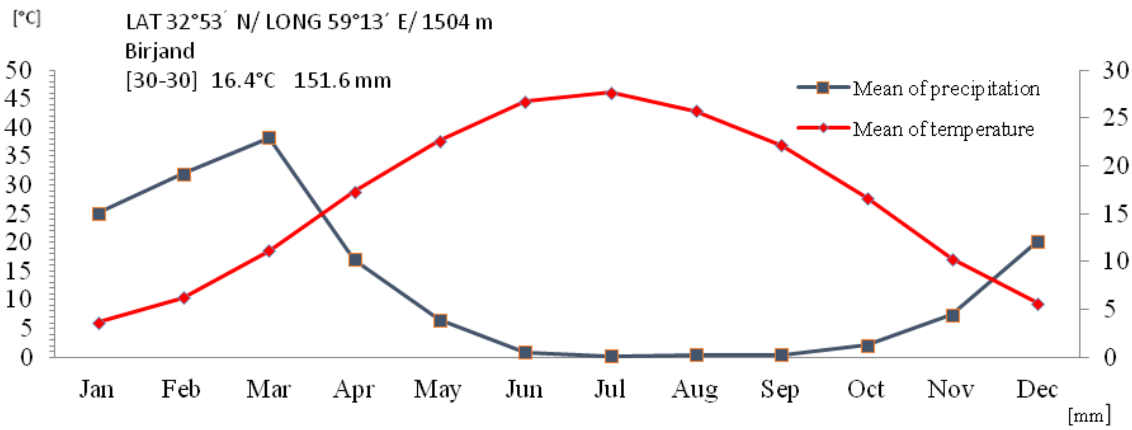

Figure 2. *Ombrothermic diagram from monthly mean temperatures and precipitation data collected by the Birjand meteorological station (1987- 2016).*

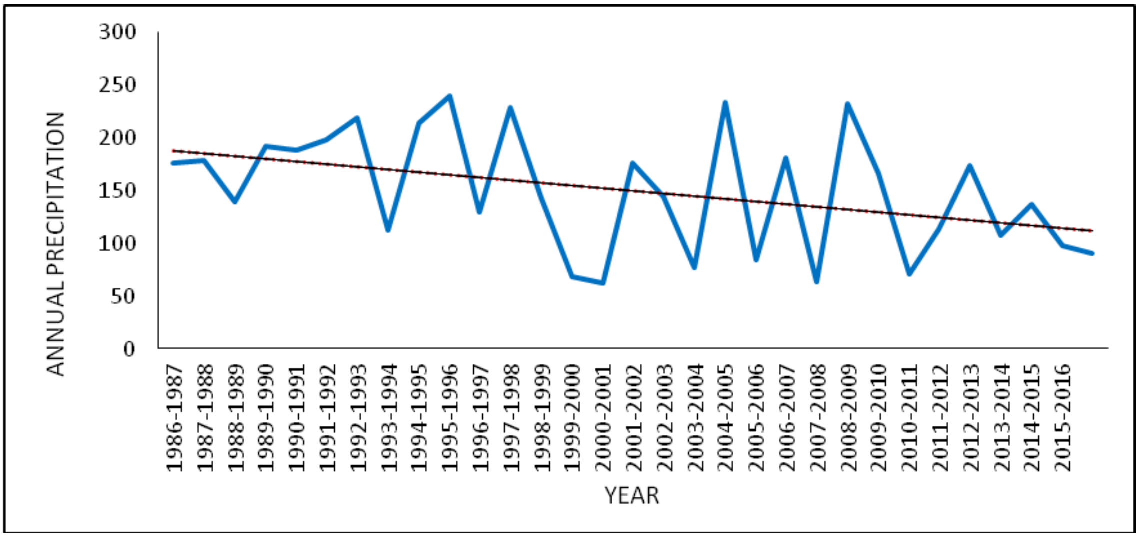

Figure 3. *Time series of changes in precipitation in Birjand Township within the a 30 year period*

Methodology

This study is an applied research study; it is a descriptive–analytical work aided by the data collection method, which was carried out based on structural equation modeling (SEM). The statistical population of this study was composed of all farmer households living in rural areas of Birjand Township in Southern Khorasan Province of Iran

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(N=6057) who were influenced by droughts during the years 2006–2015 (farmers engaged in agricultural activity in the region for at least the past 10 years and have faced droughts in the region). To determine the sample size, 2016 census data was used in accordance with which 6,057 farmer households work in rural areas of the studied township. As many as 200 households were selected as samples using Bartlett's table (2001). In total, 20 villages were considered as the target using a stratified random sampling process (districts were as the study classes).

Data collection tools in this study was provided by a questionnaire including queries in two parts in such a way that the former contains personal and professional characteristics of farmers including age, gender, level of education, history of agricultural activity, with the latter encompassing some items about farmers' perception of economic (eight items), social (eight items), and environmental consequences of drought (eight items) as well as their perspectives (attitude) on drought (10 items). SEM was used for data analysis in the Amos Graphics software. SEM mainly focuses on the hidden (latent) variables that have been defined by markers or observed variables (Rezaei, 2013). Content validity and construct validity were used to determine the validity of the research tools. Content validity was validated using the comments of university faculty members. Moreover, composite reliability was used to assess the reliability of instruments.

Results

Descriptive results

Most of the respondents (59%) stated that 100% of household income is to be derived from agricultural activities. As many as 87.5% of the people surveyed represented farming as their main job, while 62.6% were employed only in farming. The average age of respondents was 49 years with a standard deviation of 12; the youngest and the oldest farmers were aged 25 and 72 years. In total, 22% of the farmers were illiterate, 21% had an elementary level of education, 14% had a degree of guidance school, 28% had high school degree/diploma, and 15% had higher qualification than diploma. The access to agricultural service centers was at a moderate or high level for about 68% of the respondents.

Structural equation

Checking the status of indicators (observed variables)

Table 1 contains a summary of descriptive statistics (mean and standard deviation) components of the farmers' perception of consequences of drought and the initial validity of each of the factors considered based on Cronbach's alpha coefficient.

Confirmatory factor analysis and assessing validity of scales

To create and test the validity of the three subscales, including social, economic, and environmental effects as influencing factors on the perception of consequences of drought in Birjand Township, three single-factor confirmatory factor analysis (CFA) models were drawn and analyzed in the Amos Graphics platform.

The results of measuring the adjusted model (first-order confirmatory factor analysis) on the basis of standardized coefficients showed that the items increased input prices and production costs, lowered the value of rural assets, and caused disruption in trade

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(economic consequences) and loss of the quality of agricultural land, destructed animal habitats and wildlife in the area, reduced the variety and quality of plant species in the area (environmental consequences), increased insecurity and crime, reduced aid and cooperation among villagers, and lowered human values and disorder of beliefs (social consequences). These were related to the independent latent variables and some items like "there is no way to deal with drought and must be surrendered to fate, with the drought it is better to leave the villages by producers" (farmers perspective of the consequences of drought) were related to the dependent variable (latent) that were excluded from the measurement model due to lower factor loading 0.5. Factor loadings of other variables were greater than 0.5. Also, the results in *Table 1* show, the values of average variance extracted (AVE) for three hidden factors are higher than 0.5. In addition to these two indicators, the results showed that the composite reliability values obtained for the factors evaluated in the measurement model, higher than 0.7 (*Table 1*). In general, given the results obtained based on these three criteria, the research tools can be concluded to have a suitable convergent validity (a high internal convergence among the studied items). Regarding the reliability of the present study, since the composite reliability values obtained for the diverse (latent) hidden variables were greater than 0.7, it can be noted that the survey instrument enjoys an adequate reliability.

As can be seen in *Table 2*, the measurement model has goodness of fit and logical relationships are established between variables.

The fitting indices used in *Table 2* including normed chi-square (X^2/df), incremental fit index (IFI), root mean square residual (RMR), comparative fit index (CFI), goodness of fit index (GFI), parsimony ratio (PRATIO) and root mean square error of approximation (RMSEA) are the criteria to verify the formulized theoretical models by using the field data collected in the present study.

The second-order three-factor confirmatory factor analysis model for latent variable analysis of farmers' perception of consequences of drought

After carrying out the first-order confirmatory factor analysis, the second-order confirmatory factor analysis model was used in this section to understand the casual effects of our conceptual models and to assess the significance of the impact of the main latent variables. This model was also used to rank these variables according to their effects on forming and articulating farmers' perception of droughts in rural communities of Birjand. In doing so, the results of the second-order analysis are presented in *Figure 4* and *Tables 3* and *4*. The standard path coefficients between the latent variables and each other, along with the observed variables and the latent variables, which, in fact, are the most important and main portion of our analysis, can be seen in *Figure 4.*

The correlation coefficients between the farmers' perception of consequences of drought in Birjand rural communities and the three components of economic consequences, environmental consequences, and social consequences were derived as 0.341, 0.261, and 0.138 respectively. Moreover, the results of standard error estimations, critical ratios, and significance levels show that all these standard estimates are significant at a level of 99%. The obtained final model-fitting indices, along with standard values for their evaluation, are presented in *Table 3*. *Table 4* presents the standardized regression coefficients (direct and indirect effects) for the final observed variables and the obtained subscales on the main latent dependent variable.

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Table 1. *Description of items and observed indicators (final variables) of the subscales affecting the perception of the consequences of drought*

Subscales affecting perception of the consequences of drought		Items	Regression weight	Critical rate(C.R)	The standard error (S.E)	Composite reliability	The average variance extracted	Mean	Standard deviation	Cronbach's alpha
Economic consequences	E.C1	Reduced purchasing power	1			0.786	0.765	3.36	1.17	**0.851**
	E.C2	Reduced revenue	0.896	5.238***	0.171			3.38	1.23	
	E.C3	Reduced incentive to invest due to increased risk	0.936	4.589***	0.204			3.34	1.3	
	E.C4	Increased debt to the banks	0.477	5.238***	0.091			3.23	1.14	
	E.C5	Low wages and lack of employment areas	0.626	5.741***	0.109			3.23	1.22	
Environmental Consequences	En.C1	Dried wells, aqueducts and springs	1			0.798	0.693	3.75	0.911	**0.809**
	En.C2	Reduced quality of water resources	0.288	4.434***	0.065			3.24	0.948	
	En.C3	Reduced surface and groundwater resources	0.586	6.892***	0.085			3.18	1.29	
	En.C4	Soil erosion and desertification	0.894	7.451***	0.12			3.65	1.01	
	En.C5	Pests and diseases	0.587	6.525***	0.09			3.42	0.732	
Social consequences	S.C1	Resorting to false and illegal jobs	1			0.812	0.713	3.46	1.02	**0.791**
	S.C2	Mental and emotional tensions	0.648	4.533***	0.143			3.51	0.885	
	S.C3	Increased migration of villagers to cities	0.892	8.035***	0.111			3.32	1.09	

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	S.C4	Increased dissatisfaction and suspicion to governmental policies	0.819	8.106***	0.101			3.14	1.03	
	S.C5	Increased local disputes at the village level	0.808	4.253***	0.19			3.75	0.911	
Attitude toward drought	$A.D_1$	Harmful effects of drought can be managed by optimal control and exploitation of water resources	1			0.809	0.71	3.36	1.17	**0.819**
	$A.D_2$	Accurate management ways of coping with drought are easy to handle	0.538	5.331***	0.101			3.41	0.98	
	$A.D_3$	State supports plays a key role in the drought management	0.787	5.579***	0.141			3.51	1.3	
	$A.D_4$	Drought can be distanced by secondary employments	0.875	5.238***	0.167			3.23	1.14	
	$A.D_5$	Drought in the future will be considered as a threat to production in the agricultural sector (combination of two items)	0.858	5.761***	0.149			3.23	0.99	
	$A.D_6$	Drought in the future will be considered as a threat to water resources (combination of two items)	0.532	4.469***	0.119			3.61	0.871	

Source: research findings, 2016. ns: non-significant, * confidence level of 95%, ** confidence level of 99%*** error of 0.001

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Table 2. *Compliance of the measurement model with fitting indices*

Indexes	X^2/df	IFI	RMR	CFI	GFI	PRATIO	RMSEA
Proposed criterion *	≤3	0.9≤	≤0.08	0.9≤	0.9≤	0-1	≤0.08
The reported value for measurement model Economic consequences**	2.28	0.919	0.075	0.98	0.915	0.74	0.07
The reported value for measurement model Environmental consequences**	2.36	0.931	0.074	0.93	0.905	0.69	0.069
The reported value for measurement model Social consequences**	1.94	0.91	0.07	0.95	0.941	0.77	0.071
The reported value for measurement model Farmers' attitude**	2.61	0.927	0.07	0.93	0.934	0.73	0.074

Source: * Ghasemi, 2010; ** findings, 2016

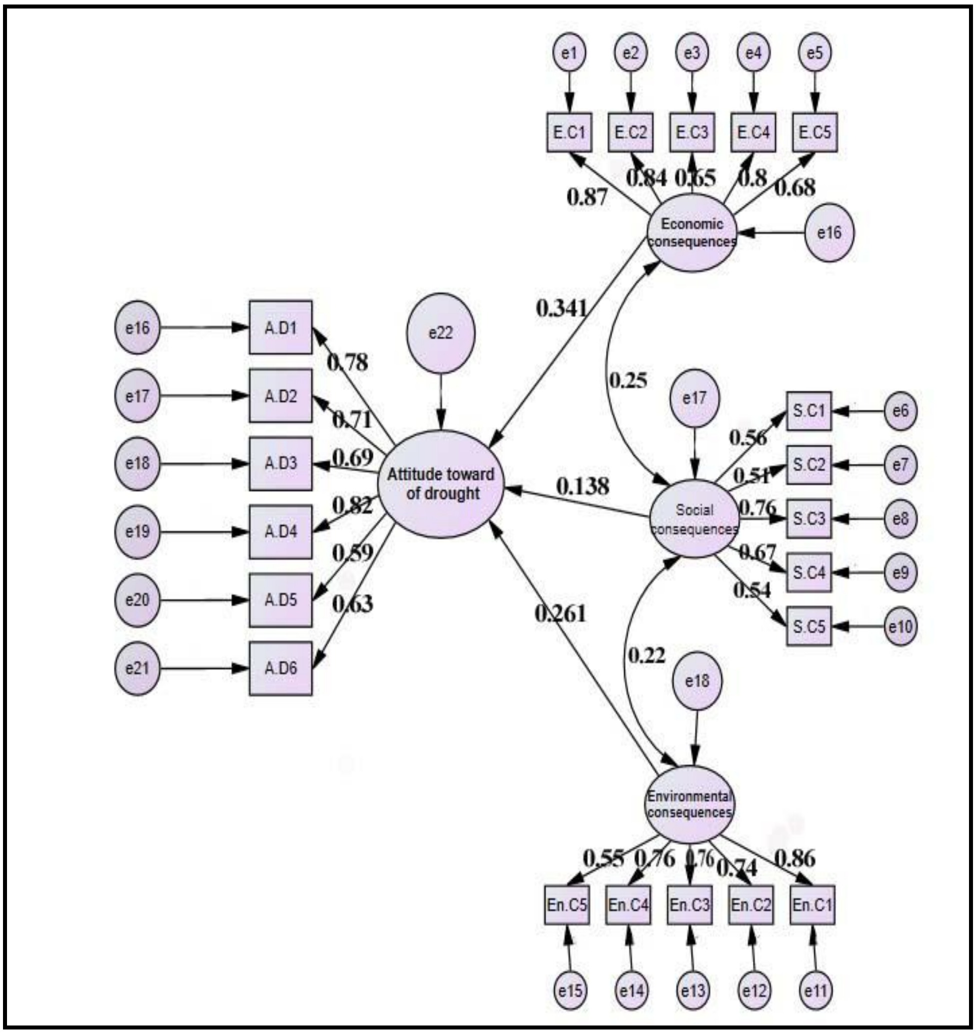

Figure 4. *The second-order three-factor confirmatory factor analysis model for latent variable analysis of farmers' perception toward the three components of economic, environmental, and social consequences*

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***Table 3.** The results of the compliance of the structural model with fit indices*

Indexes	X^2/df	IFI	RMR	CFI	GFI	PRATIO	RMSEA
Proposed criterion *	≤3	0.9≤	≤0.08	0.9≤	0.9≤	0-1	≤0.08
Reported value**	2.05	0.91	0.067	0.97	0.945	0.81	0.065

Source: * Ghasemi, 2010; ** findings, 2016

The results of *Table 4* show that the highest indirect effects on the farmers' perception of consequences of drought (economic, social, and environmental consequences) in Birjand Township are related to variables such as reduced revenue (E.C2), increased debt to banks (E.C4), reduced purchasing power (E.C1) and dried wells, and aqueducts and springs (En.C4), while the lowest ones are concerned with mental and emotional tensions (S.C2), increased local disputes at the village level (S.C5), and resorting to false and illegal jobs (S.C1). Owing to these coefficients, the lowest and highest impacts are associated social and economic consequences respectively.

***Table 4.** Overall effects of the observed variables and the subscales on the evaluation of the farmers' perception of consequences of drought*

	Evaluation of farmers' perception of consequences of drought	Economic consequences	Environmental consequences	Social consequences
Economic consequences	0.341	-	-	0.25
Environmental consequences	0.261	-	-	0.22
Social consequences	0.138	0.25	0.22	-
E.C1	0.265	0.87	-	-
E.C2	0.286	0.84	-	-
E.C3	0.221	0.65	-	-
E.C4	0.272	0.8	-	-
E.C5	0.208	0.68	-	-
En.C1	0.224	-	0.86	-
En.C2	0.193	-	0.74	-
En.C3	0.2	-	0.769	-
En.C4	0.198	-	0.76	-
En.C5	0.143	-	0.55	-
S.C1	0.077	-	-	0.56
S.C2	0.07	-	-	0.51
S.C3	0.104	-	-	0.76
S.C4	0.092	-	-	0.67
S.C5	0.074	-	-	0.54
$A.D_1$	0.78	-	-	-
$A.D_2$	0.71	-	-	-
$A.D_3$	0.69	-	-	-
$A.D_4$	0.82	-	-	-
$A.D_5$	0.59	-	-	-
$A.D_6$	0.63	-	-	-

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Discussion

The present study was conducted to investigate farmers' perception of consequences of drought in Birjand by using structural equation modeling, followed by a review and description of the most important results. Based on the findings, droughts have a direct negative impact on production of crops and livestock as well as an indirect impact on the incentive to invest due to the increased risk and decreased fields of employment in the agricultural sector. These have resulted in reduced revenue and consequently a lower purchasing power for farmers– the concepts which clearly appeared in present work. This finding is in line with the work of Singh et al. (2014). Therefore, given the considerable decline in rural incomes, it makes sense to adopt such approaches and policies as payment of subsidies and supporting the investment and entrepreneurship among the regions affected by drought to create employment and income, restore rural livelihoods and create small and early-return workshops in villages, development and support of production of handicrafts like carpets, rug, and jajim (woolen cloth), which were traditional craft in Southern Khorasan rural regions, and generally, allowing for activities that would pave the way for the restoration of rural incomes.

Based on our findings, the lowered value of rural assets and increased debt to the bank are concepts extracted by the results of the present study. This finding is consistent with Bostani et al. (2015). The convenience granting of diverse, sufficient, low-interest, and gratuitous banking facilities and credits and extension of agricultural loans repayment can be fruitful efforts to reduce the mentioned damages.

The results have shown that continuous droughts in recent years have dwindled surface and groundwater resources, thereby drying and reducing the quality of their water resources. The use of such water, in turn, lowers the quality of agricultural land in such a way that some salt-sensitive products are no more arable in these lands. This finding is in line with the work of Panda et al. (2007). Some of the strategies that can reduce the undesirable consequences of droughts in the region are governmental oversight on water withdrawals from groundwater sources, controlled withdrawals from wells by installing smart meters, effective water-pricing policies, prevention of digging of new wells, development of infrastructures and irrigation and water supply networks compatible with local conditions, reuse of wastewater, and restoration of returned waters into the cycle of consumption, and identification and promotion of products compatible with saline and unconventional waters.

Livelihoods of rural households affect many social relations and interactions; these have forced villagers willing to take up illegal jobs. Economic problems and the disruption of many of the relationships and social interactions have provided an unsuitable and unsafe environment for villagers. Consequently, many rural families were forced to migrate to towns and cities with the hope of obtaining better working conditions and providing welfare for their families. This result is in line with the work of Shewmake (2008). Some effective actions to reduce such damages include development of support services and social security for rural communities, development and promotion of drought insurance, and creating appropriate legal institutions and authorities for conflict resolution regarding the use of water and pasture.

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ENVIRONMENTAL IMPACT ASSESSMENT (EIA) OF ALTERNATIVE POTATO CROPPING SYSTEMS IN HAMADAN PROVINCE, IRAN

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Abstract. Potato is a staple food crop and a most important agricultural commodity in Hamadan Province, Iran. However, the province is facing such problems as water scarcity, nitrate pollution, and plant disease epidemics. This study explored the environmental impact assessment of potato cropping systems in Hamadan Province, Iran. To this purpose, 8 agri-environmental indicators were used for the EIAs of 4 potato cropping systems. These indicators included environmental potential risk indicator for pesticides, water use efficiency, nitrogen leaching, CO_2 emission, energy use, biodiversity, golden potato cyst nematode, and land use. The 4 systems included traditional potato system, quasi-industrial potato system, industrial potato system, and government-promoted potato system and 5 groups of features were used to describe the mentioned systems: (1) irrigation methods; (2) seed placement; (3) machinery use; (4) agrochemical use; and (5) crops rotation. The results revealed that the traditional potato system had the lowest negative impact on the environment. Of the potato production systems studied, the government-promoted potato system, quasi-industrial potato system, and industrial potato system had successively fewer negative effects on the environment. Finally, some strategies are recommended for designing a new benchmark encompassing environmental thresholds using agri-environmental indicator scores and their relationships.
Keywords: *sustainable food consumption, sustainability, Agri-Environmental Indicators (AEIs)*, *agricultural inputs, irrigation methods*

Introduction

Environmental impacts are increasingly resulted from agri-food supply chains (Sala et al., 2017). The agricultural sector accounts for about 11% of anthropogenic greenhouse gas emissions (Porter and Reay, 2016; Sellitto et al., 2017). In Iran, 3.8% of total energy, more than 90% of groundwater, and 67.5% of surface water are consumed in this sector (Najafi Alamdari, 2016). Although alternative cultivation and improved agronomic practices are generally proposed for the mitigation of environmental impacts in agriculture (Tasca et al., 2017; Smith et al., 2017), to achieve a sustainable food system, Environmental Impact Assessment (EIA) as a multi-dimensional process of an intrinsically complex evaluation (Ramanathan, 2001) is essential. Thus, to effectively assess environmental impacts, multiple objectives based on local and global effects and effect-based indicators should be directly considered in the evaluation methods, while the means or practices should be selected by farmers (Repar et al., 2017; van der Werf and Petit, 2002). Consequently, AEIs were most comprehensively developed by the Organization for Economic Cooperation and Development (OECD) (OECD, 1999a, b,

2001) for evaluating the environmental impacts. These indicators were defined by McRae et al. (2000) as a measure of the key environmental conditions, changes, or risks caused through agriculture or the related management practices utilized by producers. Therefore, agroecosystem is influenced by farming practices, the impacts of which can be estimated by AEIs at various decision-making levels. By employing them at the farm level, farmers can be environmentally friendly via an adaptation of their practices. Also, at the wider regional or national planning levels, policy decisions and assessments can be directed by AEIs (Glenn and Pannel, 1998). The 5 different topics of agri-environmental policies, including landscape, greenhouse gas emissions, agricultural nutrient loading, pesticide use, and species diversity were mentioned by Yli-Viikari et al. (2007). Based on the management of agricultural practices, numerous effects on the environment, such as the emissions of environmentally hazardous substances, ecological matter and energy turnover, soil productivity, and biodiversity can be assessed by AEIs. Yet, few indicators have been developed for each area by the scholars so far. Some indicators have been applied for models, such as those of the potential risks of nitrogen leaching (Lidon et al., 2013) and pesticide use (Pawelzik and Möller, 2014), as well as CO2 emission or global warming (He et al., 2016) and some like (EU), LU, WUE of irrigation, and EBZ have been directly measured (Brentrup et al., 2004; Geri et al., 2010; García-Feced et al., 2015; Larkin, 2016; He et al., 2016; Fandika et al., 2016).

The global production of potato crop is most dramatically increasing in the developing world. Until the early 1990s, most potatoes were being grown in the Russian Federation, Europe, and North America. Also, in Asia and Africa, potato production has been strongly increased (Birch et al., 2012). Due to this growing interest in potato, increasing the performance of this valuable food crop by decreasing its environmental impacts as future challenges is essential (Pawelzik and Möller, 2014). Accordingly, the potential of an integrated farming system of a moderate intensity was reported by Stavi et al. (2016) to sufficiently sustain environmental and ecosystem service qualities besides maintaining global food security. Notably, even the traditional systems persisting on the sustainability of high biodiversity have an advantage in some contexts (Hahn and Orrock, 2015; Uchida et al., 2016). Because of the political condition of the Iranian government, reliance on food imports will endanger food security. Potato cultivation is considered despite the water problems in Iran, because potatoes have a high nutritional content and can substitute for a large portion of wheat imports (DeFauw et al., 2012). In this regard, by using agri-environmental indicators, the current study aimed at addressing and comparing the environmental effects of the 4 mentioned systems to meet the environmental challenges of growing interests for potato production in Hamadan Province, Iran. To this goal, the features of the different potato cropping systems and their components were first described and then, their environmental impacts were assessed and compared based on the agricultural inputs used during the potato cultivation. Finally, some strategies were designed for the environmental thresholds as new benchmarks.

Materials and methods

The study area

This case study was conducted in Hamadan and Bahar Counties in Hamadan Province, Iran (*Figure 1*). Hamadan Province has a population of 1.7 million residents with 93935 farmers, who produce about 21% of the country's potato production

(Anonymous, 2016). Hamadan region has an area of 1,949,400 ha and the farming area is 1,008,038 ha (51.7%) (Ghasemi Mobtaker, 2010). The mean annual precipitation over the last 50 years has been 334.1 mm. The region is characterized as having a cold rainy season between November and April and a warm dry period from May to October. The regional inter-annual rainfall variability is high, which strongly affects the yields based on a predominantly rain-fed agriculture (Veisi et al., 2015).

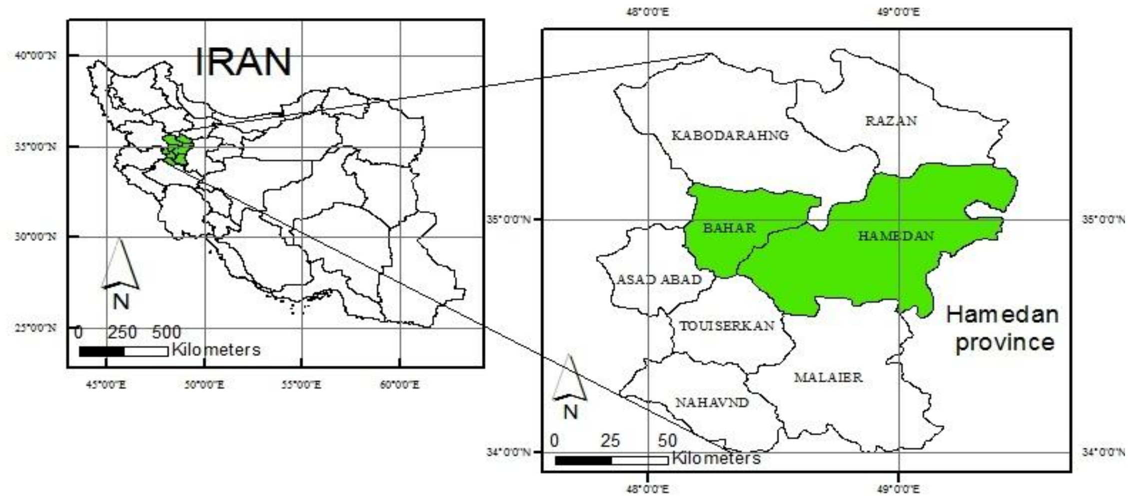

***Figure 1.** The study area location in Hamadan Province, Iran*

Description of the assessed potato production systems

A *potato cropping system* refers to the management techniques, such as pest management, soil fertility strategies, and water source and irrigation practices, which are used on a particular field for a period of years. Hamadan-Bahar regions in Iran were selected for the practices of the potato cropping systems defined in this context. The regions have got a semiarid climate with a mean annual temperature of 11.3°C and mean annual precipitation of 324.5 mm. The regional mean elevation is 2038 m above mean sea level (Akhavan et al., 2010). The major source of water supply for the agricultural sectors in the region is groundwater, which is also used for drinking and domestic and industrial activities. As a result, the groundwater level has continuously reduced in recent decades (Balali et al., 2011). In this research, the following 4 potato cropping systems were assessed by using AEIs during 2014-2015.

Traditional Potato System (TPS)

Historically, the potato cropping systems in the northwestern city of Hamadan typically included continuous potatoes and short-term rotations of 2 or 3 years with garlic (*Allium sativum*), cucumbers (*Cucumis sativus*), and vegetables, such as carrot (*Daucus carota*) and green beans (*Phaseolus vulgaris*). Many farms in the TPS had got a small size (0.5-1 ha). Rotations along with the extensive tillage and minimal crop residue returns during the potato phase of the rotation were the characteristics of this production system. Weeds were managed by hand and livestock manure was a key fertilizer in the system. Only in this system, the irrigation water was originated from

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surface water and irrigation practices were done via flood irrigation systems (*Table 1*). These data were collected from 25 farms.

Quasi-Industrial Potato System (QIPS)

In the Quasi-Industrial Potato System, the short-term crop rotations were simplified around cash crops, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare L.*). The crop protection strategies of diseases and pests were mainly based on pesticides and the mechanical control of weeds. Dairy manure and chemical N, P, and K fertilizers were uniformly applied to the fields before planting. Water was harvested from deep and semi-deep wells and distributed through a system of pipes by pumping. In the QIPS, a sprinkler irrigation system was used for irrigation (*Table 1*). These data were collected from 36 farms.

Industrial Potato System (IPS)

In the Industrial Potato System, crop rotation was often removed and the crop management was intensive: high use of pesticides and fertilizers, high sowing rates and usual sowing dates, lack of a mechanical weeding, and weed control through the use of a number of active substances. Therefore, herbicide rates were higher than average in this system. The drip irrigation systems were installed after the potato seeds were planted. A thin-wall drip tape was placed on the soil surface at the center of the raised beds (*Table 1*). These data were collected from 21 farms.

Table 1. *Comparison of the 4 potato cropping systems in Hamadan Province*

Attributes	potato production systems			
	TPS	QIPS	IPS	GPPS
Irrigation methods	Flood irrigation	Sprinkler irrigation	Drop irrigation	Drop irrigation
Seed placement	Flat ground	A row in the stack	Two rows on the stack	Two rows on the stack
Use of machinery	Substrate preparation	Substrate preparation, Sowing, out tubers from the soil	Substrate preparation, Sowing, out tubers from the soil or combine harvester	Substrate preparation, Sowing, combine harvester
Use of mineral fertilizers	Limited	Almost too much	Too much	Controlled
The use of pesticides	Almost Limited	Almost too much	Too much	The emphasis is on organic pesticides
Rotation	short-term	simplified	Without rotation	Recommended

Government-Promoted Potato System (GPPS)

A government project called as Government-Promoted Potato System (GPPS) aimed at reducing the environmental impact of potato production in the region from the early stages of implementation. The irrigation system used here was similar to that of the IPS except that water management was further emphasized in GPPS. An integrated control of pest management was developed by employing a number of various compatible control measures to minimize the harmful effects on the wider environment. In this method, water was mainly saved by drip irrigation. Furthermore, a limited use of fertilizers and pesticides was evaluated in this 4th method (*Table 1*). This potato cropping system was applied to all the 5 farms under study.

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Study methodology

The method used in this research was based on 6 stages (*Fig. 2*).

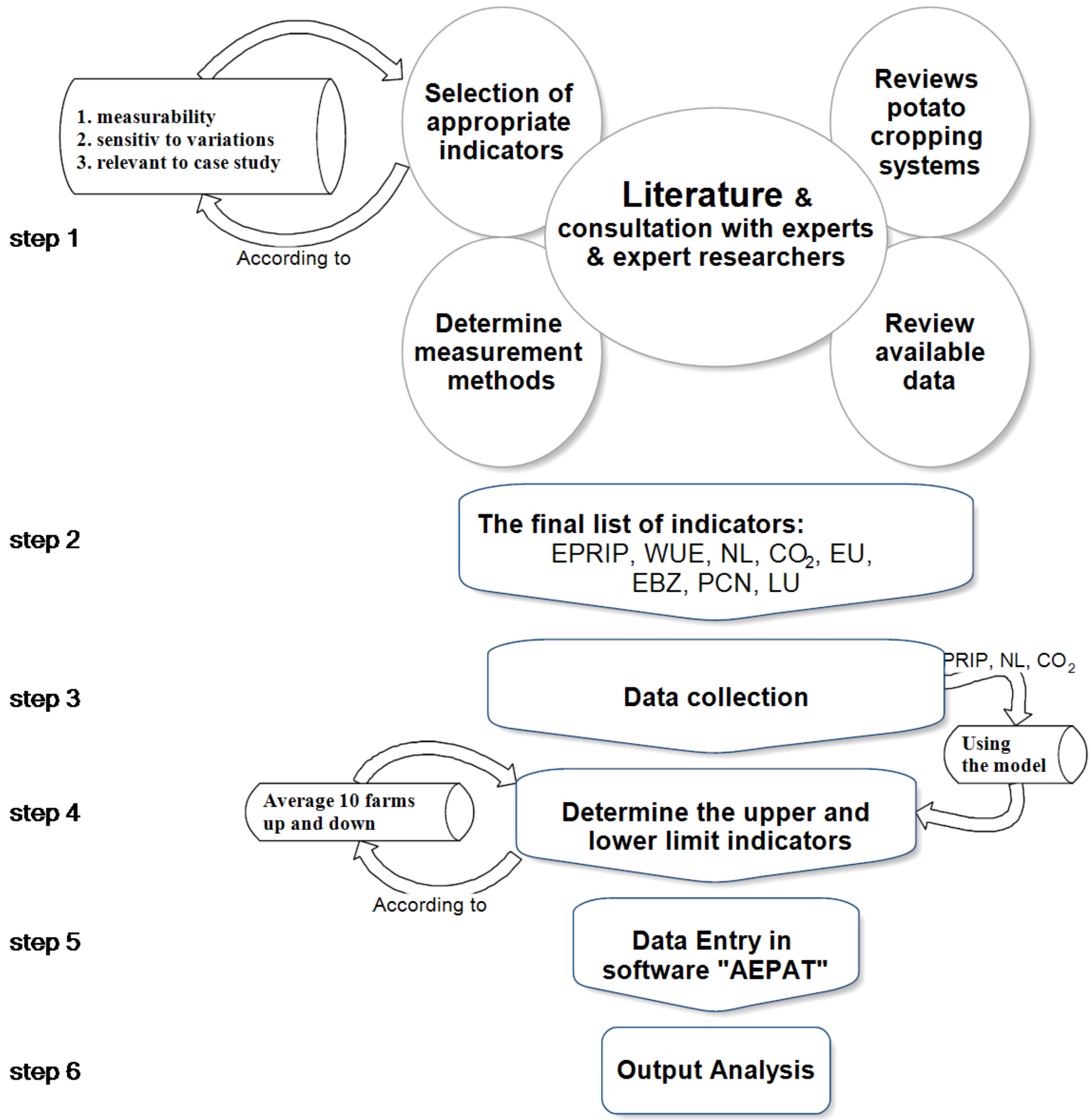

Figure 2. *Study methodology framework for EIA of alternative potato cropping systems*

The first stage included 4 sections that were almost preceded at the same time. The sections included reviews of the potato cropping systems and available data, selection of appropriate indicators, and determination of measurement methods. All the 4 sections were consulted with the relevant experts and expert researchers. In this step, the geographic and time scale boundaries, as well as the precise definitions of the study scenarios were discussed. In the 2nd step, the final lists of indicators and measurements were determined and an appropriate model was presented for measuring each indicator if necessary (The first 2 steps were adopted from the study of van Asselt et al., 2015). In step 3, the required data and information were collected via a direct measurement. With

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the second-stage samples, a stage sampling method was used to select farms. The first stage involved determining the cropping systems on farms, and the second stage included selecting target farms in a square system. This approach ensured that the selected farms represent all farms. However, since the GPPS was only tested (experimentally) on 5 farms with good dispersal in the region, all farms under this cropping system were studied. In step 4, the average upper and lower limits of 10 farms were determined for each indicator and a total of 87 farms were obtained. In step 5, the data were entered into "Agro-Ecosystem Performance Assessment Tool" (AEPAT) software to obtain each farm score and position. "AEPAT" software was used for assessing the agronomic and environmental performances of the management practices in agro-ecosystem experiments. This software is a computer program to assess the relative sustainability of management practices using agronomic and environmental data through performance-based indicators to derive the relative agro-ecosystem performance ranking for the functions and indicators (Liebig et al., 2004). Finally, "F" tests were employed to analyze the data and test the differences between the mentioned potato production cropping systems. When the results of the "F" tests were significant, Tukey's HSD (honest significant difference) test was used to compare pairs of mean values (with $p < 0.05$ as the level for statistical significance). The EIA framework for the potato production cropping systems is presented in *Figure 2*.

Agri-Environmental Indicators (AEIs)

Environmental Potential Risk Indicator for Pesticides (EPRIPs)

As a most susceptible crop in arable crop rotations, potato is potentially influenced by some pests like aphids and nematodes, viruses, and diseases (Pawelzik and Möller, 2014). Potentially hazardous pesticides for soil, groundwater through leaching, surface water via drift and run-off, and air by volatilization can be evaluated by EPRIPs. Although some leaching information is provided by Groundwater Ubiquity Score (GUS), drift and run-off and volatilization are disregarded. The accepted EPRIP values are within a range of 1-625 points, which correspond to a number of risk classes indicating "None" to "Very large" risks to the environment by man (Pacini et al., 2009, Merante et al., 2015). Separate scores are given for varied environmental compartments by EPRIPs, which can be combined into a total score (Reus et al., 2002). In this work, the EPRIP variables were adopted from a previous study conducted by Ramezani and Heydari (2013) in the same area.

Water Use Efficiency (WUE)

Potato high sensitivity to drought stress is due to its shallow and sparse root system. Hence, the high yielding of this crop is dependent on irrigation (Birch et al., 2012). Rodriguez et al. (2015) found the water footprint of potato production reaching 324 m^3/ton and concluded that an average value of 65 liter of water was consumed by potato assuming that one potato was 0.2 kg. Each plot yield (t ha^{-1}) was recorded in each pick. WUE is potato effectiveness for using water during its complete period of growth. It is expressed as the ratio of total yield of marketable tuber to the total depth of water applied to the crop (Yaghi et al., 2013; Fandika et al., 2016). An effective rainfall during a complete period of growth is also incorporated in the total water. Marketable and non-marketable tubers are >55 g without any defects and <55 g with defects, respectively.

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WUE (t ha^{-1} mm^{-1}) is equal to CY/WA, where CY and WA indicate the total marketable tuber yield (t ha^{-1}) and total depth of water applied (mm), respectively.

Nitrogen Leaching (NL)

In autumn, potato leaves the highest nitrate (N) amount after harvesting and is thus considered as a crop of highest residual nitrate level (Pawelzik and Möller, 2014). In this area, potato irrigation with groundwater is conducted with the simultaneous uses of fertilizers and agrochemicals. LEACHN model is a valid tool for assessing irrigation and N management effects on nitrate leaching (Lidon et al., 2013). Using Richards' equation, one-dimensional water flow can be described in unsaturated zones by this model. The main process of solute transport in the nitrogen module, including mineralization, nitrification, denitrification, and volatilization, is modeled by the convection-dispersion equation (Lidon et al., 2013). Nitrogen leaching amount was estimated via this model in this article.

CO_2 emission

Atmospheric radiative forcing is caused by carbon dioxide (CO_2) as a part of greenhouse gases. Management highly determines the flux of these gases from agroecosystems. Radiative forcing can be lowered by agricultural management via enhancing soil organic carbon (Mosier et al., 2003; Liebig et al., 2005). CO_2 cost of production was calculated by the Cool Farm Tool-Potato (CFT-Potato). The cost of producing 1 ton of potatoes by calculating CO_2 equivalent amount is determined via CFT-Potato spreadsheet program (Sandaña and Kalazich, 2015; Haverkort et al., 2014). CO_2 emission is equal to its total emission per ton of potatoes (kg CO_2 eq t^{-1})

Energy Use (EU)

Potato production is based on the Energy Uses (EUs) of seed potatoes, uses of fertilizers and agrochemicals, tractor operations, electricity (for irrigation), grading, storage, and store loading (Haverkort and Hillier, 2011; Haverkort et al., 2014). By calculating the primary EU factors, the data of fertilizer and pesticide productions were specified (Camargo et al., 2013). To this end, the potato production inputs, including electricity, irrigation water, farmyard manure, chemical fertilizers, pesticides, herbicides (biocides), fungicides, machinery, diesel fuel, and human labor were determined. Potato tubers were considered as the outputs. Upon calculating energy productivity, the input data computed per hectare were multiplied by the energy equivalent coefficient (Ghasemi Mobtaker et al., 2010; Mohammadi et al., 2008; Saad et al., 2016). Thus, EU was equal to potato tubers output (kg ha^{-1}) divided by energy input (MJ ha^{-1}).

Biodiversity

Certain forms of biodiversity loss are often caused by LU (Chen et al., 2016). Pest control and crop yield can be biologically promoted by the diverse strips of wildflower for farmland biodiversity (Tschumi et al., 2016). However, biodiversity in arable lands have been significantly declined by LU changes and simplification of cropping systems and chemical inputs for farming, while leading to the drastically reduced elements of Ecological Buffer Zones (EBZs), such as trees, wet zones, etc. (Bockstaller et al., 2011; Hole et al., 2005) Semi-natural habitats in the surrounding farms are highly enriched by

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biodiversity, especially in field edges (Lüscher et al., 2014). These habitats are mainly grasslands, shrub and agro-forestry areas, and the vegetation not used for crop production like hedgerows, buffer strips, field margins, and wood lots (García-Feced et al., 2015; Geri et al., 2010). Thus, EBZs are equal to the percentage of the semi-natural land surface.

Golden Potato Cyst Nematode (PCN)

The golden PCN *(Globodera rostochiensis)* is globally known as a most serious biotic constraint for potato production (Hajihassan et al., 2013). Several studies have addressed the relationship of reduced growth and yield parameters with the initial population density of potato cyst nematodes (Hajihassan et al., 2013; Greco and Moreno, 1992). To identify the suspiciously infested land area with nematodes based on symptoms like low growth and yellowing, the samples of nematode babies and eggs collected in June were examined in terms of numbers by using hand-binoculars and microscope (Gitty et al., 2001). Therefore, golden PCN was equal to the surface whit percentage of more than 15 babies or eggs per gram of soil.

Land Use (LU)

The functioning of ecosystems and natural resources can be positively or negatively influenced by LU (Taelman et al., 2016). To compare agricultural systems, LU productivity as the main component of land evaluation and supported LU planning must be determined (Huiyi, 2013; Sombroek, 1992). Assessment of LU productivity was done by calculating the total marketable potato weights with the diameters of more than 4.8 cm per 20 m of rows and converting them into the equivalent potato values of Kg per ha (Larkin, 2016; Brentrup et al., 2004). Therefore, LU was equal to potato tubers (t ha^{-1}).

Results and discussion

EPRIP

The results analyzed by AEPAT software revealed the profound difference of TPS and IPS as the former provided management choices based on EPRIP with a value of 25 and a score of 0.9, while the latter with a value of 103 and a score of 0.4 led to the mismanagement of pesticides used in the fields ($P<0.01$). In the TPS, mostly the farmers controlled weeds, pests, and diseases through cultivation techniques, while the main reason for the lowered score in IPS was related to the use of nematicidal compounds. On the other hand, a low difference was found between QIPS with a value of 78 and a score of 0.56 and GPPS with a value of 83 and a score of 0.52, while both standing on the average (*Table 2* and *Figure 3*). Chemical pesticide type is also very important. Several studies conducted in Iran and around the world, including those of Aghilinejad et al. (2008) and Koureas et al. (2012) have proven the positive relationship between health problems and exposure to pesticides. Soil fertility can be adversely affected by pesticide accumulation in the soil, which leads to the crop contamination (Komárek et al., 2010).

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Table 2. *The environmental performances of the Traditional Potato System (TPS), Quasi-Industrial Potato System (QIPS), Industrial Potato System (IPS), and Government-promoted Potato System (GPPS)*

Indicators	Abbreviation	Unit	Weight (%)	Farming systems				*P-value*
				TPS	QIPS	IPS	GPPS	
Environmental Potential Risk Indicator of Pesticide Use	EPRIP	Score	12.5	25.54 ±14	78.31 ±28	103.43 ±42	83.40 ±17	4E-13
Water use efficiency	WUE	t ha^{-1} mm^{-1}	12.5	0.0234 ±0.002	0.0316 ±0.004	0.0420 ±0.004	0.0442 ±0.004	8E-30
Nitrogen leaching	NL	kg ha^{-1}	12.5	35.67 ±17	49.80 ±21	44.56 ±22	36.76 ±8.8	0.0497
CO_2 emission	CO_2	kg CO_2 eq t^{-1}	12.5	40.01 ±12	52.12 ±16	54.86 ±14	46.09 ±15	0.004
Energy use	EU	kg MJ^{-1}	12.5	0.319 ±0.04	0.291 ±0.03	0.288 ±0.03	0.299 ±0.01	0.007
Biodiversity (ecological buffer zones)	EBZ	Percent (%)	12.5	3.23 ±0.9	2.82 ±0.8	1.53 ±0.7	1.46 ±0.4	4E-10
Potato golden cyst nematode	PCN	Percent (%)	12.5	0.008 ±0.04	0.141 ±0.2	0.203 ±0.2	0.100 ±0.1	0.008
Land use	LU	Kg ha^{-1}	12.5	30.14 ±4.7	33.79 ±4.3	34.86 ±3.5	34.33 ±3.4	0.001
Ecological sustainability	**Ec.Su**	**Score (0-1)**	**100**	**0.65 ±0.10**	**0.55 ±0.14**	**0.53 ±0.14**	**0.62 ±0.08**	**0.01**

Water Use Efficiency (WUE)

Water Use Efficiency (WUE) was affected by the 4 cropping systems (P<0.01) (*Table 2*). Its indicator showed that GPSS with a production of 0.044 tons of tuber per hectare by consuming 10 m^3ha^{-1} of water and gaining a score of 0.9 had a better behavior than the other systems. With the irrigation management, IPS and QIPS resulted in lower WUE values (0.042 and 0.0316 ton ha^{-1} mm^{-1}, respectively) compared to GPSS, which in turn was lower than TPS (0.023 ton ha^{-1} mm^{-1}). In the drip irrigation, 79-88% less water was used compared to GPSS and IPS, thus obtaining higher WUE compared to TPS surface irrigation. Drip irrigation has been shown to be an effective method for high potato yields (Wang et al., 2011; Onder et al., 2005). A 48-88% increase in WUE with drip irrigation compared to surface and seepage irrigations was reported by Reyes-Cabrera et al. (2016) for potato production. Therefore, the scores obtained from the cropping systems of GPPS, IPS, QIPS, and TPS were equal to 0.9, 0.8, 0.4, and 0.1, respectively.

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Nitrogen Leaching (NL)

The average Nitrogen Leaching (NL) indicator scores for GPPS, IPS, QIPS, and TPS were 0.77, 0.65, 0.57, and 0.78, respectively. In QIPS, NL was the highest (49.8 kg ha^{-1}), whereas TPS (35.67 kg ha^{-1}) had the lowest NL ($p<0.05$). NL in GPPS (36.76 kg ha^{-1}) was lower than that of the IPS (44.56 kg ha^{-1}). In general, NL increases with a larger amount of irrigation water (Giletto and Echeverria, 2013) and a longer irrigation interval (Woli et al., 2016), while leading to an enhanced N rate since a more quantity of N is available in the soil for leaching (Cambouris et al., 2008). However, it shows a smaller amount with the clay soil compared to the lighter soils (Woli et al., 2016). QIPS and TPS used larger amounts of irrigation water, while QIPS and IPS had the greatest N rate uses. TPS and GPPS received relatively less amounts of nitrogen, whereas TPS had a clay soil. IPS had the longest irrigation interval, at least for the seasonal period.

CO_2 emissions per tons of potatoes

Among the cropping systems, CO_2 emission in IPS (54.86 kg CO_2 eq t^{-1}) was the highest, which accounted for over 37% of that of the TPS (40.01 kg CO_2 eq t^{-1}). QIPS (52.12 kg CO_2 eq t^{-1}) and GPPS (46.09 kg CO_2 eq t^{-1}) were ranked to be on the second and third positions. The previous studies conducted in the Netherlands (Haverkort and Hillier, 2011), southern Chile (Sandaña and Kalazich, 2015), and Iran (Pishgar-Komleh et al., 2012) represented CO_2 emissions for potato production systems to be 77 kg CO_2 eq t^{-1}, 41-72 kg CO_2 eq t^{-1}, and 992.88 kg CO_2 eq ha^{-1}, respectively. Overall, the highest average score for CO_2 emission per tons of potatoes was found in TPS (0.74), while GPPS, QIPS, and IPS scores were 0.63, 0.52, and 0.47, respectively (*Fig. 3*). The highest values of CO_2 emission belonged to chemical fertilizer, diesel fuel, water for irrigation, and machinery.

Energy Use (EU)

Energy Use (EU) was highest in TPS (0.319 kg Mj^{-1}) due to the lower energy consumptions of chemical fertilizers (mainly nitrogen) and diesel fuel or electricity consumption for land preparation, sowing, and irrigation, whereas IPS had the lowest EU (0.288 kg Mj^{-1}) ($p<0.01$). IPS consumed 11%, 4%, and 1% higher energies to produce a potato unit compared to TPS, GPPS (0.299 kg Mj^{-1}), and QIPS (0.291 kg Mj^{-1}), respectively. Overall, the highest energy use score was found in TPS (0.68), while those of the GPPS, QIPS, and IPS were 0.53, 0.47, and 0.44, respectively (*Fig. 3*).

The previous studies conducted in Ardabil Province in northern Iran (Mohammadi et al., 2008) and Hamadan (Rajabi Hamedani et al., 2011) and Esfahan provinces in the center of Iran (Pishgar-Komleh et al., 2012) demonstrated that the EUs for the mentioned potato production systems were 0.35, 0.9-0.31, and 0.37-0.59 kg Mj^{-1}, respectively. Pishgar-Komleh et al. (2012) showed that with an additional 1% use of seed, water for irrigation, chemical fertilizer, and diesel fuel energies, the yields of potatoes raised to 0.36, 0.42, 0.12, and 0.04%, respectively. Rajabi Hamedani et al. (2011) showed that nitrogen fertilizer (39%) had the highest consumption followed by diesel fuel (21%) and seed (14.9%). In their study, Park and Kremer (2017) concluded that EU was the most practical and useful indicator among all the 55 environmental sustainability indicators extracted from the extant literature.

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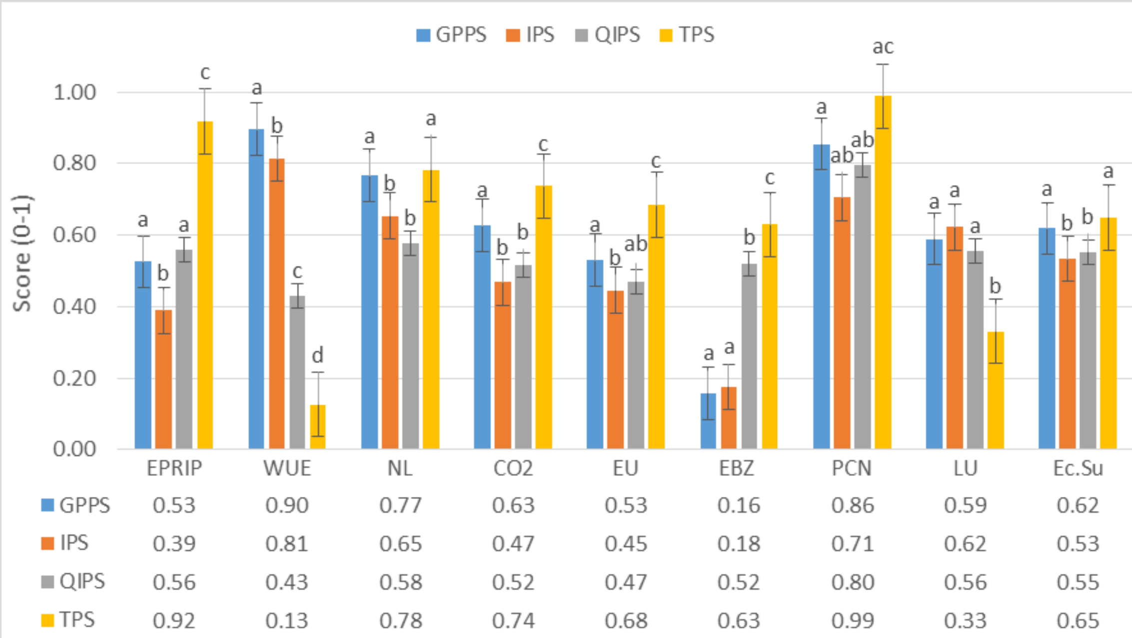

Figure 3. *The environmental impacts of the Traditional Potato System (TPS), Quasi-Industrial Potato System (QIPS), Industrial Potato System (IPS), and Government-Promoted Potato System (GPPS); values with different letters indicate significant differences among the potato cropping systems at 5% by Tukey's HSD test.*

Ecological Buffer Zones (EBZ)

Ecological Buffer Zones (EBZ) indicator represented that only 1.46% of the semi-natural surface occurring in the GPPS had been conserved, while 98.54% of this system had been involved in agriculture and urban areas. However, 1.53, 2.82, and 3.32% of the semi-natural surfaces occurring in the IPS, QTPS, and TPS had been conserved, respectively. Based on the Common Agricultural Policy (CAP) legislation (2014–2020), at least 5% of the arable lands holding for most farms with an arable area of larger than 15 hectares must be dedicated to the ecological areas mainly composed by semi-natural vegetation features (García-Feced et al., 2015). The rapid loss of semi-natural lands caused by advanced agriculture area has been proven (Liu et al., 2016). Overall, the highest EBZ score was found for TPS (0.63), while those of the QIPS, IPS, and GPPS were 0.52, 0.18, and 0.16, respectively (*Fig. 3*).

Golden Potato Cyst Nematode (PCN)

In TPS, only 0.008% of the surface (in one of the surveyed potato fields) had more than 15 babies or eggs of golden Potato Cyst Nematode (PCN) (*Globodera rostochiensis*) per gram of soil, while 0.2% of the surface was affected by more than 15 babies or eggs of golden PCN per gram of soil in IPS. The nematode infection in GPPS (0.1% of surface) was slightly lower than that of the QIPS (0.14% of surface) ($p<0.01$). Currently, nematicides classified as organophosphates, carbamates, and soil fumigants are somewhat used for nematode control. The challenge to control nematode pests is, however, becoming more difficult as Class I red-band nematicides are progressively withdrawn from world markets (Fourie et al., 2016). For example, among the most rigorous and non-discriminative measures that can be taken to control soil-borne pests

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like plant-parasitic nematodes, soil fumigants have been already banned in many countries or will be banned in the near future (Vervoort et al., 2014). The management of nematode pests is rarely successful in the long term when single strategies, such as chemical control, host plant resistance, crop rotation, and/or other methods are applied (Fourie et al., 2016). In this regard, the highest score was found for TPS (0.99), while the scores of GPPS, QIPS, and IPS were 0.86, 0.80, and 0.71, respectively (*Fig. 3*).

Land Use (LU)

Land Use (LU) for the yield of marketable potato tubers was the highest (34.86 t ha^{-1}) in IPS, whereas TPS had the lowest LU (30.14 t ha^{-1}) ($p<0.01$), which accounted for over 14% of that of the TPS based on GPPS. Overall, GPPS (34.43t ha^{-1}) and QIPS (33.79 t ha^{-1}) were ranked on the second and third places. The previous studies conducted in Ardabil Province in northern Iran (Mohammadi et al., 2008) and Hamadan (Rajabi Hamedani et al., 2011) and Esfahan provinces in central Iran (Khoshnevisan et al., 2014) showed the average yields of potato production farms to be 28, 35, and 23 tons per hectare for the above-mentioned systems, respectively. Regardless of whether significant differences were found between GPPS and IPS together with QIPS, the highest score was found iforn IPS (0.62), while the scores of GPPS, QIPS, and TPS were 0.59, 0.56, and 0.33, respectively (*Figure 3*).

Ecological Sustainability (Ec. Su.)

Generally, this study represented clear environmental benefits associated with a transition from IPS to GPPS. In particular, GPPS offered great potentials to decrease the use of high-risk pesticides and chemical fertilizers. There was also a more efficient use of irrigation water. Yet, TPS indicated a minimum impact on the environment. Although TPS made inefficient uses of water and land, it was less dependent on fertilizers and pesticides. Moreover, biodiversity was considered in this system. Among the cropping systems studied, the highest ecological sustainability score was obtained for TPS (0.65), which accounted for over 22%, 18%, and 5% of those of the IPS (0.53), QIPS (0.55), and GPPS (0.62), respectively. However, no significant differences were found between GPPS with TPS and QIPS with IPS. Finally, it should be noted that in calculating the ecological sustainability score, an equal share was given to each indicator (12%). In the previous studies, Böhringer and Jochem (2007) applied a self-restraint weighting that was believed to be generally associated with subjective judgments, while He et al. (2016) used 8 weighing indicators between 0.15 and 0.09. The self-restraint weighting was also confirmed by Singh et al. (2009).

Conclusion

The aim of the paper was to conduct an assessment of the environmental impacts of 4 different types of potato cropping systems in Hamadan Province, as well as comparing the variations of such impacts with the current programs of agri-environmental indicators. In this regard, 8 indicators, i.e., EPRIP, CO_2 emissions, N leaching, …, were used to measure the environmental performance. The results revealed that TPS had better environmental performance than GPPS, QIPS, and IPS. Among all, GPPS had the potential to reduce the environmental impacts by increasing the proportion of semi-natural areas. Certain benefits of GPPS, such as a suitable rotation due to its role in

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controlling diseases like nematodes, reduction of indirect energy consumption and carbon dioxide emissions, and wisely management of soil and nutrients were evidenced. For instance, compared to the other systems, IPS that was being strongly expanded in the middle plain areas had lower environmental performance because of possessing the lower scores of EPRIP, EU, and CO_2 emissions, thus needing fundamental changes due to being involved in reduced water availability. We should conclude that farmers and agronomists will find useful information in this article to reduce the negative agricultural impacts on the environment through better decisions and provision of more efficient and sustainable potato production systems. Based on methodology, a more precise analysis can be provided since the AEIs used here were drawn from several indicators, including the direct environmental indicators. Therefore, the environmental performances of different types of farms can be more comprehensively evaluated. In general, it was demonstrated that the differences in the environmental performance can be readily and potentially detected by applying AEI methodology to potato cropping systems and more broadly to other crops in a standardized way.

We recommend that the relationship between total 8 agri-environmental indicators be calculated by study in research farms. In this case, the minimum score of one indicator that cannot be compensated by sufficiently high score of others indicators can be considered as environmental thresholds. For example, if the EBZ is reduced of a certain amount, it will increase the use of pesticides and thus reduce the scores in the EPRIP, EU, and CO2 emissions, but before this threshold it can be compensated by the increase in LU score. Also, achieve highest scores in treatments with optimal and acceptable yield can be considered as sustainability marker at the farm level. Accordingly, we have a early warning system because if the farm consumes higher irrigation water or energy consumption of the calculated amount in the above method, these farms need to review their management practices. Of course, some environmental health rules should be considered for indicators such as NL and EPRIP, regardless of desirable performance. Regarding access to irrigation water, which is the biggest problem in the study area, it is necessary to establish the environmental sustainability thresholds at the regional level by calculating the amount of allowable harvest water of the region. It is also necessary to carry out research and enforcement measures regarding the control of the potato crop extent in order to meet the domestic needs of the country and prevent the export of this crop. Its necessary that the export product selected to based on regional advantage and have maximum compliance with the regional climate.

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ESSENTIAL OIL COMPOSITION AND PHYTOTOXICITY EFFECTS OF *SOLANUM NIGRUM* L. ON SEED GERMINATION OF SOME VEGETABLES

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Abstract. The allelopathic activities of *Solanum nigrum* shoot and root aqueous extracts on cabbage, spinach and tomato seed germination were investigated. Treatment concentrations were 0, 2, 4, 6, 8 and 10 mg ml^{-1}. The results indicate that the shoot extracts did not affect the germination of tomato and spinach seeds but 2 mg ml^{-1} slightly hindered the germination of cabbage seeds. The highest root extract concentration (10 mg ml^{-1}) led to a significantly lower germination percentage in tomato seeds in comparison with the control. The highest extract concentration (10 mg ml^{-1}) in both shoot and root extracts slowed down germination time in tomato seeds. The mean germination time indicates that cabbage seeds germinated faster than tomato and onion. These results indicate that *S. nigrum* shoot and root extracts pose no hindering effect on the germination of cabbage and onion seeds, although increased root extract concentrations may hinder germination in tomato seeds. A total of 16 and 12 compounds were respectively identified from the shoots and roots of *Solanum nigrum* analysed by GC-MS. The major shoot compositions were Citronellol (11.98%), Cyclohexanol, 2-methyl-5-(1-methylethenyl) (5.84%), Geraniol (3.76%) and Geranyl tiglate (3.53%). In the roots, Hexacosane (35.62%), 1H-Indole, 1-methyl-2-phenyl (12.66%), Hexadecanal (8.11%) and Tetrasiloxane, decamethyl (7.58%) were the major components.
Keywords: *allelopathy, wild vegetable, seed viability, chemical compositions, GC-MS*

Introduction

The domestication of wild vegetables as a means of combatting food insecurity has been a topic of interest in South Africa for over a decade. Various aspects of wild vegetables have been widely documented including their agronomy and more commonly their nutritional compositions (Edmonds and Chweya, 1997; Odhav et al., 2007; Flyman and Afolayan, 2008; Atta et al., 2010; Mahala et al., 2012). Although the cultivation of wild vegetables for food is common in some African countries such as Botswana, Nigeria, Ghana, Zambia and Zimbabwe, this practice is not widespread in South Africa (Gbile et al., 1988; Mushita, 1997; Madisa and Tshamekang, 1997; Maroyi, 2011; Aju et al., 2013). Since wild vegetables are commonly viewed as weeds, intercropping them with conventional crops and/or vegetables is one of the options that need to be explored in order to make maximum use of the land in the fight against food insecurity. However, there are fears that wild vegetables may cause growth problems or nutritional stress to exotic vegetables growing alongside them.

The stimulatory or inhibitory effects plants have on the growth of other plants within the same ecosystem through a mechanism known as allelopathy is well known. Bio-chemicals known as allelochemicals/allelochems are released from plant parts by leaching, root exudation, volatilisation, residue decomposition and other processes in both natural and agricultural systems (Fraenkel, 1959; Stamp, 2003). The

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allelochemicals are secondary metabolites like terpenoid and phenolic compounds such as flavonoids, anthocyanins, lignin as well as tannins and these have specific actions (Khanh et al., 2007). In recent years, there have been interests in the allelopathic activities of wild plants. Ataollahi et al. (2014) reported that increasing the concentration of the *Eucalyptus globulus* leaf extract had a strong allelopathic effect on *S. nigrum* seed germination, shoot and root length growth. In another study, Sadeghi et al. (2010) reported that the water extract of *Helianthus annuus* inhibited the germination of *S. nigrum* seeds. The roots of *Tithonia diversifolia* have been found to stimulate the growth and chlorophyll content of some *Solanum* species (Otusanya et al., 2014). Sabh and Ali (2010) found *S. nigrum* extracts to hinder dicotyledonous seedling growth and chlorophyll in comparison with monocotyledonous seeds. *S. nigrum* has also been reported to reduce germination, root and shoot length growth of onion (Baličević et al., 2015). Some allelochemicals such as alkaloids that are produced by *S. nigrum* have been found responsible for suppressing growth of other plants (Sabh and Ali, 2010).

Although *S. nigrum* is an important wild vegetable of the Eastern Cape Province, its allelopathic activity on some important vegetables has not been investigated. Cultivating wild vegetables alongside traditional vegetables in farming operations is an option being explored in order to reduce food insecurity. The current experiment was therefore conducted to investigate the effect of *S. nigrum* root and shoot aqueous extracts on the germination of cabbage, spinach and tomato seeds. Understanding plant on plant interactions is important in agricultural production especially in relation to intercropping. The detrimental effects of inclusion of wild vegetables into the existing farming operation on the ecology, environmental degradation and conservation of genetic biodiversity require a better understanding. This study was therefore conducted to help know the stimulatory or inhibitory effects of the residues of *S. nigrum* on the germination of these important traditional vegetables. Also, a comprehensive understanding of the essential oil composition of *S. nigrum* is vital for proper understanding of its value as both a nutritional and pharmacological plant. The essential oils of the fresh roots and shoots were therefore also analysed for their chemical compositions.

Materials and methods

Plant collection

S. nigrum plant samples were previously identified in 2011 at the University of Fort Hare and the voucher specimen (BVE 11/017) kept at the Giffen herbarium. Fresh samples were collected by uprooting the whole plant from around Alice in August 2015 in the wild and washing with distilled water to remove impurities. Cabbage (Starke Ayres - Cabbage: Drumhead), tomato (Starke Ayres- Hybrid Tomato: STAR 9003) and spinach (Starke Ayres - Swiss Chard: Fordhook Giant) seeds were purchased from a local supermarket.

Preparation of aqueous extracts

The method of Badmus and Afolayan (2012) was used to prepare the extracts of the plant samples. The root and shoot samples were separately dried to a constant weight in the oven at 35°C. The dry matter yields were ground in an electric hammer mill fitted with 1 mm sieve and stored separately in tightly sealed vial bottles in the refrigerator at

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4°C till further use. About 50 g of each powdery material was extracted in 1.5 L of distilled water and agitated on an orbital shaker for 12 h at room temperature. The filtrate was freeze dried at -50°C under the vacuum (RVT4104, USA) and reconstituted in distilled water to obtain the desired concentrations of 10, 8, 6, 4 and 2 mg ml^{-1}.

Viability test

Viability tests were conducted using the Tetrazolium Chloride Technique. Following the method described by Peters (2000), 25 seeds were imbibed in water overnight at 22.5 ± 2.5°C in triplicates. The seeds were then cut along the margin without damaging the embryo and soaked in colourless 0.1% solution of 2,3,5- triphenyltetrazolium chloride (TTC) for 24 h at 22.5 ± 2.5°C in the dark. The pH of the TTC solution was 6.61. The seeds were removed from the TTC solution, washed in distilled water and soaked in 95% ethanol to permit direct observation of the embryo under the microscope. Embryos of viable seeds appeared reddish in colour.

Seed germination trials

To evaluate the effects of the five shoot and root aqueous extract concentrations on the vegetable seeds, the method of Badmus and Afolayan (2012) was adopted with modifications. In triplicates, 25 seeds of each vegetable were placed evenly in 9 cm Petri dishes lined with two Whatman filter papers and moistened with the respective extract concentrations and distilled water in the control. The petri dishes were laid out in a Randomised Complete Block Design on a laboratory work bench under ambient temperature conditions (between 19 and 25°C). The seeds were examined on a daily basis and considered germinated when the radicle was visible.

Gas chromatography mass spectrometry (GC-MS)

Using the modified method of Okoh and Afolayan (2011), 100 g of the fresh leaf and root samples of *S. nigrum* were subjected to a DRYDIST Milestone manufactured (2007) Solvent-free Microwave Extraction (SFME) Labstation apparatus for 30 min. The extracted oil was kept in tightly sealed vial bottles in a refrigerator at 4°C till further use. GC-MS analyses were performed on the Agilent 5977A MSD and 7890B GC System, Chemetrix (pty) Ltd; Agilent Technologies, DE (Germany) with a Zebron-5MS column (ZB-5MS 30 m x 0.25 mm x 0.25 um) (5%-phenylmethylpolysiloxane) apparatus. The column and temperature conditions which were used were as follows: GC grade helium at a flow rate of 2 ml/ min and splitless 1 µl injections were used. The oven temperature, injector and source temperatures were set at 70, 280 and 280°C respectively. The ramp settings were; 15°C/ min to 120°C, then 10°C/ min to 180°C, then 20°C/ min to 270°C and held for 3 min.

Identification of components

The identification of the chemical constituents of the essential oil was determined by their GC retention times, percentage composition (Area %) and retention indices. The interpretation and identification of their mass spectra was confirmed by mass spectral incorporated library. The identification was further confirmed by search using the National Institute of Standards and Technology (NIST) database (NIST/EPA/NIH mass spectral library 2014) and comparing with those of published data.

Statistical analysis

Where applicable, the data were subjected to statistical analysis using MINITAB Release 12.22. A one way analysis of variance was used to compare seed germination as influenced by the extract concentration. Means were compared using Duncan's multiple range test. The means were treated as significantly different at $p < 0.05$.

Results and discussion

Seed viability and germination

Viability in the purchased cabbage, spinach and tomato seeds was 99, 92 and 83% respectively. In *S. nigrum* (belonging to the same family as tomatoes) seeds stored at room temperature in a period ranging from 1 to 9 years, Roberts and Lockett (1978) reported 99, 100, 96, 98, 91, 73, 27, 2 and 0% viability. This indicates an inverse relationship between seed viability and time. However, the viability and germination results are in close agreement.

The effects of the aqueous extract of *S. nigrum* shoot on seed germination of cabbage; tomato and spinach are shown in *Table 1*.

Table 1. *Percentage inhibition on germination of vegetable seeds as influenced by the aqueous extract of S. nigrum shoot extract*

	Vegetable seeds		
	Cabbage	**Tomato**	**Spinach**
10 mg ml^{-1}	97±0.58^{ab}	83±2.08	65±3.06
8 mg ml^{-1}	97±0.58^{ab}	92±1.73	80±1.00
6 mg ml^{-1}	100±0.00^{a}	92±0.58	64±2.00
4 mg ml^{-1}	100±0.00^{a}	99±0.58	72±4.58
2 mg ml^{-1}	96±0.00^{b}	89±0.79	68±3.61
Control	100±0.00^{a}	95±2.31	79±1.16

Values shown are MEAN ± SD
Different letters down the same column represent significant differences at $p < 0.05$

The results indicate that the shoot extract did not significantly ($p < 0.05$) affect the germination of tomato and spinach seeds. However, 2 mg ml^{-1} significantly lowered the germination of cabbage seeds although this was not significantly lower than 10 mg ml^{-1}, the highest concentration. The root extract of *S. nigrum* did not significantly affect the germination of cabbage and spinach seeds but significantly hindered the germination of tomato seeds (*Table 2*).

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Table 2. *Percentage inhibition on germination of vegetable seeds as influenced by the aqueous extract of S. nigrum root extract*

	Vegetable seeds		
	Cabbage	**Tomato**	**Spinach**
10 mg ml^{-1}	98±0.58	13±1.73^{b}	42±1.16
8 mg ml^{-1}	96±0.58	87±1.73^{a}	80±1.73
6 mg ml^{-1}	96±0.58	62±4.62^{a}	60±3.31
4 mg ml^{-1}	98±0.58	80±2.00^{a}	44±3.06
2 mg ml^{-1}	100±0.00	87±0.00^{a}	40±4.58
Control	100±0.00	84±1.53^{a}	56±5.03

Values shown are MEAN ± SD
Different letters down the same column represent significant differences at $p < 0.05$

The highest concentration (10 mg ml^{-1}) significantly lowered tomato seed germination. The Mean Germination Time (*Figs. 1* and *2*) indicates that cabbage seeds germinated faster than spinach and tomato seeds. However, 10 mg ml^{-1} of both extracts significantly slowed down tomato seed germination time. Numerous allelopathic effects of *S. nigrum* on germination and growth have been performed on a wide range of crops and scantily on some important horticultural crops such as tomato, cabbage and onion. For example, Agarwal et al. (2002) reported that *S. nigrum* strongly inhibited plumule growth by 100% and radicle growth by 92% in some wheat cultivars. In the same study, *S. nigrum* strongly inhibited seedling growth as compared to other plants. *S. nigrum* extracts have also been reported to hinder the growth of chickpea as well as some tomato and onion cultivars (Kadioglu et al., 2004). Girija et al. (2008) as well as Marinov-Serafimov (2010) also reported some allelopathic effects of *S. nigrum* on some legumes. Although, studies on other species indicate that *S. nigrum* strongly hinders the growth of some important species, the present results indicate that this plant does not pose the same effects on some important horticultural crops such as tomato, cabbage and spinach.

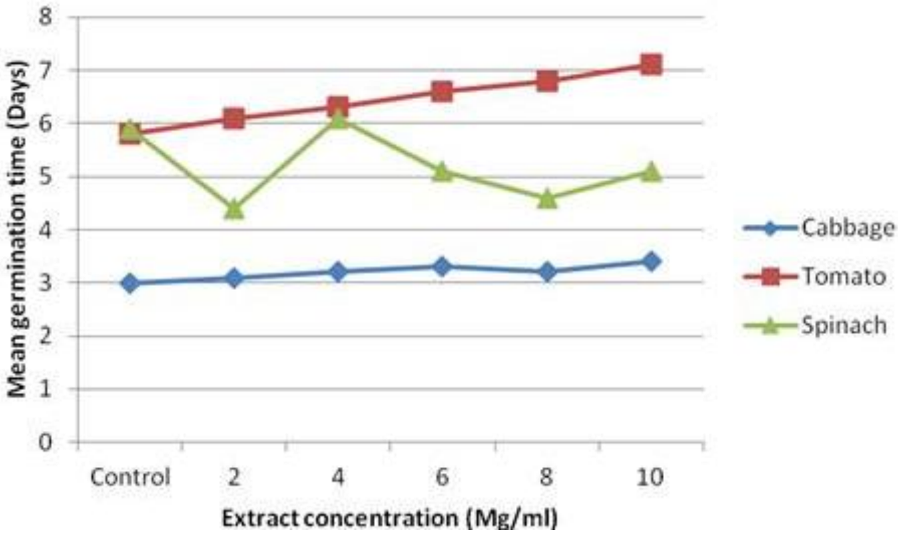

Figure 1. *Effect of S. nigrum aqueous shoot extract on seed germination time of vegetables*

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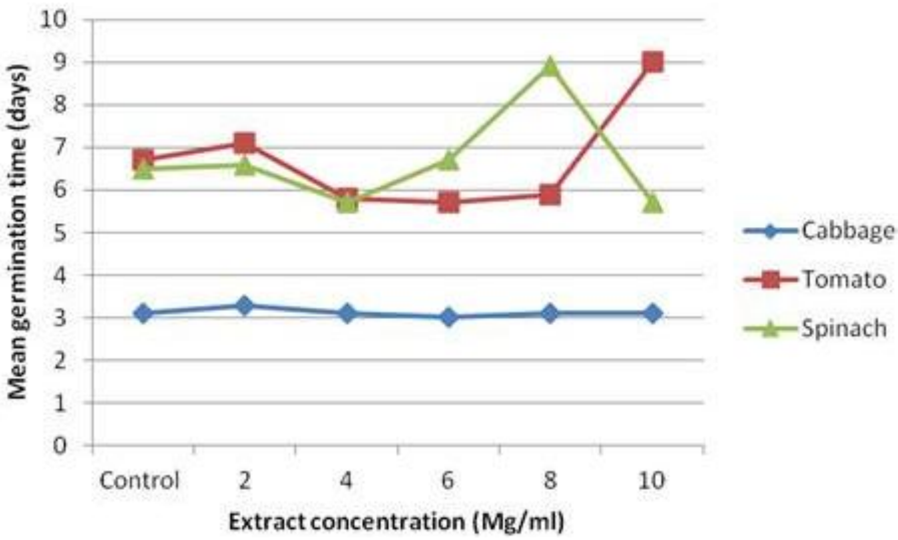

***Figure 2.** Effect of S. nigrum aqueous root extract on seed germination time of vegetables*

Essential oil compositions

Both the shoot and root samples yielded a colourless oil with a pungent scent after extracting with the microwave. A total of 16 compounds were identified from the shoot oil extract (*Table 3*) and 12 were identified from the root oil extract (*Table 4*).

***Table 3.** Essential oil chemical composition of S. nigrum shoots*

Name	Molecular formula	RT	KI	Area (%)
α-farnesene	$C_{15}H_{24}$	8.085	1580	2.57
.beta.-iso-Methyl ionone	$C_{14}H_{22}O$	8.779	1673	2.63
1-Benzothiazol-2-yl-3-(4-methyl-benzoyl)-thiourea	$C_{16}H_{13}N_3OS_2$	7.893	2334	0.93
2-[2-Thienyl]-4-acetyl quinoline	$C_{15}H_{11}NOS$	7.736	1545	0.75
3-(4-Fluoro-phenyl)-4-methyl-2-(2, 2,2-trifluoro-1-methoxycarbonyl-ethylidene)-2,3-dihydro-thiazole-5-carboxylic acid ethyl ester	$C_{17}H_{15}F_4NO_4S$	9.121	1596	2.02
Benzamide, 4-methoxy-N-[4-(1-methylcyclopropyl)phenyl]-	$C_{18}H_{19}NO_2$	10.955	1729	0.66
Chola-5,22-dien-3-ol, (3.beta.,22Z)-	$C_{24}H_{38}O$	8.477	-	1.91
Citronellol	$C_{10}H_{20}O$	6.249	1368	11.98
Cyclobutanecarboxylic acid, 2-phenylethyl ester	$C_{13}H_{16}O_2$	8.717	1690	2.71
Cyclohexanol, 2-methyl-5-(1-methylethenyl)-	$C_{10}H_{18}O$	11.766	1920	5.84
Cyclopentaneethanol, 2-(hydroxymethyl)-.beta.,3-dimethyl-	$C_{10}H_{20}O_2$	8.242	1567	0.98
Geraniol	$C_{10}H_{18}O$	6.452	1388	3.76
Geranyl isobutyrate	$C_{14}H_{24}O_2$	7.936	1594	1.36
Geranyl tiglate	$C_{15}H_{24}O_2$	9.318	1703	3.53
p-Anisaldehyde 4-[1-adamantyl]-3-t hiosemicarbazone	$C_{19}H_{25}N_3OS$	8.845	1506	1.92
Silane, chlorodimethyloctadecyl	$C_{20}H_{43}CLSi$	8.440	1534	0.87

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In the shoots, the major compositions were Citronellol (11.98%), Cyclohexanol, 2-methyl-5-(1-methylethenyl) (5.84%), Geraniol (3.76%) and Geranyl tiglate (3.53%). In the roots, Hexacosane (35.62%), 1H-Indole, 1-methyl-2-phenyl (12.66%) and Hexadecanal (8.11%) were the major components. Four compounds, *viz,* α-farnesene, Citronellol, Geraniol and Geranyl tiglate were identified in both the shoot and root extracts. Although some authors (Ogundajo et al., 2013; Sivakamasundari et al., 2013; Taherpour, et al., 2013; Aburjai et al., 2014; Huda et al., 2015) have reported various compounds in the leaves of *S. nigrum*, none have reported the findings of this current work. Aliero et al. (2006) reported the presence of Geraniol in the leaves of *Solanum pseudocapsicum* and this corresponds with what was found in both the shoot and root extracts of the present project.

Table 4. *Essential oil chemical composition of S. nigrum roots*

Name	Molecular formula	RT	KI	Area (%)
1,2,4-Benzenetricarboxylic acid, -dodecyl dimethyl ester	$C_{23}H_{34}O_6$	13.008	-	0.43
1H-Indole, 1-methyl-2-phenyl-	$C_{15}H_{13}N$	14.360	2207	12.66
2-Phenylethyl tiglate	$C_{13}H_{16}O_2$	8.711	1689	1.83
α-farnesene	$C_{15}H_{24}$	8.081	1579	2.38
Citronellol	$C_{10}H_{20}O$	6.249	1368	11.98
Eicosane	$C_{20}H_{42}$	12.191	1910	2.78
Geraniol	$C_{10}H_{18}O$	6.452	1388	3.76
Geranyl tiglate	$C_{15}H_{24}O_2$	9.318	1703	3.53
Hexacosane	$C_{26}H_{54}$	11.305	-	35.62
Hexadecanal	$C_{16}H_{32}O$	9.380	1672	8.11
Nonadecane	$C_{19}H_4O$	10.335	1655	4.45
Phthalic acid, 2-(1-adamantyl)ethyl methyl ester	$C_{21}H_{26}O_4$	8.774	-	2.24

Conclusion

The current study indicates that the viability and germination results are in close agreement. The shoot extract hindered the germination of cabbage while the root extract hindered that of tomato. Also, an increase in the shoot and root extract concentration slowed down the germination time of tomato seeds. However, the results indicate that *S. nigrum* does not pose a severe hindering effect on the germination of cabbage, spinach and tomato and as such possibilities of cultivating the wild vegetable alongside these exotic vegetables remain open. *S. nigrum* shoots and roots also possess a diverse range of chemicals in their essential oils. A total of 16 and 13 compounds were respectively identified by GC MS in the shoot and root extracts of the plant.

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MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CULTURABLE EPILITHIC AND ENDOLITHIC BACTERIA FROM ROCKS OF AYUBIA (MURREE), LOWER HIMALAYA, PAKISTAN

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Abstract. Murree and Ayubia hills are parts of the lower Himalaya Mountain range in Pakistan. The rocks of these hills are younger and mostly limestone in nature. In this study, geomicrobiological investigation of the selected samples collected from Murree and Ayubia hills was carried out. The rock samples were spread on nutrient agar plates and endolithic and epilithic bacteria were isolated on the basis of colony morphology and studied further. All the isolates were screened for different industrially important hydrolytic enzymes. Study isolates were identified through 16S rRNA gene sequencing. These endoliths and epiliths were assayed for antimicrobial activities against clinical isolates *S. aureus, P. aeuroginosa* and *E. coli*. Total 31 bacterial isolates were recovered of which 15 were endolithic and 16 were epilithic. 16S rRNA gene sequencing revealed major culturable groups colonizing these rocks were clustered in four major groups, Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes which inlcude *Alcaligenes, Lysinibacillus, Actinobacteria, Pseudomonas, Pusillimonas, Streptomyces, Fluviicola, Serratia, Flavobacterium, Stenotrophomonas* and *Brevindomonas* species. The sequences were deposited in NCBI for acquisition of accession numbers. The bacterial isolates were efficient producers of oxidase, catalase, protease, amylase and gelatinase. The endolithic isolates N4 (*Pseudomonas* sp. KT223616) and N28 (*Streptomyces* sp. KT004386) exhibited good activity against all the three clinically isolated target strains. The study revealed the rocks of Murree and Ayubia hills have a rich microbial ecology that besides having an important role in weathering and mineralization processes may also be potential source of biotechnological applications.
Keywords: *Murree hills, Pakistan, antimicrobial production, endolith, epilith, microbial mineralization, weathering*

Introduction

Many microorganisms can survive and thrive in physically and geochemically extreme conditions that were previously considered hostile to life. These microorganisms are known as extremophiles (Sharma et al., 2012) and grow inside the rocks and on the outer surface of rocks. Such rock inhabiting organisms are categorized as endolithic, epilithic and hypolithic (Golubic et al., 1981) on basis of position of their colonization on the rocks.

Several types of rocks have been studied for microbial diversity such as rocks of igneous origin (both glassy and crystalline) by Thorseth et al. (1992), Villar et al.

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(2006), Herrera et al. (2009), sandstones, salts and limestones (sedimentary rocks) by Matthes et al. (2001), Wierzchos et al. (2006), Weber et al. (1996), and gneisses and granites (metamorphic rocks) by Ríos et al. (2005) and Cockell et al. (2002).

These rock dwellers are reported from different habitats on earth, those dwelling in mountains (Walker and Pace, 2007; Horath and Bachofen, 2009), in deserts (Friedmann and Galun, 1974; Friedmann and Ocampo, 1976), from deposits of tsunami (Cockell et al., 2007), from the floor of sea (Mason et al., 2007), from the impact crater (Cockell et al., 2002, 2005) from springs having temperature above 60°C (Walker et al., 2005), from the subsurface of deep sea (Amy et al., 1992; Pedersen, 1997), from monuments of cultural heritage (Scheerer et al., 2009), Daughney et al. in 2004 also reported bacteria thriving in deep hydrothermal vents. The colonization of microbes has been reported In glassy and crystalline igneous rocks (Thorseth et al., 1992; Villar et al., 2006; Herrera et al., 2009); in sandstones, salts and limes stones of sedimentary rocks (Weber et al., 1996; Matthes et al., 2001; Wierzchos et al., 2006) and gneisses and granite of metamorphic rocks (Cockell et al., 2002; De-los Ríos et al., 2005).

Endoliths and epiliths, inhabiting the extreme environments, produce molecules which help microbes in their adaptation to extreme life conditions and are considered as a new important source of moieties of biological origin (Sánchez et al., 2009). Accordingly, these microorganisms are important targets as a source of variety of bioactive compounds such as secondary metabolites and other industrially important enzymes like amylase, protease and gelatinase. Microbes play key role in weathering and mineralization of rocks. They attach to mineral surfaces and bring about physical and chemical changes in the structure. They create microenvironment of the complex nature at mineral-water interface, where they produce organic acids, metabolically catalyzed redox reactions and complexing agents lead to change in pH and concentration gradient, which is different from bulk solution. This helps in thermodynamic state which enhances weathering. The extracellular polysaccharides (EPS) also serves as a site for secondary mineral precipitation, which is different in morphology from those precipitated in bulk solution inorganically (Konhauser, 2007). The antimicrobial compound production seems to be the general phenomenon for most bacteria. A commendable array of defense, i.e. broad-spectrum antibiotics, lytic agents such as lysozyme and metabolic byproducts such as organic acids are produced by many types of bacteria. In addition, several other types of bacteriocins, proteins and exotoxins are also produced which are biologically active peptide moieties, with bactericidal mode of action (Riley et al., 2002; Yeaman et al., 2003). Secondary metabolites are produced in response to nutrient exhaustion, biosynthesis or addition of an inducer and growth rate decrease (Demain, 1998). The misuse of antibiotics lead to drug resistance, so the only solution is to explore new environments in terms of its diversity that will not only lead to the discovery of new forms of life, but also the secondary metabolites and other industrially important enzymes. Currently, extremozyme production is the demand of recent markets. These extremozymes can withstand more tough conditions in various industrial processes. The microbes inhabiting such dry and hard environments may be a good source of different hydrolytic enzymes.

In the current research our focus was to isolate and characterize the extremophiles (endoliths and epiliths) from natural rocks of Ayubia (Murree), Khyber Pakhtunkhwa (KPK) Pakistan. All the isolates were characterized on molecular basis by 16S rRNA gene sequencing. The isolated endoliths and epiliths were screened for industrially important hydrolytic enzymes and also for antibacterial activity against clinical isolates.

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Methodology

Sampling

The rocks and soil samples were collected from Ayubia, District Abbottabad, Khyber Pakhtunkhwa (KPK), Pakistan, (Elevation 2600 meters), (34°4'20"N, 73°23'55"E) (*Figure 1*). The rock samples were collected in pre-sterilized (ultra violet treated for 20 minutes) polyethylene zipper bags. The temperature at the time of sampling was 20°C while pH of both samples was 5. All the samples were proceeded to laboratory and stored at 4°C.

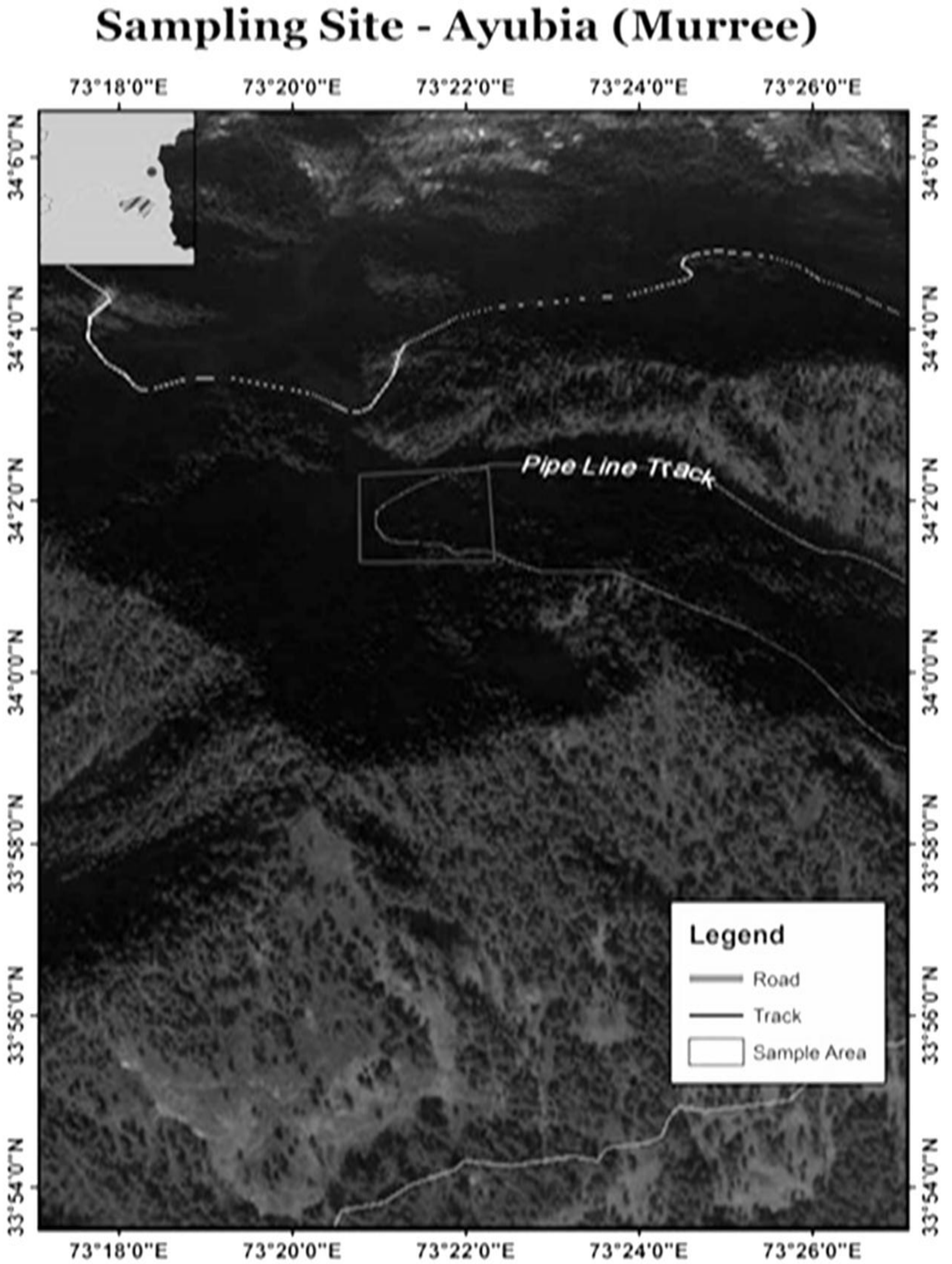

***Figure 1.** Sampling site: Ayubia Muree lower Himalaya, Pakistan*

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Rock analysis

The collected rocks belong to Lockhart formation/Lockhart Limestones, which are derived from Paleocene era and are around 65.5 million years old (Akhtar and Butt, 2000). Along Murree-Nathiagali road, near Changlagali the Lokhart limestones are very well exposed, and there it overlies the early to middle Paleocene Hangu Formation and underlain by Patala Formation. Lithology of the rock sample consists of highly fossiliferous limestone with infrequent calcite filled fractures followed by 0.8 meter thick band of dark gray colored calcareous shale. Bedded in between is thick fossiliferous nodular limestone of gray to dark gray in color.

Isolation of endolithic and epilithic bacteria

For the isolation of endolithic and epilithic bacteria, Nutrient Agar/Broth and Luria-Bertani Agar/Broth were used. For epilithic bacterial isolation, outer surface of rocks were directly swabbed with sterile cotton swabs. While for isolation of endolithic bacteria, rocks were broken down with sterile hammer under aseptic conditions. Then the freshly exposed inner surface was swabbed with sterile cotton swabs. The swabs were then immersed and shaken in 1 ml sterile water, the suspensions were then spread on nutrient agar plates (Difco, Detroit, USA) and incubated at 30°C for 3 days and cell count (CFU/ml) was determined for all samples. For purification, visible colonies were picked on the basis of morphological differences and cultured on nutrient agar plates separately.

Identification of bacteria by morphological and biochemical analysis

Identification of isolated strains was carried out morphologically and biochemically. Bergey's Manual of Determinative Bacteriology (9^{th} Edition) was used for identification of isolates. Culture characteristics of the isolated strains were observed. While the microscopic analysis was done through Gram staining procedure.

Molecular characterization

Further confirmation of the isolated bacterial strains was done by molecular characterization through 16S rRNA gene sequencing. The genomic DNA of all bacterial strains was extracted by DNA extraction kit (QIAGEN). The concentrations of DNA were qualified and quantified by spectrometry, NanoDrop 2000 (Thermo Scientific).

PCR amplification

PCR amplification of extracted DNA was done using universal bacterial primers 27F′ (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R′ (5-CTACGGCTACCTTGTTACGA-3′) bacterial primers. The PCR mixture consisted GoTaq®Green Master Mix Promega (25 µl), primer 27F′ (1 µl), primer 1492R′ (1 µl), DNA extract (2 µl), and *Nuclease-Free* Water (50 µl). MJ mini Personal Thermal Cycler was used to perform the PCR reaction (BIO RAD) the cycling conditions consisted of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. At 72°C the final extension was performed for 7 min. Electrophoresis was done for the PCR product analysis with 1% agarose gel.

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Sequencing and phylogenetic analysis

Sequencing of the amplified PCR product was done by Macrogen Service Center (Geunchun-gu, Seoul, South Korea). BLAST tool was used for the identification of nearest relatives of the sequences in the NCBI database, and homologues were analysed for phylogeny using Molecular Evolutionary Genetic Analysis (MEGA) version 6. Maximum Likelihood method was used for the construction of phylogentic tree (Tamura and Nei 1993), and diversity among endoliths and epiliths extremophiles were studied (Tamura et al. 2013). Finally, the sequence was submitted to the National Center for Biotechnology Information (NCBI) GeneBank for assignment of an accession number. The 1000 bootstrap replicates were used to estimate the significance of product tree.

Antimicrobial and biochemical potential

Antimicrobial potential of isolated strains

Three multi-drug resistant clinically isolated pathogens were used as target subjects, i.e. *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*. The antimicrobial activity of the strains was evaluated by using the point inoculation method. The test microbial colonies were transferred into sterile tubes containing normal saline and the turbidity was adjusted to 0.5 McFarland standard solution. Homogenous lawns were prepared on Muller Hinton agar plates under aseptic conditions and inoculated with the test strains.

Intracellular and extracellular enzymes

All the isolated bacterial strains were screened for the production of both intracellular and extracellular enzymes. Intracellular enzymes, including catalase, oxidase and hydrogen sulfide production and extracellular enzymes including amylase, protease and gelatinase were screened according to the protocol described by Murray et al. (1981).

Results

A total of 31 different endolithic and epilithic isolates were reported in the present study. All these different strains were isolated on the basis of distinct morphological characteristics. Morphological features such as size, shape, color, margins were checked and recorded.

Epilithic and endolithic bacteria

Among 31 isolates, 15 were epilithic named P3, P4, P5, P10, P10i, P11, P12, P17, P18, P19, P20, P21, P23, P24 and P26. while 16 were endolithic named NI, N3, N4, N5, N6, M6, N10, N12, N14, N21, N22, N26, N27, N28, N30 and N40. Colony morphology features are in *Table 1b*. Gram's staining results show that out of 31 strains, 3 were Gram positive and the rest were Gram negative.

The pH of the site was 5 to 6 and temperature was 20°C. Shrubs and tall pine trees were present around the sampling site. Insects, snakes, monkeys and different birds were seen there. A total of 31 different endolithic and epilithic isolates were reported in the present study. Microscopic, morphological and biochemical characteristics of the isolates were studied by comparing these characteristics with Bergey's Manual of Determinative Bacteriology (9^{th} Edition). 16S rRNA gene sequences of the isolates

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were submitted in databank and were assigned the Accession numbers by the NCBI (*Table 1a*). Isolates N22, N21, P21, N14, N27 and P24 were identified as *Alcaligenes* spp., P17 and N40 were identified as *Lysinibacillus* spp., P20 was identified as *Brevindimonas* spp., P23 and N30 were identified *Bordetella* spp*., P4, P10, P10i, P11,* P26, P19, N4 and N10 were identified as *Pseudomonas* spp., N12 as *Pusillimonas* spp. N26 as *Fluviicola* spp., N28 as *Streptomyces* spp. and isolate P18 identified as *Parapusillimonas* spp., P12 was identified as *Serratia* spp., P5 belonged to *Stenotrophomonas* spp., and P3 was identified as *Flavobacterium* spp.,while N1,N6 and M6 were identified as *Actinobacteria* spp. (*Figures 2 a,b*).

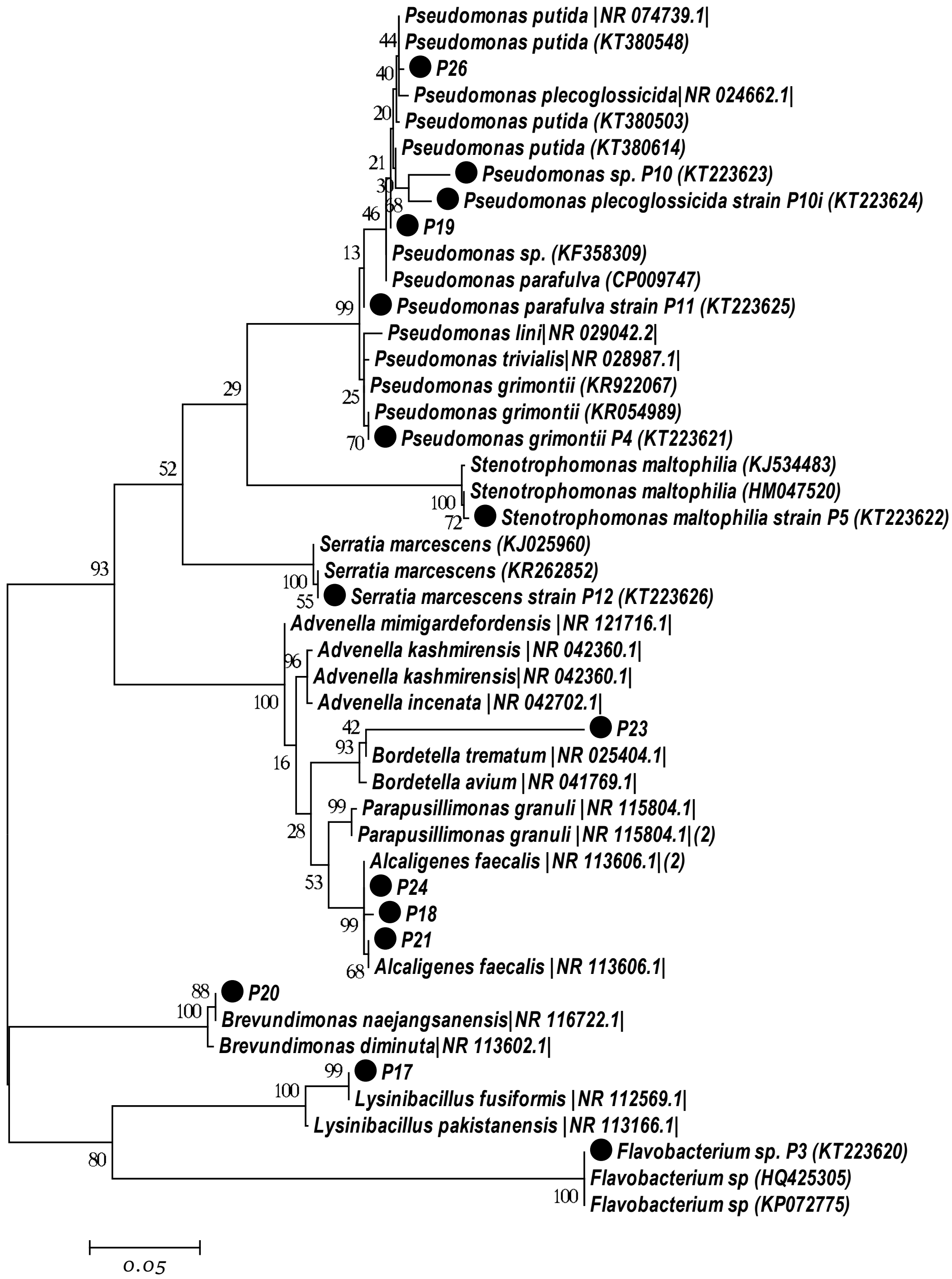

Figure 2 (a). *Molecular phylogenetic analysis by Maximum Likelihood method*

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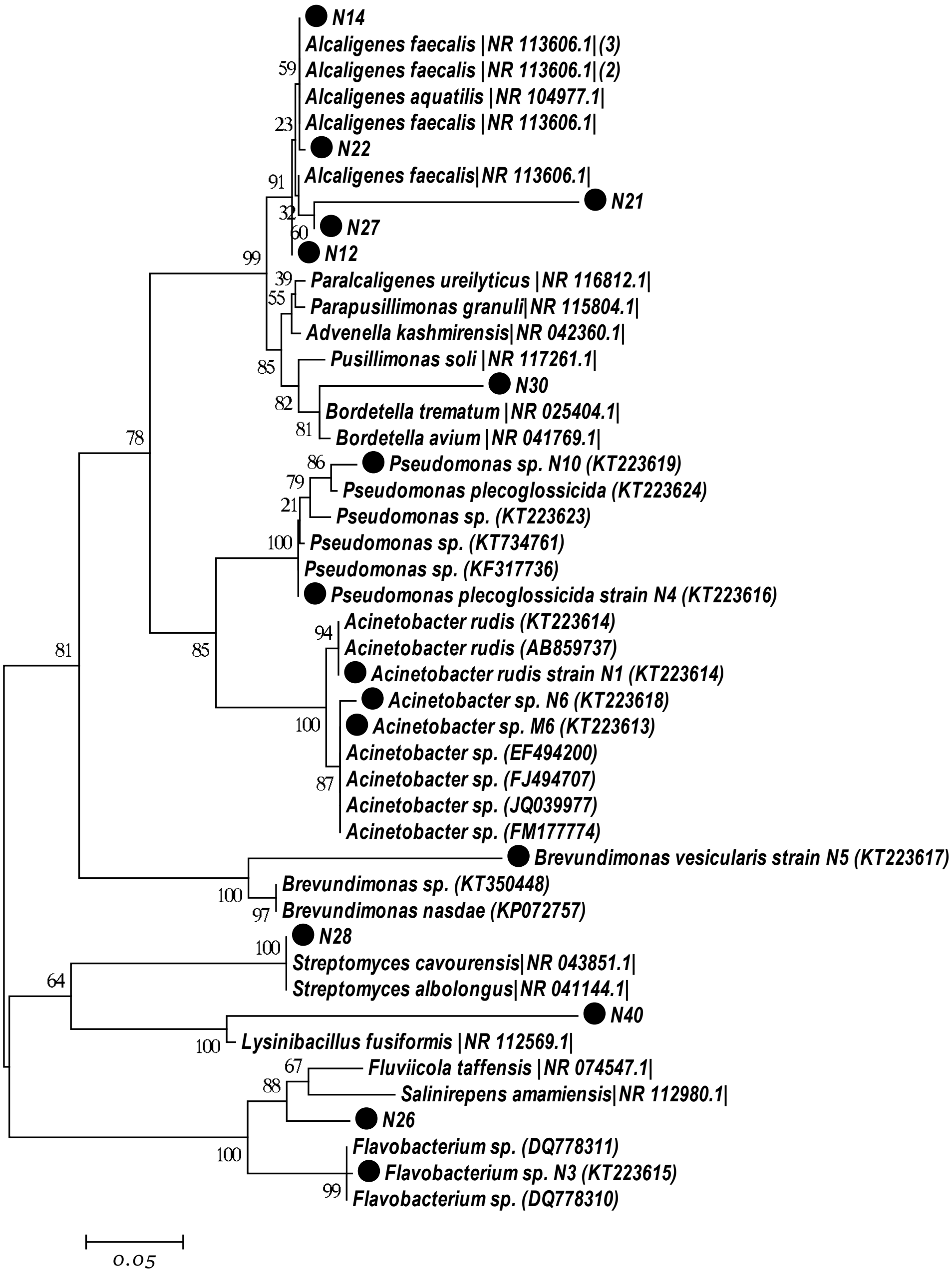

Figure 2 (b). Molecular phylogenetic analysis by Maximum Likelihood method Tamura-Nei model was used for the construction of evolutionary history using the method of maximum likelihood (Tamura K. and Nei M.1993). Next to the branches are the percentage of trees which show the associated taxa clustered together. For heuristic search intial tree(s) were automatically obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated by Maximum Composite Likelihood (MCL) method. MEGA6 was used for evolutionary analysis (Tamura et al. 2012).

Evaluation of antimicrobial potential of the isolated strains

Good antibacterial activity was shown by all the isolated bacterial strains. The endolithic bacteria *Pseudomonas* spp. N4 and *Streptomyces* spp. N28 exhibited good activity against all the three clinically isolated strains, i.e. *Pseudomonas aeruginosa,*

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Staphylococcus aureus and *Escherichia coli* Among epilithic bacteria *Brevindomonas* sp. P20 and *Bordetella* spp. P23 showed activity against the 3 human pathogenic test strains (*Tables 2 a, b*).

Table 1a. *Isolated strains with their accession numbers, query coverage and identity*

S. No.	Isolated strains	Homologous species	Accession number (Assigned)	Query coverage (%)	Identity (%)
1	P17	*Lysinibacillus spp.*	KT004373	100	99
2	N22	*Alcaligenes spp.*	KT004374	100	99
3	N30	*Bordetella spp.*	KT004375	99	89
4	N21	*Alcaligenes spp.*	KT004376	99	86
5	N40	*Lysinibacillus spp.*	KT004377	98	82
6	P21	*Alcaligenes spp.*	KT004378	100	99
7	P23	*Bordetella spp.*	KT004379	99	90
8	P24	*Alcaligenes spp.*	KT004380	100	99
9	P26	*Pseudomonas spp.*	KT004381	100	99
10	N12	*Pusillimonas spp.*	KT004382	100	97
11	N14	*Alcaligenes spp.*	KT004383	100	99
12	N26	*Fluviicola spp.*	KT004384	98	93
13	N27	*Alcaligenes spp.*	KT004385	100	98
14	N28	*Streptomyces spp.*	KT004386	100	100
15	P19	*Pseudomans spp.*	KT004387	100	98
16	P20	*Brevundomonas spp.*	KT004388	100	100
17	P18	*Parapusillimonas spp.*	KT004389	100	95
18	M6	*Acinetobacter spp.*	KT223613	100	99
19	N1	*Acinetobacter spp.*	KT223614	100	94
20	N3	*Flavobacterium Spp.*	KT223615	100	99
21	N4	*Pseudomonas Spp.*	KT223616	100	100
22	N5	*Brevundomonas spp*	KT223617	100	97
23	N6	*Acinetobacter spp.*	KT223618	100	95
24	N10	*Pseudomonas Spp.*	KT223619	99	86
25	P3	*Flavobacterium Spp.*	KT223620	100	96
26	P4	*Pseudomonas Spp.*	KT223621	99	96
27	P5	*Stenotrohpomonas Spp.*	KT223622	98	90
28	P10	*Pseudomonas Spp.*	KT223623	100	100
29	P10i	*Pseudomonas Spp.*	KT223624	100	100
30	P11	*Pseudomonas Spp.*	KT223625	99	99
31	P12	*Serratia Spp.*	KT223626	100	99

Table 1b. *Colony morphology and microscopy of isolated straisn*

S. No.	Strain	Colony Morphology	Microscopic examination
1	P17	Small, circular, cream color, flat, opaque	Gram +ive , long rods
2	P18	Small sized, raised colonies, opaque , pin pointed colonies	Gram -ive, rods
3	P19	Off white in color, flat in shape, small size, circular	Gram –ive, short rods
4	P20	Grows in net form, off white in color, long threads type	Gram –ive, long rods
5	P21	Off white color, flat colonies, small in size	Gram -ive, rods
6	P23	Large in size, sticky, irregular in shape, cream	Gram –ive, short rods

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		color, raised colonies with irregular margins	
7	P24	Small in size, circular shape, off white color, flat colonies, opaque	Gram –ive, rods
8	P26	Small in size, shiny appearance, opaque,	Gram –ive , rods
9	N12	Off white color, small in size, flat colonies	Gram –ive, rods
10	N14	Small in size, irregular in shape, cloudy type in appearance, off white in color	Gram -ive, rods
11	N21	Small in size, flat colonies, entire margins, off white color	Gram -ive, short rods
12	N22	Small in size, raised colonies, yellowish in color, circular in shape	Gram -ive, rods
13	N26	Orange color, small in size, flat colonies, irregular in shape	Gram –ive, short rods
14	N27	Transparent type, small in size, irregular in shape, off white in color	Gram –ive, rods
15	N28	Milky white in color, dry, small in size, circular in shape, raised colonies.	Gram +ive, rods
16	N30	Large in size, flat colonies, sticky type, off white color	Gram –ive, rod
17	N40	Medium size, yellowish, circular in shape, flat colonies,	Gram +ive, rods
18	P3	Orange color medium colonies, shiny,raised, round, opaque	Gram –ive, long rods
19	P4	off white color Small in size, irregular , translucent, flat colonies,	Gram –ive, rods
20	P5	Brownish dry large colonies, raised centers, irregular opaque	Gram –ive, rods
21	P10	Small offwhite colonies, round, flat, dry, opaque	Gram –ive, rods
22	P10i	Off white medium colonies, irregular, translucent	Gram –ive, rods
23	P11	Small offwhite colonies, round, flat, dry, opaque	Gram –ive, long rods
24	P12	Cherry red, raised colonies, shiny, round, opaque	Gram –ive, rods
25	N1	offWhite small colonies, hard, raised, round, dry, opaque	Gram –ive, rods
26	N3	Yellowish shiny medium colonies, round, raised, opaque	Gram –ive, rods
27	N4	Off white, small colonies, dry, opaque	Gram –ive, rods
28	N5	Offwhite, spread like a network, fibrous, dry, hard, opaque	Gram –ive, rods
29	N6	Off white small colonies, irregular, flat, shiny, translucent	Gram –ive, rods
30	N10	Brownish small colonies, pin pointed raised at center, dry, irregularly shaped, opaque	Gram –ive, long rods
31	M6	offWhite small colonies, hard, raised, round, dry, opaque	Gram –ive, rods

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Table 2a. *antibacterial activity of isolated endolithic strains*

S.No	Isolates	Activity against ATCC		
		***Pseudomonas aeruginosa* (15442)**	***Staphylococcus aureus* (6538)**	***Escherichia coli* (10536)**
1	N1	++	+	+
2	N3	+	++	-
3	N4	+++	++	++
4	N5	-	+	++
5	N6	+	-	++
6	M6	-	-	+
7	N10	-	-	-
8	N12	+++	+	++
9	N14	++	-	++
10	N21	++	+++	-
12	N22	+	-	+
12	N26	+++	-	-
13	N27		+++	-
14	N28	+++	+++	+++
15	N30	-	+++	-
16	N40	+	-	-
Key - = No Activity, + = Weak activity, ++ = Moderate Activity, +++ = Strong Activity				

Table 2b. *antibacterial activity of isolated epilithic bacterial strains*

S.No	Isolates	Activity against ATCC		
		Pseudomonas aeruginosa	***Staphylococcus aureus***	***Escherichia coli***
17	P3	++	+	+++
18	P4	+	-	++
19	P5	-	++	++
20	P10	+	-	++
21	P10i	-	+	+
22	P11	+	++	-
23	P12	+	-	-
24	P17	++	+	+
25	P18	+++	-	-
26	P19	+++	-	+
27	P20	++	+++	++
28	P21	-	-	++
29	P23	++	++	+++
30	P24	-	-	+
31	P26	+	-	++
Key - = No Activity, + = Weak activity, ++ = Moderate Activity, +++ = Strong Activity				

Intracellular and extracellular enzyme production

The bacterial isolates were efficient producers of oxidase and catalase enzymes. Almost all the 31 endolithic and epilithic strains produced catalase and oxidase enzymes. None of the strains produced Hydrogen sulfide gas. Amylase was produced by 7 endolithic and 9 epilithic bacterial strains, gelatinase was produced by 11 endolithic and 11 epilithic strains and 7 endolithic and 5 epilithic strains were positive for protease production (*Tables 3 a, b*).

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Table 3a. *Production of intracellular and extracelluar enzymes by isolated endolithic strains*

S. No	Isolates	Extra cellular enzymes activities			Intracellular enzymes activities		
15	N1	+	+	-	-	+	+
16	N3	-	+	+	-	+	+
17	N4	+	-	+	-	+	+
18	N5	+	+	+	-	+	+
19	M6	+	+	-	-	+	+
20	N6	-	-	-	-	+	+
21	N10	-	-	+	-	+	+
22	N12	-	+	-	-	+	
23	N14	+	+	+	-	+	+
24	N21	-	+	-	-	+	+
25	N22	-	-	-	-	+	+
26	N24	+	+	-	-	+	+
27	N26	-	+	+	-	+	+
28	N27	-	-	-	-	+	+
29	N28	-	+	+	-	+	+
30	N30	+	+	-	-	+	+
31	N40	-	-	-	-	+	+

Table 3b. *Production of intracellular and extracellular enzymes by isolated epilithic strains*

S. No	Isolates	Extra cellular enzymes activities			Intracellular enzymes activities		
1	P3	+	+	+	-	+	+
2	P4	+	+	+	-	+	+
3	P5	-	+	-	-	+	+
4	P10	+	-	+	-	+	+
5	P10i	-	+	+	-	+	+
6	P12	+	+	-	-	+	+
7	P17	+	+	+	-	+	-
8	P18	+	-	-	-	+	+
9	P19	+	+	-	-	+	+
10	P20	+	+	-	-	+	+
11	P21	-	-	-	-	+	+
12	P23	-	+	-	-	+	-
13	P24	-	+	-	-	+	+
14	P26	+	+	-	-	+	+

Discussion

Extreme environment of rocks have long been thought to have limited microbial diversity and activity. Isolation and characterization of endolithic and epilithic bacteria give an insight into Ayubia rocks, and production of secondary metabolites as potential drugs and enzymes for industrial use. In the past century, many endolithic ecosystems were studied which focused on cyanobacterial and algal diversity, using microscopic and culture techniques for identification (Horath and Bachofen, 2009). Variation in morphology, color and size may be induced by different stress factors which are present on endolithic sites, but one cannot rely on morphological properties after cultivation or

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in situ. Taxanomic identification is substantially mislead by morphological information alone (Norris and Castenholz, 2006).

Using 16S rRNA gene sequencing our isolates were clustered into four different groups that were predominated by Proteobacteria. One possible reason might be the slow metabolic activities of this group in order to prolong its survivability in extreme niches. In addition to this, the endolithic microorganisms differ from the known epilithic ones which is a questionable attribute at the subsurface level. According to the studies conducted by Walker and Pace (2007), the endolithic microbes in the Rocky Mountain range of Antarctica possess restricted diversity and relatively simple systems in comparison to the terrestrial ecosystems, e.g. soil.

The rocks are mostly dominated by Proteobacteria, which includes alpha, beta and gamma Proteobacteria. Other phyla were also found like Firmicutes, Bacteroides and Actinobacteria The endolithic microbial community of Ayubia is different from that of Tibet the endolithic Bacteriodetes like *Flavobacterium* (Wong et al., 2010). The epilithic and endolithic community reported by (Wierzchos et al., 2006) is almost similar to the bacterial community isolated from Ayubia. Which consists mostly of Proteobacteria with Firmicutes and Actinobacteria. Acidobacteria were not found in our study. Endolithic bacterial seven strains of *Pseudomonas* were isolated from Ayubia both endolithic and epilithic and *Pseudomonas grimontii* were also isolated from impact-shocked rocks from Haughton impact structure, Devon Island, Nunavut, Canadian High Arctic (Fike, 2002).

The bacterial similarities identified in these diverse environments pave the path to question the definite origin of these communities. The prior question which could arise is that whether these bacteria are truly multicultural or they reside prevalently in a particular environment and they are only transported into other environments.

Bacteria from lower Himalaya rocks have not yet been reported for antimicrobial agents. Secondary metabolites, mainly new and novel antibiotics are in great demand with the increase in multi-drug resistant pathogens. The microbe driven natural products from these environments have largely been ignored by scientists. All the isolated strains were screened for antimicrobial secondary metabolites. Our studies reveal that these extremophiles are good producers of antibiotics, with potent activities against the tested Gram positive and Gram negative clinical isolates. Endoliths and epiliths produce these active metabolites to prolong its survivability in these extreme conditions. *Streptomyces* sp. strain N28 showed maximum activity against all the tested isolates. In our study the endolithic strain N28 *Streptomyces* spp. (KT004386) also able to produce satisfactory zones of inhibition against both Gram positive and Gram negative bacteria. Other strains, i.e. N4 *Pseudomonas* sp. also screened positive against all the three pathogenic strains. *Streptomycetes* have greater ability to synthesise and secrete antibiotics. Recent findings showed presence of four volatile compounds which might be responsible for diverse biological activity of the secondary metabolites from *Streptomyces* sp. (Zothanpuia et al., 2017).

In actinobacteria and other microorganisms the biosynthetic systems like PKS, NRPS, and *phz*E are considered to be responsible for the production of a large number of biologically active compounds (Yuan et al., 2014).

Considerable changes occurred In the last decades regarding what scientists consider the limits of habitable environmental conditions. For every extreme environmental condition investigated, a variety of microorganisms have shown they can tolerate these conditions, but they also often require these extreme conditions for survival

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(Rampelotto, 2010). The global market for industrial enzymes was nearly US$ 4.8 billion in 2013, and it is expected to reach US$ 7.1 billion by 2018, with a compound annual growth rate (CAGR) of 8% over the five-year period, according to BCC Research (BCC Research, 2014). Endoliths and epiliths represent largest reservoir of biodiversity on the planet, and have a great potential for development of new natural products including enzymes. Endoliths and epiliths participate in biogeochemical cycling, polar food web and produce a wide variety of enzymes including amylases, cellulases, peptidases, lipases, xylanases and other classes of enzymes (Gadd, 2007).

Our result demonstrated that these endoliths and epiliths are of great importance in terms of enzyme production. Most of the bacterial isolates were able to produce both intracellular and extracellular enzymes. At extreme conditions the enzymes become more rigid, which is of great importance. *Pseudomonas* sp. N8 was a good producer of protease and amylase, which were according to the findings of Liu et al. (2007). The understanding of specific factors that confer the ability to withstand extreme habitats on such enzymes has become a priority for their biotechnological use (Dalmaso et al., 2015). It is important to study and understand these microorganisms in order to be able to use the biochemical, ecological, evolutionary and industrial potential of these endoliths and epiliths microbes.

Conclusion

According to our results, a total of 31 isolated strains have been analyzed by rRNA sequencing, of which 16 proved to be endolithic and 15 epilithic. This study comes with implication that such unique environment is for the first time explored for bacterial diversity in Himalayan range Pakistan.

In our study the endolithic strain N28 *Streptomyces* spp. (KT004386) also able to produce satisfactory zones of inhibition against both Gram positive and Gram negative bacteria. Other strains i.e. N4 *Pseudomonas* sp. also screened positive against all the three pathogenic strains. *Streptomycetes* have greater ability to synthesis and secrete antibiotics. Endolithic bacterial seven strains of *Pseudomonas* were isolated from Ayubia. The bacterial similarities identified in these diverse environments pave the path to question the definite origin of these communities. The prior question which could arise is that whether these bacteria are truly multicultural or they reside prevalently in a particular environment and they are only transported into other environments. In the last decades, substantial changes have occurred regarding what scientists consider the limits of habitable environmental conditions. These endoliths and epiliths have a great potential to produce secondary metabolites and other extremozymes. Further studies are required to produce highly pure compounds of great commercial use.

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RISK ASSESSMENT OF SAND DUNE DISASTER IN RELATION TO GEOMORPHIC PROPERTIES AND VULNERABILITY IN THE SADUQ-YAZD ERG

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Abstract. Sand dunes occupy a vast expanse of desert area in Iran and they are considered as a threat for urban areas and agricultural lands. Erg is an area of desert covered with shifting sand dunes. Saduq (Ashkezar) city, located in the west and northwest of the Yazd City, Iran, is surrounded by these mobile sand dunes. The aim of this study was the risk assessment of sand dune disaster in relation to geomorphic properties and vulnerability. The methods of satellite imageries, multi-layer perceptron (MLP) neural network, and geographic information system (GIS) were used. Sand dunes changes were mapped, and field surveys were carried out to resolve any ambiguities. The results show that the sand dunes in the center of Erg have moved closer to the city and agricultural lands. The dunes displacement (movement) was dependence to size, cover and shape and direction of them. These analyses can help to city governor and mayor for preventing of dunes offense toward the city.
Keywords: *neural network, sand dunes, satellite image, remote sensing, Yazd, Iran*

Introduction

Aeolian dune geomorphology is a product of the processes of wind erosion and deposition on land surface (Xiao et al., 2015). Thus, the formation and development of these landforms depend on windblown sand movement (Wu, 2003). The windblown sand movement is affected by the landscape characteristics, including vegetation, mulch, topography, wind regime and other regional factors. Studies in the history of aeolian geomorphology have enhanced the knowledge of dune morphology, formation and development conditions, dynamic processes of formation and evolution, and regional differentiation and regularity (Lancaster, 1995; Li and Ni, 2000; Livingstone et al., 2007). Studies in traditional dune geomorphology tend to focus on the formation and evolution of dunes that are in regions unaffected by complex terrain (Pye and Tsoar, 1990; Tsoar and Blumberg, 2002); however, sand dune formation also occurs in complex terrain. This research focuses on using new methods and softwares for visualization the trend and drift of the Erg and dunes that can be a danger for settlers. Tavakkolifard et al. (2013) were determined the risk of sand transportation to residential

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areas around Kashan-Iran Erg using anemometry data analysis without geographic information system (GIS) and remote sensing. Sandy landforms are morphologically divided into sand seas (Ergs), isolated dunes, dune fields, sandy plains, and sheets. At several locations, sand encroachment causes hazards to farmlands, highways, population centers and other infrastructures. Remote sensing techniques could be used effectively to monitor sand dune movements by comparing the multi-temporal satellite images (Pye and Tsoar, 1990) in Saduq having Erg, dune fields, and sandy plains. Bars, mulch, vegetation cover, or complex topography can decelerate, accelerate, and otherwise control the direction of the wind or windblown sand flow, particularly on the leeward side of trees and bushes. These changes in windblown sand movement lead to different erosion and deposition near the complex terrain, forming a variety of sand dunes, including small dunes, single dunes and string dunes (Evans, 1962; Cooke and Warren, 1973; Tsoar, 1983; Elbelrhiti et al., 2005; Elbelrhiti, 2012). Past studies in this area have documented local changes in bedform type (Al-Masrahy and Mountney, 2013) and associated spatial variation in aeolian lithofacies distributions in champ dune fields (Breed and Grow, 1979; Sweet et al., 1988; Kocurek and Lancaster, 1999; Saqqa and Atallah, 2004; Baas, 2007; Bullard et al., 2011). However, relatively few researchers have endeavored to measurably substantiate the form of spatial variability of the dune and interdune morphology from the center of aeolian dune-field systems to their borders (Jamali and Abdolkhani, 2009; Kocurek and Ewing, 2005; Wilkins and Ford, 2007; Ewing and Kocurek, 2010a; Kocurek et al., 2010; Hugenholtz and Barchyn, 2010). In modeling desert sites, Mili (2016) studied sites where the desert merges with wet coastal zones in Tunisia. Mili used GIS and modeling between water storages and some social factors in aquifer and floodplain sites merging of desert sites as important natural water storage and as natural protection against flooding. Meyer and Crews Meyer (2016) conducted a study to examine the suitability and fragmentation analysis in the Central Kalahari Game Reserve desert region, using GIS, remote sensing and GPS . the suitability analysis revealed three suggested corridors for protection Wildlife and Tourism. Potter (2016) has studied the analysis of desert sand dune migration patterns using Landsat image time series in the Southern California Desert. The predominant direction of sand dune movements was correctly mapped from the satellite imagery, as well as the migration distances of the leading edges of sand dunes over the entire 1992-2010 monitoring period. Some studies concentrated on extraction of sand dune encroachment by GIS (Dakhla Oases, the western desert of Egypt) (Ghadiry et al., 2012).

McCulloch and Pitts (1943) created a computational model for neural networks based on mathematics and algorithms called threshold logic. This model paved the way for neural network research to split into two distinct approaches. One approach focused on biological processes in the brain, and the other concentrated on the application of neural networks to artificial intelligence. In this study, some methods were composed to obtain sand dune movement. By using intelligence method such as multi-layer perceptron (MLP) and using a statistical method, we can find land changing and trend in Erg and we can control sand dune moving according to their movements.

The aim of this study is to model sand dune movement related to their shape properties (Dune's stoss and lee side, mulch, dune size) and vicinity threat in the Saduq-Yazd Erg. The specific objective is to evaluate effective factors in sand dune movement and its vulnerability using neural networks, statistical modeling and field surveys. Hypotheses are: (i) Sand dunes with different shapes are going to move, and change

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differently, and (ii) Erg movement towards the city depends on the wind direction and relative location of Erg.

Materials and methods

Study area

This survey was conducted on Yazd Erg dune groups in the center of Iran. The Erg sand dune lies near the Saduq (Ashkezar), and it is 20 km away from Yazd city. The area surrounded by some vulnerable land use such as road, farmland, and villages. The height of Yazd plain from the see level is 1200 m with the general slopes of less than 2%.The plain is located in north of the Shirkouh Mountains (4040 m above sea level), which is the highest mountain range in central part of Iran. The Erg has been covered with flowing sands, its mean rainfall in the past thirty years was 61.7 mm the average annual temperature was 18.1°C and maximum, and minimum temperatures are +46.5°C and -15.5°C, respectively. This area has a cold and dry climate, according to Emberger climate classification, and according to the Demarton classification, its climate is ultra-dry cold. The study area has some loose row and single vegetation cover such as *Haloxylon sp.* and *Tamarix sp.* (Baghestani et al., 2015; *Figs. 1* and *2*).

Methods

Following methods in literature (Tsoar, 1983; Tang et al., 2011), we studied the morphology of dune types (e.g. parabola and linear dunes) by using a variety of methods and measured parameters including slope, length, height and direction (Xiao et al., 2015). In the present study, the morphology and movement of sand dunes would be investigated using a variety of material and methods, including satellite imagery, remote sensing, intelligence GIS and statistical methods to find their possible threats for city and its settlers; thus the Landsat 5 Thematic Mapper (TM) acquired in Jun 1986, The Advanced Spaceborne Thermal Emission and Reflection Radiometer (ASTER) acquired in Jun 2001, Jun 2010, and Landsat 8 Operational Land Imager (OLI) and google earth remote sensing images acquired in Jun 2016, were used to identify the dunes,dunes transition and their geomorphological parameters. These images have been used to analyze the morphology and topography of the sand dunes. Wind data recorded by Iran Yazd Meteorological Information Center has been used. Wind data included daily maximum wind speed and direction (*Figs. 4* and *5*). The sand dune boundaries and ridges which were clearly defined in the remote sensing images were imported in remote sensing software. These temporal satellite images were used for trending dunes by a modern method such as MLP while the classical remote sensing techniques for monitoring sand dune migration are carried out comparing multi-temporal images and aerial photographs (Gad, 2016; Gad and Ali, 2011; Kamel et al., 1982; Hereher, 2000; Elbana, 2004). By using the GIS software, the continuous polygons and polylines to represent the areas and ridges of the sand dunes were extracted from the georeferenced, remote sensing images. Some ground control points (GCP) were used for monitoring and selecting the sand dunes shapes and properties and then for doing statistical analysis in statistical software (*Fig. 2*). Some infrastructure and the available data were considered for predicting modeling. Digital elevation model (DEM) data are acquired from the US National Aeronautics and Space Administration's ASTER Global Digital Elevation Model Version 2 (GDEM V2) with 30-m horizontal and 20-m vertical

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resolution. also other infrastructure layers that can affect the transition of dunes, such as road networks, villages, and city location, were prepared. These layers and classified land uses of the region were imported into GIS software. For predicting the dune movement, it was assumed a static condition for GIS layers in transition sub model. The artificial neural network (ANN) operation uses some layers for trend changes. In this research ANN was run with default options in GIS software (e.g. 10000 iterations, 25000 sample, 50% for test and 50% for training, stop at 0.01 Root Mean Square Error (RMSE)). Then decision making was made for future programs based on statistical and GIS method results. The flow chart of steps is shown in (*Fig. 3*).

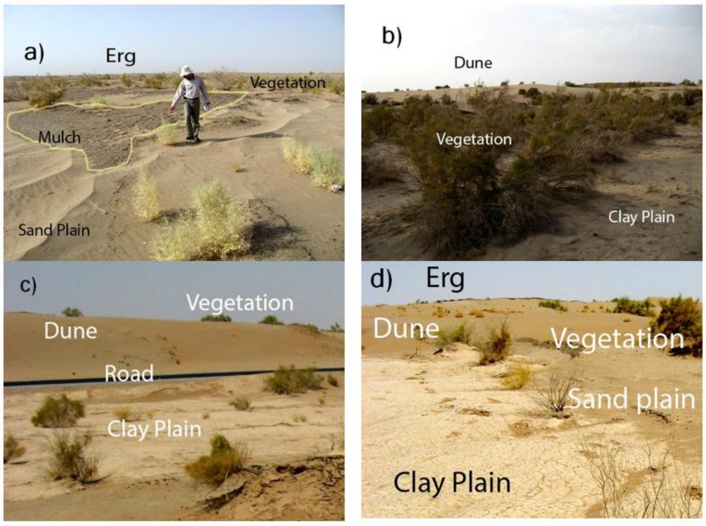

Figure 1. *Photo of Erg and dunes a) some mulch in the area b) Taq vegetation c) road next to dune d) clay and sand plain*

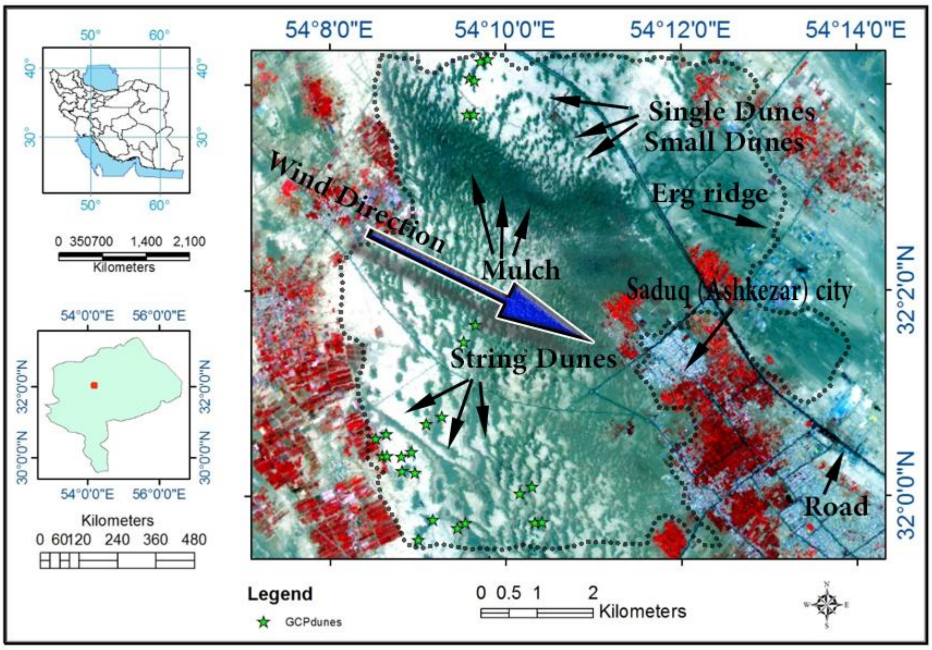

Figure 2. *Location of the study areas, stars shows ground control points (GCP) on the ASTER 2010 image Red, Green, Blue (RGB) 432*

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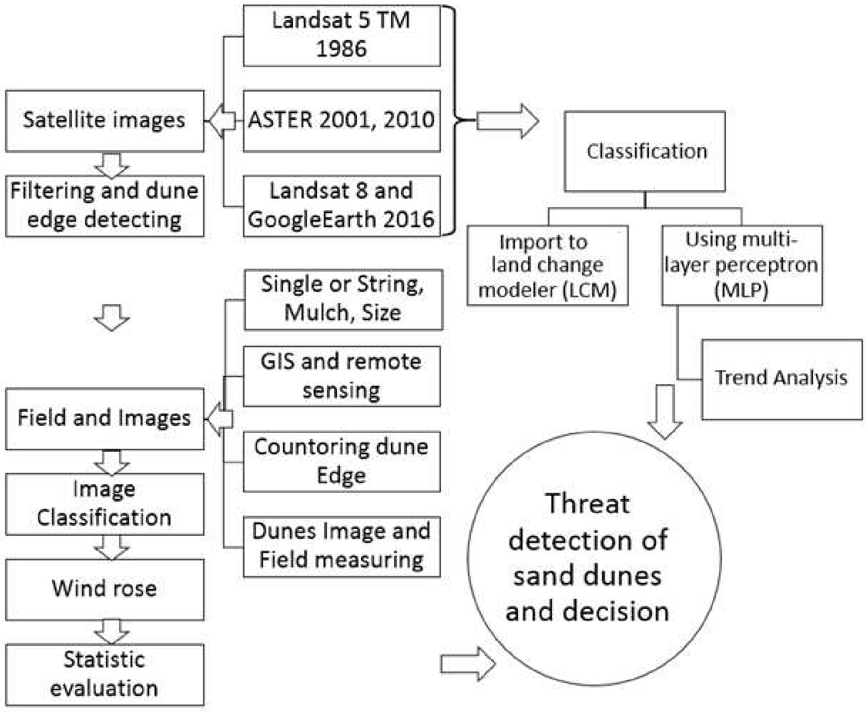

Figure 3. *Flow chart of study*

Results and discussion

Wind and dune movement

The statistical results show that the characteristics of dunes are greatly different in different sand formation progresses and environmental characteristics. Dunes kurtosis, collection, and movement, reflect the influence of intensive winnowing processes downwind of obstacles. Results obtained from the Wind Rose Plot (WRPLOT) software analysis indicated that the direction of prevailing winds in Yazd Erg was generally from west to northwest. Interpretation of aerial photos and satellite imagery show that sand dunes were formed along these predominant winds. These kinds of dunes have two wind sides with different slopes. The low slope side (the upwind side) shows the direction of the predominant wind. Among the anemometric data employed in the research, the frequency percentage of calm winds with a velocity of less than one knot (0.54 m s^{-1}) was 0.59%, and average wind speed was 6.83 m s^{-1}. Based on the storm rose, the frequency of winds of a velocity less than 6 m s^{-1} (threshold velocity) is 93.79% as observed from Yazd meteorological station, and the frequency of dust storm winds is reduced to 6%. The analytical results of the grain size in Erg sand dunes show that the mean grain sizes vary between 180 and 300 µm (about 280 µm), and the values of sorting and Skewness are 1.4 and -0.17, respectively (Mesbahzadeh and Ahmadi, 2012). *Figures 4* and *5* shows that the dominant wind has come from the northwest, thus the dunes were expected to move toward southeast.

Movement

The dunes were selected from the satellite images for the statistical survey during 1986 to 2016 (30 years). A comparison was made for size in two groups, small and big

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dunes. These samples were independence, and nonparametric test Man-Whitney U test was used for this comparison. *Figure 5* shows more movement in small dams, so that about 12 dunes had about 50m movement or drift. The result shows more movement in small dunes. The other comparison was conducted about the presence of mulch on the dunes (*Figure 5*). The dunes with mulch and without mulch have 38.3 m and had 42.7 m movement, respectively. We understood the dunes with mulch are more stable. The mulch is the fuel that is used around 1980 in the region and its protective effect is acceptable.

In another set of dunes comparison was statistically dunes and single-threaded. The two groups were significantly different from the dunes. During the time that the satellite images were compared to string and collected dunes have more than a single movement (*Figure 5*).

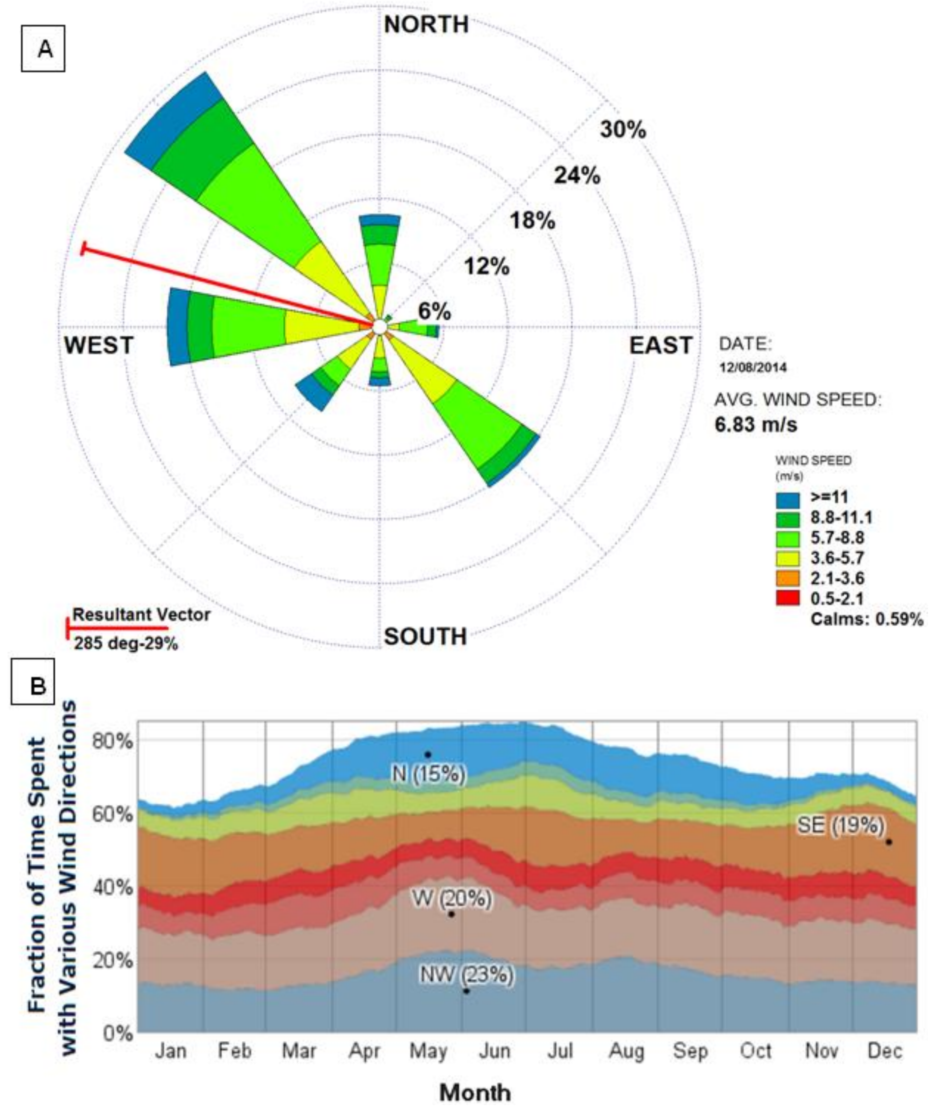

Figure 4. *A: Ten years Annual WRPLOT of Yazd region (2004 to 2014). B: The fraction of time spent with the wind blowing from the various directions on a daily basis. Stacked values do not always sum to 100% because the wind direction is undefined when the wind speed is zero (2006 to 2012) (https://weatherspark.com/averages/32839/Yazd-Ir) 12/0/2016*

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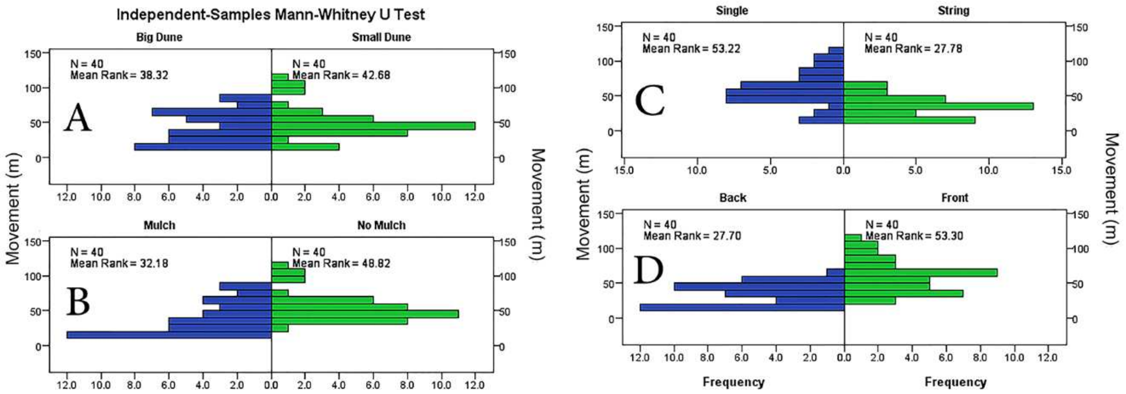

Figure 5. *Comparing displacement (movement) A) big and small dunes, B) Using mulch and no mulch, C) single and string dunes, D) Dune's stoss (front) and lee (back) side relative to wind direction*

Movement and vulnerability trends

Sand dunes were sampled using satellite imagery and field surveying. *Figure 6* shows four regions which were selected for measuring and monitoring sand dune movement base on their geomorphic properties.

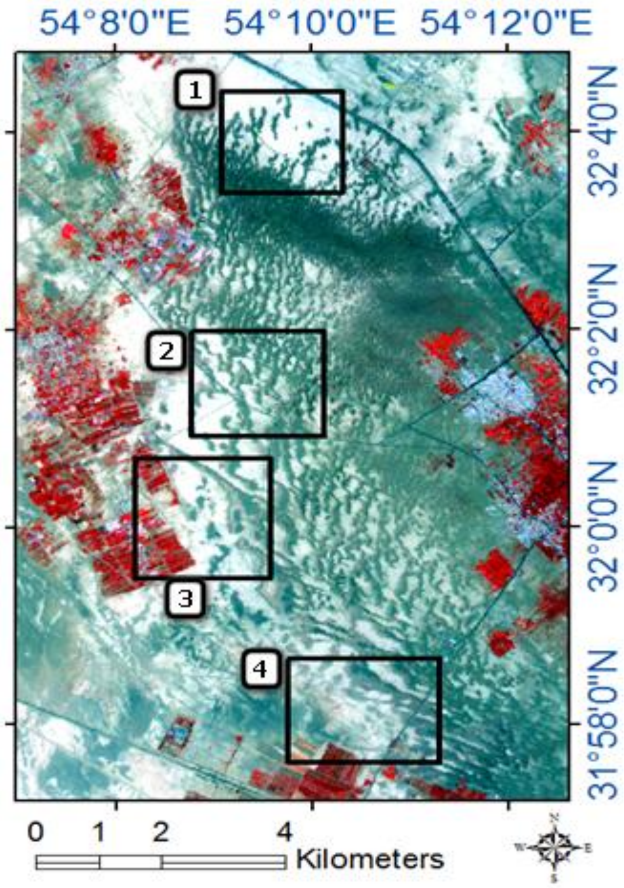

Figure 6. *Sand dunes sampling 1 to 4 for Figure 7*

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Figure 7 shows Sand dunes movement measuring and making comparison by size using GCP and satellite images, row2 comparison by Mulch row3 comparison by shape (single and string) row4 comparison by shape (stoss (front) and lee (back) sides; *Fig. 7*).

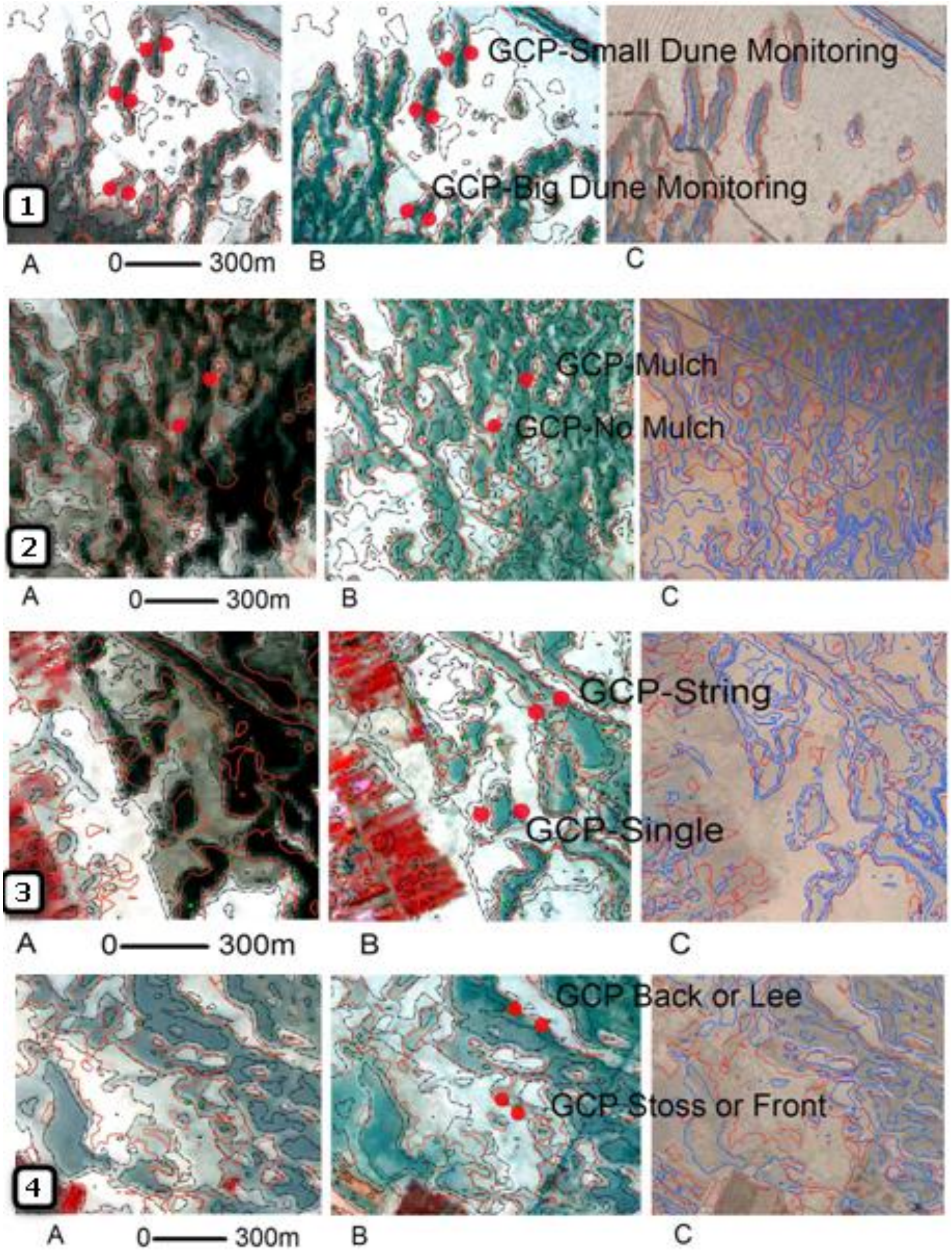

Figure 7. *Row1: Sand dunes movement measuring by size from GCP (ground control point) and image: big and small sand dunes, left to right TM1986, ASTER2010 and Google Earth (GE) (lines shows recent border and edge of dunes in past), Row2:Sand dunes movement measuring by Mulch from GCP and image: Mulch and no mulch on sand dunes, left to right TM1986, ASTER2010 and GE, Row3: Sand dunes movement measuring by shape from GCP and image: Single and String sand dunes, left to right TM1986, ASTER2010 and GE, Row4: Sand dunes movement measuring by shape from GCP and image: Dune's stoss (front) and lee (back) sides, left to right TM1986, ASTER2010 and GE*

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The dune transition and trends could be achievable by using GIS software and MLP neural network. MLP neural network analysis results in preparing four maps of dune trends: A: Potential for the transition from dune to vegetation (west of the region has more potential); B: Potential for the transition from dune to sand plain (west of the region has more potential); C: Potential for the transition from sand plain to vegetation (west of the region has more potential); D: Potential for transition from sand plain to clay plain (east part of the region has more potential).

It can be found that dunes are stabled in this region, and residents would change land uses to vegetation. Trend and transfer were assessed for vegetation to sand dunes. The city and villages are threatened with the direction east to west, and most unsuitable changes occurred near the city and where there are intensive road networks (*Fig. 8*).

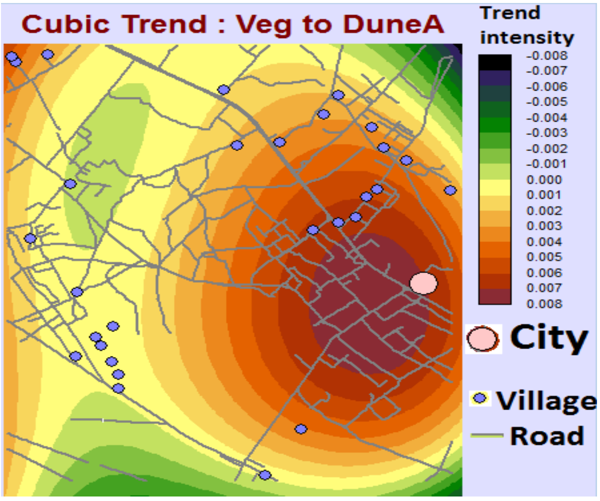

Figure 8. *Trend and transfer for vegetation to sand dunes; the city and villages are threatened by this trend east to west, and more severe change is near the city and where it is road intense*

The vulnerability is a weakness of an asset or group of assets that can be exploited by one or more threats (International Organization for Standardization/International Electrotechnical Commission (ISO/IEC), "Information Technology-Security Techniques-Information security risk management" ISO/IEC FIDIS 27005:2008). In this region, road networks, agricultural and residential land uses are vulnerable, and sand dune movement is a hazard factor that should be controlled. The settlers, villagers, and government are expanding vegetation covers to reduce the vulnerability of land uses now.

In the past (about 1980) black oil mulch was used on the part of Erg near villages and cities, but today's using vegetation cover and biological controlling is the main policy of government because it is safe and healthy. Settlers were encouraged to plant more row trees perpendicular to the predominant wind by distribution sidles by Saduq (Ashkezar) and Yazd Natural Resources and Watershed Management Office.

Some settlers and other people in a 50-year period should leave their assets, lands, and homes because of the sand dunes onset toward their lands. This problem should be studied by modern methods such as remote sensing, GIS, and MLP to show the settlers and the decision makers the future of sand dune movements and their threats. In anticipation of the neural network and trend study it was found that sand dunes will migrate to the areas with denser road networks and residential (city and villages) areas. Rural and urban development and road networks, as well as the prevailing wind from the

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west to the east, could be the main reasons for this trend. Reduced groundwater levels and declining rainfall can also reduce vegetation during these recent years, and it has caused the development of the dunes. Vast areas with 30 km far from the Yazd Erg in the west has no vegetation cover, thus this region also can be a sand source that the wind can bring them into the Erg. This sand source is also caused growing the sand dunes and incursion them toward residential areas. Using MLP neural network, GIS, and statistical surveys could show the discussed changes and trends in the status of sand dunes by measuring in time series satellite images as well as filed measures. Mirhosseini et al. (2016), Julien et al. (2011), Emami et al. (2014), Zhang et al. (2014), Hegazy and Kaloop (2015) also confirm this trend and changes in the Erg and dunes related the variation land uses. Considering these results, municipal managers can decide better for urban development by preventing further conversion of vegetation into the dunes and moving dunes toward urban areas.

Main findings related to wind effects revealed that predominant wind direction is from northwest to southeast.

Conclusion

According to the findings,sand dunes move toward the vulnerable area in southeast and east of Erg such as Saduq (Ashkezar) city and roads. Small, single and without mulch dunes moves more than big, strong and mulch covered sand dunes.

The trend of movement base on wind direction is toward the city and land change from dune to vegetation, such that west of the region has more potential and show villager positive activity in planting. Another trend shows a transition from sand plain to vegetation so that west of the Erg region has more potential and Erg is moving from west to east following the prevailing wind direction. The dunes displacement (movement) was dependence to size big or small, cover mulch or without mulch and shape and direction of them. These analyses can help to city governor and mayor for preventing of dunes offense toward the city. Today's primary policy is using vegetation cover and biological controlling by the government. Settlers were encouraged to plant more row trees perpendicular prevailing wind by distributing seedlings by population centers.

Future extensions of this research for future researches: It is proposed such as using other spatial factors for logistic regression to find effective factors on Erg expansion and sand dune movement. The relation between groundwater, rainfall, and Erg changes can be studied. Installing of some wind break and effect of the sand movement during years can be considered.

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IDENTIFICATION OF THREAT AND PRESSURE FACTORS ON PROTECTED AREAS USING RAPPAM METHODOLOGY (CASE STUDY: KHUZESTAN PROVINCE, IRAN)

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Abstract. Given the significance of conserving and improving lands management with a high conservation value, chosen for conserving and recovering plant and animal habitats as protected areas on a large scale, this study was conducted with the aim of identifying threat and pressure factors of protected areas in Khuzestan Province, Iran. In this study at 2015, using Delphi method, the threat and pressure factors of protected areas in Khuzestan Province were identified. Next, using RAPPAM methodology presented by WWF organization for managerial effectiveness of protected areas, the greatest threat and pressure factors of regions were investigated by individual regions and collectively for all of the regions. The results of this study suggest that five major factors of collective threat and pressure across all protected areas in Khuzestan Province in Iran are overgrazing (405.07), dam building (374.25), shortage of workforce and equipment (338.22), conversion of land use (283.23), cutting of trees (243.72). In contrast, the lowest threat and pressure factor was the effects caused by immigration of nomads (43.88). Among the protected areas studied in Khuzestan Province, the greatest collective threat and pressure factor is related to Dez protected area (674.41), while the lowest threat and pressure factor is related to Koraii protected area (342.51). Thereafter, attempts have been made to present some suggestions to solve or mitigate the effects of these factors given the pressure and threat factors present in the protected areas of Khuzestan Province. Among these solutions can one mention offering the license of grazing to local communities in the area in a periodical fashion, consideration of incentive measures for the area's staff in order to enhance motivation and aid in conservation of the area, holding training courses for the staff suited with the new needs of the area, recovery of the boundaries of the protected areas to prevent to transgression to the area and alteration of land use.
Keywords: *overgrazing, biodiversity, dam building, deforestation, Delphi method*

Introduction

Protected areas are regions of land or sea that are kept specifically for conserving and maintaining biodiversity, natural resources, and contract resources in order to be managed and conserved legally through common traditional methods (Giuliani, 2007; Rana et al., 2010). Today, these regions are vital instruments for preserving biodiversity in the face of the global crisis of extinction of species and losing universal natural capacity for supporting human habitat. Furthermore, these regions are crucial for guaranteeing a healthy society which provides diversity of life by preserving the species and habitats. They also help survival of living creatures, each of which is a product of millions of years of evolution on the earth (Secretariat of the Convention on Biological Diversity, 2008). Suitable management of protected areas requires full understanding of the existing conditions, accurate implementation and planning as well as regular

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supervision. Sometimes there is a need to develop some changes in the management of a protected area if required (Nolte et al., 2010).

In 2010, a report was published across Europe entitled evaluation of the effectiveness of management of protected areas. In this study, RAPPAM [Rapid Assessment and Prioritization of Protected Area Management] method is known as one of the international and valid methods for evaluating the effectiveness of management of protected areas (Hockings et al., 2010). RAPPAM method, WWF [Wildlife World Federation] which is one of the persistent attempts for developing a special evaluation instrument congruent with the framework of WCPA [World Commission on Protected Areas] , is one of the most common methods of evaluation of effectiveness of management of protected areas in the world. Through this method, over 1600 protected areas have been examined in 49 countries (Leverington et al., 2010).

In studies conducted on the pressures and threats of protected areas using RAPPAM methodology in different countries, various factors have been identified as threat and pressure factors. According to RAPPAM methodology, pressure refers to activities and processes that incur harmful damages to the integration of the protected area such that it results in decreased biodiversity or biocapacity or damage to natural resources. This pressure might be caused by legal or illegal activities either directly or indirectly. Threat refers to a potential pressure as a result of which destructive effects might take place in the protected area (WWF, 2003).

In a study conducted in Russia, hunting, tourism, and natural disasters were identified as the most extensive threat and pressure factors of protected areas, where these factors have been detected in over 90% of areas (Tyrlyshkin et al., 2003). In South Africa (2003), in all areas, the attack of foreign plants has been the most important pressure and threats factor of areas due to alteration of land-use in protected areas to agriculture and other usages (Goodman, 2003). In Butane (2003), the major threat and pressure factors in protected areas in the country are caused by illegal hunting, overgrazing, road construction, and construction in general (Tshering, 2003). In Georgia (2003), hunting and overgrazing are among the major factors of threat and pressure for the protected areas (Zazanashvili, 2003). In China (2003) and among the serious threat and pressure factors for areas are increased tourism, illegal hunting, and commercial use of forests (apart from timber cutting) (Li, 2003). In a study conducted in Cambodia (2004), it was found that 53% of threat factors of the country's protected areas is related to forest clearing (cutting of trees) and the most important pressure factors of the areas in the country are caused by illegal cutting of trees, illegal hunting, fishing, and extraction of nontimber forest products (Lacerda et al., 2004). In Mongolia (2005), illegal hunting in the wildlife claimed the highest score among the pressure and threat factors in the protected areas in this country (Batsukh and Belokurov, 2005). In Nepal (2006), overgrazing has the highest score of pressure and threats factor for protected areas in Nepal (Nepali, 2006). In Romania (2006), the considerable threat factors in areas are legal and illegal harvest of timber trees, altered land-use, and illegal hunting. The important pressure factors are altered land-use and entrance to the system and inappropriate management of wastewater as well as construction (Stanciu and Steindlegger, 2006). In a study carried out on the effect of urbanization in tropical Mangrove forests in Fortalza, Brazil (2008), hunting, fishing, entrance to areas, harvest of wood, collection of nontimber wood, activities in land adjacent to protected areas, and tourism have been identified as the most serious threats in protected areas of this region (Leverington et al., 2008). In Brazil (2010), the greatest threat and pressure

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factors introduced to the areas of this country are caused by the activities related to living creatures in the area (Simões et al., 2010). In a study performed for rapid evaluation of the pressure factors in protected areas along with the threat factors in national parks, the greatest threat factors of national parks were related to grazing, hunting, harvest of timber trees, and fishing. These threat and pressure factors are directly related to the vulnerability of the area (Nchor et al., 2012). In a study dealing with analysis of the threat and pressure factors in wetlands of South of Iran using RAPPAM method in Khuzestan Province (2014), it was found that extensive changes in land use into residential and agricultural uses, exhaustion and fracture of oil pipes and oil products in the wetland, legal and out of season hunting claimed the highest scores of pressure and threat factors, respectively (Sabzghabaei et al., 2014). In a study carried out on the evaluation of the effectiveness of management in Mangro protected areas in a 10-year period (2016), it was concluded that across all the years, factors including animals, harvest of plants, residential areas, firing, and the hazards caused by pollution have claimed considerable effects in Environmental Protection Agency (EPA), most of which would probably be irreversible. However, in 2006 and 2012, the managers of protected areas stated that progression of residential areas is considered a threat for preserving natural resources (de Almeida et al., 2016). Protected areas in Iran have been divided into four groups by Iran's environmental management (DOE), and these areas have grown considerably over the past 10 years in Iran. In total, Iran possesses 253 protected areas accounting for around 10.12% of Iran's area (Mirkarimi, 2007). From among the 514 bird species existent in Iran (Mansoori, 2008), 285 species have been identified in Khuzestan Province (Nabavi et al., 2010). Similarly, from among 194 mammal species in Iran (Zyaii, 2008), 58 species have been identified in Khuzestan Province (Nabavi et al., 2010). The Iranian yellow deer species, on the verge of extinction, has been preserved artificially in natural and enclosed parks for many years, and Dez and Karkheh protected areas in Khuzestan Province are considered the only natural habitats for this mammal In Iran (Nabavi et al., 2010). This research has been conducted with the aim of determining the threat and pressure factors present in protected areas in Khuzestan Province and identifying protected areas in Khuzestan Province with the greatest threat and pressure factors. The scoring and prioritization of these factors have been carried out according to RAPPAM methodology.

Study area

Khuzestan Province with an area of 63633.6 km^2 is situated between 29° 57' up to 33° 0' of the northern latitude off the equator and 47° 40' up to 50° 33' of the Eastern longitude of the Greenwich Median in the South West of Iran. Khuzestan Province has 19 areas under management with an area of 733102 hectares (Gitashenasi, 2007). among the most important protected areas in this province are Dez, Karkheh, Shalo and Mongasht, Shimbar, Koraii, and Haft Shahidan protected areas. The geographical location, area and characteristics of these studied protected areas are provided in *Table 1* and *Figure 1*.

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Table1. *The geographical location and area and characteristics of studied protected area*

Protected area	North latitude	East longitude	Area (ha)	Indicator fauna	Indicator flora
Dez	32° 15′- 35° 31′	48° 21′- 48° 51′	18711	*Dama dama mesopotamica, Vulpes vulpes, Francoljnus francdinus,Turdoides altirostris*	*Vitex seudonegando, Capparis spinosa, Calotropis frocera, Rubus anatolica, Populus euphratica*
Karkhe	32° 57′- 36° 31′	48° 10′- 48° 32 ′	8600	*Dama dama mesopotamica, Mellivora capensis, Felis chaus, Canis lupus, Marmaronetta anas angustirostris, Threskiornitis aethiopicus, Ardeola ralloides, Circus macrourus*	*Lycium depressurm, Salix sp, Capparis spinosa, Calotropis frocera, Tamarix sp, Cynodon dactylon, Trifolium repens, Vitex seudonegando*
Shimbar	32°39′- 32° 9′	49° 30′- 49° 44′	54139	*Sciurus anomalus, Martes foina, Canis lupus, Herpestes edwardsii, Ursus arctos, Panthera pardus saxicolor, Capra aegagrus, Ovis orientalis, Merops aplaster, Alectoris chukar, Ammoperdix griseogularis*	*Vitex pseudo, Populus euphratica, marus alba, Ficus carica, Quercus branti, Amygdalus scoparia, Mespilus azarolus, Pistacia atlantica, Pistacia khinjuk, Myrtus communis*
Shalo and Mongasht	31°35′- 31°45′	50°15′- 50°40′	12992	*Canis lupas,Ursus arctos, Vulpes vulpes, Alectoris chukar, Ammoperdix griseogularis, Canis aureus*	*Quercus brantii, Pistacia khinjuk, Ficus carica L.subsp rupestris, Astragalus gummifer,* Crataegus *aronia, Amygdalus scoparia*
Haft Shahidan	32° 3′ – 32° 8′	49° 04′- 49° 13′	9609	*Canis aureus, Canis lupus, Vulpes vulpes, Perdicinae sp, Ammoperdix griseogularis, Streptopelia decaocto*	*Zizyphus mauritiana, Ziziphus nummularia, Pistacia khinjuk, Ficus carica L.subsp rupestris*
Koraii	31° 35′- 31° 59′	49° 00′- 49° 23′	39420	*Ovis orientalis, Canis lupus, Canis aureus, Cricetulus migratorius, Felis silvestris, Hystrix indica, Pipistrellus pipistrellus, Martes foina, Hyaena hyaena, Lepus capensis, Egretta garzetta, Upupa epops*	*Zizyphus mauritiana, Amyg dalus scoparia, Achillea wilhelmsii, Ficus carica, Pistacia khinjuk*

(Albodovirej, 1994; Dinarvand, 2015; Booklet annual report on protected areas in Khuzestan Province, 2015)

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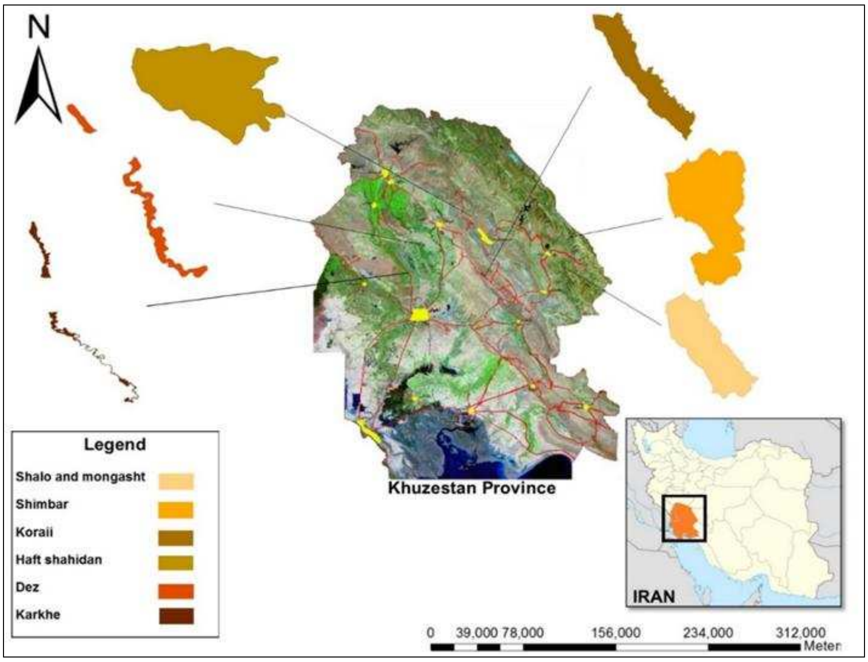

Figure 1. *Situation of protected areas under study in Khuzestan Province*

Methodology

In this research, RAPPAM methodology presented by WWF organization for rapid evaluation and protection prioritization of protected areas has been used (WWF, 2003). RAPPAM methodology has been prepared according to questionnaire method, where in this study after determining the study scope according to studies, reports, and valid evidence available on protected areas, a list of pressure and threat factors of the areas was prepared, where the factors and activities with greater effectiveness were chosen according to Delphi method. Delphi is a method based on intuitive opinions of experts in which a group of experts reach a consensus after expressing their opinions about a certain problem (Lang, 1994).

One of the reasons of choosing Delphi method is the most prominent feature of this method regarding its ability in integration of opinions among a diverse group of participants (Stitt-Gohdes et al., 2004).

Thereafter, according to RAPPAM methodology, the extent, impact, and permanence of the effects were scored by workgroups consisting of specialists and experts, managers, beneficiaries, and all stakeholders associated with studied protected areas. The extent of the effect is a range of pressure and threat factors on the region. The impact refers to the direct or indirect degree of threat and pressure on the resources of the protected area. Permanence of effect means the duration required for the resources affected by the protected area to be improved through human intervention or without it. "Improvement" denotes recovery of functions, ecological processes and structures before the occurrence of pressure or development of threat. The improvement time is referred to the duration required for mitigation or elimination of threat and pressure factors through managerial interventions or natural processes. Each of the mentioned characteristics was scored according to *Table 2*.

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Based on scoring in *Table 2*, the degree of pressure and threats is obtained through multiplication of three parameters, which can culminate in a score between 1 and 64 for each threat and pressure factor (WWF, 2003).

Table2. *Description and Scoring For Threats and Pressures*

Features threat and pressure	The range of threats and pressure	Score	Description
Extent	Throughout	4	Activity occurs in 50 per cent or greater of its potential range
	Widespread	3	Means occurrence in between 15 and 50 per cent
	Scattered	2	Occurs in between 5 and 15 per cent
	Localized	1	Occurs less than 5 per cent of its potential range
Impact	Severe	4	Impact is serious damage or loss to protected area resources
	High	3	Impact is significant damage to protected area resources
	Moderate	2	Impact is damage to protected area resources that is obviously detectable, but not considered significant
	Mild	1	Impact is damage that may or may not be easily detectable, and is considered slight or insignificant
Permanence	Permanent	4	Damage is damage to a resource that cannot recover, either by natural processes or with human intervention, within 100 years
	Long term	3	Damage can recover in 20 to 100 years
	Medium term	2	Damage can recover in 5 to 20 years
	Short term	1	Damage can recover in less than 5 years

(WWF, 2003)

Results and Discussion

In this research, according to Delphi method, 20 questionnaires were given to the determined workgroups in order to specify the threat and pressure factors in the studied protected areas. The number of threat and pressure factors in the protected areas obtained based on Delphi method included 14 factors in Dez protected area, 14 factors in Karkhe protected area, 14 factors in Shimbar protected area, 13 factors in Shalo and Mongasht protected area, 15 factors in Haft Shahidan protected area, and 12 factors in Koraii protected area, provided in *Table 3*. Thereafter, according to RAPPAM methodology, scoring was conducted and the results obtained from scoring of the threat and pressure factors in the studied protected areas are provided in Diagrams (*Figure 2* and *Figure 3*).

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Table 3. *List of Pressures and Threats used in the Evaluation of Protected Areas in Khuzestan Province, Iran*

Threat and pressure factors	Cumulative threats	Cumulative pressures	Dez	Karkheh	Shimbar	Shalo and Mongasht	Haft Shahidan	Koraii
Drought	133.44	143.19				✓	✓	✓
Fire	56.7	56.87	✓	✓	✓	✓	✓	✓
Overgrazing	205.98	201.07	✓	✓	✓	✓	✓	✓
Cutting tree	113.43	130.29	✓	✓	✓	✓	✓	✓
Conversion of land use	137.77	145.46	✓	✓	✓	✓	✓	✓
Shortage of workforce and equipment	152.96	185.26	✓	✓	✓	✓	✓	✓
Hunting	101.17	104.77	✓	✓	✓	✓	✓	✓
Turism effectiveness	65.97	60.61	✓	✓	✓	✓	✓	✓
Not having a clear boundary with adjacent land	95.28	77.9	✓	✓	✓	✓	✓	✓
Air pollution by natural and unnatural	73.81	68.92	✓	✓	✓	✓	✓	✓
Dam building	197.79	176.46	✓	✓	✓	✓	✓	✓
Flora pests	78.61	72.03	✓	✓	✓	✓	✓	✓
Fauna pests	29.49	32.1	✓	✓				
River pollution and the forest effectiveness	42.94	34.8	✓	✓				
The effects caused by immigration of nomads	24.5	20.33			✓			
Industrial- Seismography projects	25.28	25.85					✓	
Oil exploration and operation	32.57	37.71					✓	
Establish the Roads	91.49	88.44			✓	✓	✓	

Protected areas are among the most important instruments for protecting the biodiversity and services of ecosystems (Scharlemann et al., 2010; Klein et al., 2007; Coad et al., 2008) and are the basis of forest protector policies in developing countries (Assessment, 2005). This has resulted in development of protected areas of over 15.4% of the earth surface in every land section (Juffe-Bignoli et al., 2014). In spite of this extensive coverage, biodiversity is still declining (Butchart et al., 2010; Tittensor et al., 2014), since protection from areas is only one aspect of the performance and effectiveness of the protected areas. In other words, areas require a legal framework and suitable administrative structure to aid in halting loss of biodiversity (Leverington et al., 2010; Watson, 2014).

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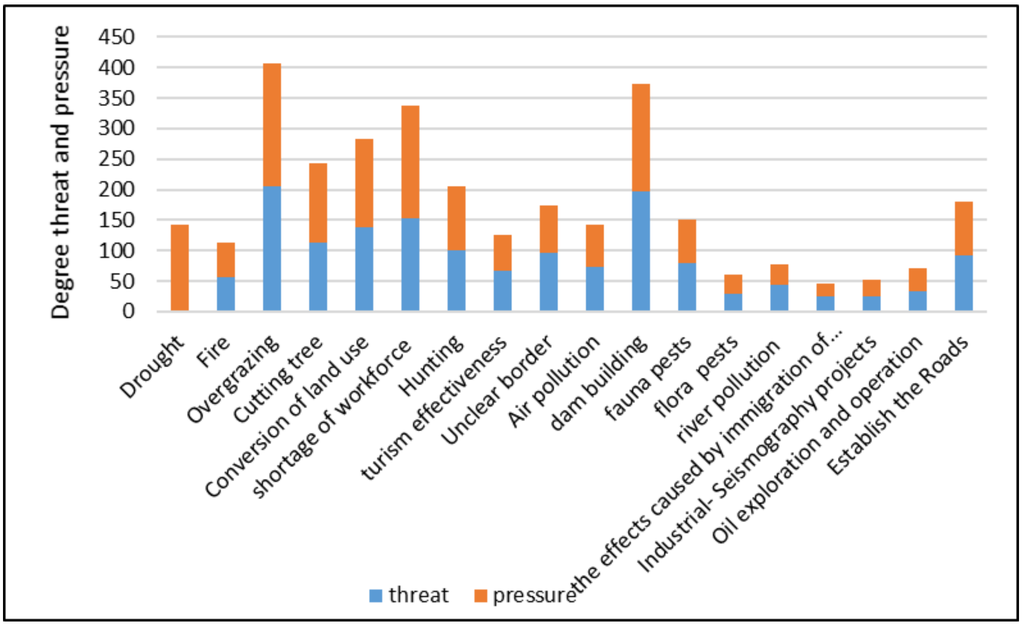

Figure 2. *Cumulative degree of pressures and threats of protected areas under study in Khuzestan Province,Iran*

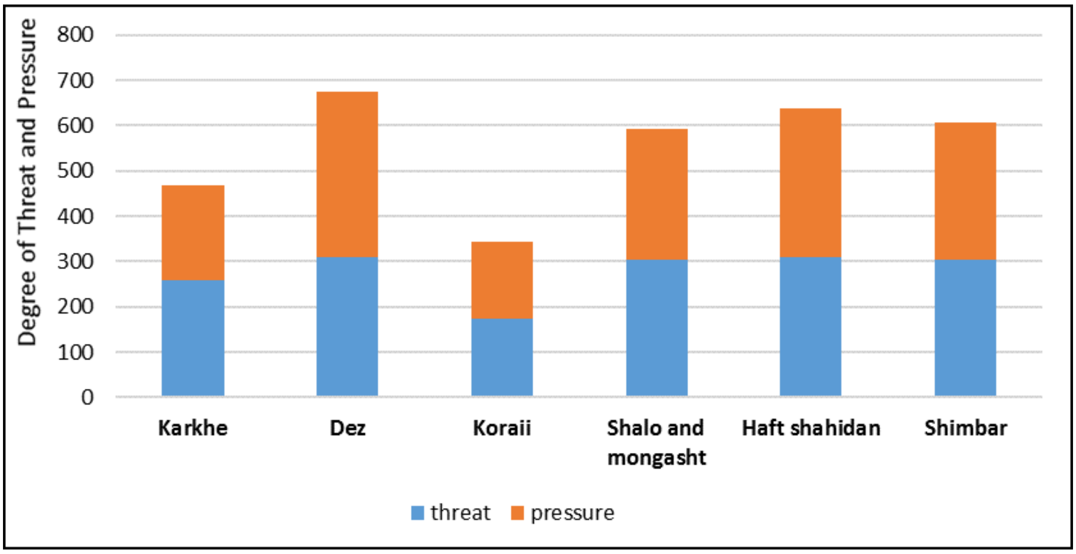

Figure 3. *Cumulative degree of pressures and threats of protected areas under study in Khuzestan Province.Iran*

Protective measures need the support of local communities and if a national park does not receive political and financial support from the central government, application of protective measures that realizes the goals of the local community becomes difficult (Hadjibiros and Sifakak, 2009). Due to this reason, decreased biodiversity is still taking place inside the boundaries of protected areas (Butchart et al., 2010; Tittensor et al., 2014) and the current level of management of protected areas to "stop loss of biodiversity" in a global scale is not adequate (Watson et al., 2014).

The results of this study suggest that five major collective threat and pressure factors across all protected areas in Khuzestan Province are overgrazing (405.07), dam building (374.25), shortage of workforce and equipment (338.22), conversion of land use (283.23), and cutting of trees (243.72).

According to studies conducted in Nepal (Nepali, 2006), Butane (Tshering, 2003), Georgia (Zazanashvili, 2003), and National parks (Nchor et al., 2012), overgrazing has the greatest impact and is the major threat and pressure factor in protected areas. Mountainous rangelands and Meadows are intensely affected by overgrazing, such that overgrazing diminishes food production for the wildlife and causes desertification (Li et al., 2003). Similarly, in some regions due to sensitivity of areas and lack of sufficient time for improvement, overgrazing is increasing with a greater intensity. In many areas, due to high altitude, agriculture is not possible and thus the life of local communities is dependent on grazing (Tshering, 2003). However, grazing in high altitude areas not only involves grazing but also local communities of wild animals, especially during night when night-active species are active (Nepali, 2006). Nevertheless it is clear that the impact, scale, and extent of grazing are different from park to park (Tshering, 2003). Grazing can cause natural disorders in the soil chemical processes, while simultaneously causing soil erosion. However, overgrazing, if not properly managed, often results in problems. In general, by introducing disorders into the physical characteristics and population of species, grazing affects the ecosystem. Not covering the land causes susceptibility of erosion and increased production of weeds. Vegetation helps soil in prevention from erosion and runoff during rainfall. However, when grazing takes place, the plant structure of the society changes and thus the society or biomass is destroyed (Azarnivand et al., 2011). Nevertheless, it should be noted that grazing can be benefited from as a good managerial method for preserving biodiversity and frequency in the long-term in enclosed rangelands (Wu et al., 2009).

Dam building claimed the second position among the major threat and pressure factors. Considering the long drought and effect of human, natural resources are at-risk. This has resulted in caring for the significance of the quantity and quality of water resources for proper management and exploitation of water resources in terms of sustainable development (Mouratidis et al., 2010). Until 2014, about 37641 have been constructed in the world (ICOLD, 2014). Many of the environmental impacts of dams are immediate and evident. However, other environmental effects are gradual and latent and can be hardly predicted (Johnson, 1998). These effects often develop across the entire river basin including changes in sedimentation and water flow as well as losing aquatic animals and losing or developing disorders in forests, flood plains, and other ecosystems (Fearnside, 2016). Therefore, when planning and managing water resources, the significance of adaptation of the executed plan with meteorological changes has been underscored (Khoi and Thom, 2015).

Shortage of workforce and equipment has been identified as the third threat and pressure factor for protected areas in Khuzestan Province. Overall, human and financial resources, planning, and efficient environmental management are required for proper management of protected areas (de Almeida et al., 2016). Shortage of area staff especially trained staff and the low motivation of employees as the major constraints affect the private forestry plan in Nepal (Chaudhary, 2000).

In addition to protected areas in Khuzestan Province, conversion of land use has been identified as the major factor of forest destruction and deforestation in

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Romania (Stanciu and Steindlegger, 2006) and Philippine (Verburg et al., 2006). Protected areas have been established for limiting the extent of changes in land use in areas with biodiversity and are large areas for conservation of at-risk species. Land-use and Land Coverage Changes (Lucc) can be a major threat for biodiversity, destruction of natural vegetation, segmentation or separation of areas in the nature (Bates and Rudel, 2000). Studies have shown that exploitation of lands and changing their use cause altered biodiversity (Shackleton, 2000). In management of these areas, altered land-use across the entire protected areas is very different, but nearly the development of agricultural activities and growth of residential communities in protected areas can be observed around the world (Bailey, 2015). If this elevation of agricultural lands in the proximity of protected areas is not properly managed, the resources of protected areas would be most probably adversely affected (Davis and Hansen, 2011).

The fifth threat and pressure factor is cutting of trees, accounting for 50% of the threat of areas in Cambodia (Lacerda et al., 2004). According to studies conducted in this research, the lowest collective threat and pressure factor is related to the effects caused by integration of nomads. This factor is natural according to expectations of experts, since the nomads residing in the nature have gradually adapted to the environment and prevented its destruction. This is because if these areas are destructive, their life would break down in return. In protected areas studied in Khuzestan Province, the greatest collective pressure and threat factor is related to Dez protected area (674.41), whereas the lowest pressure and threat factor is related to Koraii protected area (342.51). This can suggest unsuitability of conditions and facilities as the origin of threat and pressure factors in Dez protected area and to some extent appropriateness of these conditions and facilities in Koraii protected area in comparison with other areas.

Conclusion

This research states that overgrazing, dam construction, shortage of workforce and equipment, altered land-use, and cutting of trees are among the most important threat and pressure factors in protected areas in Khuzestan Province. It is essential that management of the areas introduce special solutions for eliminating or at least minimizing these pressure and threat factors regarding the objectives of the area. With elimination or mitigation of the effects of these pressure and threat factors, they can prevent destruction of areas. These solutions can be presented in short-term planning for removing or mitigating the pressure factors and long-term plans for eliminating threats factors of the areas in the future. Among these solutions can one mention offering the license of grazing to local communities in the area in a periodical fashion and supervising uniform distribution of grazing in this period, consideration of incentive measures for the area's staff in order to enhance motivation and aid in conservation of the area, holding training courses for the staff suited with the new needs of the area including the courses related to pests and animal diseases in the area, recovery of the boundaries of the protected areas to prevent to transgression to the area and alteration of land use, zoning of the areas and developing zone buffer, and constant monitoring of the area as well as the vegetation using remote sensing data, training of local communities residing in the areas considering the significance and functions of the protected area.

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MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSES OF PURPLE CHRYSANTHEMUM (*ASTER SPHATHULIFOLIUS*) UNDER LONG-TERM STRESS OF CALCIUM CHLORIDE AS DEICING SALT

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Abstract. Long-term research on the effect of deicers on groundcover plants can open up usage of groundcover plants for ornamental purposes on the roadside affected by deicer salt. The objective of this study was to investigate the long-term effect of $CaCl_2$ on morphological and physical responses of purple chrysanthemum (*Aster sphathulifolius*) grown in a greenhouse. Five different concentrations (0, 1, 2, 5, 10, and 15 g/L) of road deicer ($CaCl_2$ 74%) solutions (100 ml) were applied twice a week for a 5 month period. Survival rate, growth parameter, biomass, and physiological indices were measured. Increased $CaCl_2$ concentration resulted in decreased survival rate, especially at concentrations higher than 10 g/L. Exposure to increasing $CaCl_2$ concentrations resulted in dramatic decreases in growth index, number of leaves, leaf width, and leaf length at concentration higher than 2 g/L. Biomass was also negatively affected by increasing deicer salt stress, with shoot mass being reduced more than root fresh weight. Chlorophyll *b* content was decreased, while chlorophyll *a* and proline contents in leaves had a gradual increase when plants were exposed to increasing salt stress. Although a clear roadside negative effect did exist, there was no significant difference between plants under 1 g/L of $CaCl_2$ and control treatment for 5 months. Our results suggest that *Aster sphathulifolius* planting could be highly beneficial to the roadside or urban areas with mildly salt-affected soils.
Keywords: *calcium chloride; ornamental groundcovers, road deicer; soil-plant continuum; tolerance of salt stress*

Introduction

Deicing salt has been used for decades to melt snow and ice from the road to improve traffic safety in winter (Viskari and Kärenlampi, 2000). However, these salts are eventually displaced to roadside areas where they can negatively impact soils, vegetation, and water resources (ground or surface) (Devitt et al., 2014). A high amount of deicer has been used in South Korea due to its geographically distinctive winter season characterized by snowfall. On roads, $CaCl_2$ has been used as one of the most popular road deicer in South Korea (Shin et al., 2010), because it is more effective at low temperatures. Thus, at roadside areas that use $CaCl_2$, the Cl^- concentration is higher than the other sites. The use of deicing salts on roads has resulted in high concentrations of chloride (577 ~ 2,353 mg/kg) in urban roadside soil (Zhang et al., 2012). This is probably due to heavy volume of traffic at these sites (Baek et al., 2014). With increasing of salts content, a large amount of ions enter the cells, thereby morphology and physiology of plants growing under salt stress are adversely affected (Cao et al., 2012). Even though $CaCl_2$ has on plant physiology include membrane permeability and reduction in Na^+ concentration within a certain limit (Amuthavalli et al., 2012). Therefore, ways to reduce salt damage should be presented including a planting guide to match species to a site. The harmful effects of deicing salt on roadside herbs and grasses depends on the sensitivity of plant species (Ali et al., 2012). Planting salt tolerant ornamental groundcover plants can be a very effective strategy to utilize deicer salt affected soils. Salt tolerance varies widely among plant species and genotype. Plants can adapt to salinity by tolerating or avoiding salt uptake. Some plants can achieve salt tolerance by osmotic adjustment (Mao et al., 2008). Many researchers have studied salt tolerance of herbaceous plants after they are exposed to high salts. Eom et al. (2007) have shown that salt tolerance of six groundcover species is different in response to NaCl treatment (0 ~ 400 mM). It can be grouped into three categories: highly sensitive to salt treatment (*Sedum acre*), intermediately sensitive (*Achemilla mollis*, *Nepeta* × *faassenii*, *Thymus praecox*, and *Phlox subulata*), and tolerant (*Solidago cutleri*). Ali et al. (2012) have reported that the ornamental plant *Anternanthera bettzickiana* can be characterized as a salt-tolerant glycophyte. Other studies have also reported salt tolerance of other ornamental herbaceous plants, including *Suaeda salsa* (Guan et al., 2011), *Foeniculum vulagre* (Semiz et al., 2012), *Eugenia myrtifolia* (Acosta-Motos et al., 2015), Aster perennials (Wu et al., 2016), *Salvia splendens* and *Ageratum houstonianum* (Jędrzejuk et al., 2016), and *Sedum* species, *Allium* species, and a mixture of turf grasses (Whittinghill and Rowe, 2011). Salt tolerance has also been demonstrated for woody plants such as *Ardisia japonica* (Lee et al., 2008), ornamental shrubs (Cassaniti et al., 2009), *Prosopis glandulosa* (Moore et al., 2010), *Atriplex nummularia* (Alharby et al., 2014), and *Rosa rubigionsa* (Hura et al., 2017). However, to the best of our knowledge, no study has reported the salt tolerance of *Aster spathulifolius* for a long-term. Also, the effect of calcium chloride deicer on ornamental herbaceous plants compared to NaCl remains to be investigated.

Asteraceae is one of the largest plant families with many important ornamental species (Wu et al., 2016). Seashore spatulate aster, specifically, *Aster spathulifolius* Maxim, is halophyte, which typically found on the coast areas of Korea. Because the demend for road deicers continues to increase, long-term research on the effect of deicers on groundcover plants is needed. Therefore, the objective of this study was to determine the effect of $CaCl_2$ on morphological and physiological responses of *Aster spathulifolius*.

Material and methods

Plant material and growth conditions

Seedlings of *Aster sphathulifolius* were purchased from a commercial nursery (Sannea Botanical Garden, Chenonan, Chungnam, Korea). All plants with similar sizes used in experiments were 5-6 cm in height with 10-15 fully grown leaves. These seedlings were transferred to 12 cm-diameter pots filled with 0.5kg of artificial substrates (Wonjo-mix, NongKyung Inc., Korea) in March 2016. Pots were kept in the laboratory and watered every 2 days in the four weeks following transplanting. The experiment was conducted from April 2016 to October 2016 at KonKuk University, Chungju (latitude, 35°49´N; longitude, 127°08´E). Plants were grown in the greenhouse under

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natural light. During the study period, air temperature and relative humidity were monitored with a thermo recorder (SK-1260, SATO, Japan). Photosynthetically active radiation (PAR) was measured with a digital light meter (Extech 401025, EXTECH, USA). Average air temperature, relative humidity, PAR were kept at 26.2ºC, 62.7%, and 1,500 μmol· m^2/s, respectively.

Treatments

The substrate used for this study was a commercially produced ridging (Nongkyung Floricultural materials, Co., Chungbuk, Korea) with pH 6.5. Prior to transplanting, each metal was thoroughly mixed with 0.5 kg of air-dried substrate. The mixture was then used to fill the pots. To evaluate the effect of $CaCl_2$ concentrations on *Aster spathulifolius*, a completely randomized block design with five treatments was adopted (3 replications, 3 seedlings per replication, total of 45 seedlings). Based on the results of earlier studies (Zhang et al., 2012; Thouvenot et al., 2012) on roadside soil concentration of chloride ions, $CaCl_2$ powder (74% of calcium chloride, Oriental Chemical Industries, Korea) was diluted in distilled water to obtain concentrations at 0 (Control), 1 (C1), 2 (C2), 5 (C5), 10 (C10), and 15 g/L (C15) corresponding to 0.5, 7, 14, 35, 70, and 105 mM, respectively. *Aster sphathulifolius* seedlings were watered with 100 ml of $CaCl_2$ solution twice a week until the end of the experiment (for 5 months).

Measurements

The number of plants that were survived was recorded. This number was then used to estimate the survival rate (percentage). Plants that maintained 3 to 6 green or greenish and elongating leaves were scored as being alive (survived). Those with all leaves dried out were scored as dead. Survival rates were calculated with the following formula (Kanawapee et al., 2012): Survival rate = (survived plants/ total plants) × 100 (%).

The following growth parameters were observed: growth index, leaf number, leaf length, and leaf width. Growth index was measured once a month for each group of seedlings in May and September 2016 during peak growth period. Plant height (H) at the tallest point and width at the widest point in two directions (left to right and front to back: W1, W2) were also measured (Whittinghill and Rowe, 2011). Data of height and width were used to calculate growth index [(W1 + W2)/2 + H]/2 which is commonly used as an indicator of plant size (Hammond et al., 2007). Length of leaf, width of leaf, and leaf number on the stem were also measured for *Aster sphathulifolius*. These growth parameters were partially based on the relative growth rates (RGR) of plants (Thouvenot et al., 2012): RGR = (In L2- In L1)/(T2-T1), where L1 and L2 were total length at time 1 and time 2, respectively.

Each plant harvested was then divided into shoots (leaves and stem) and roots after 5 months of treatments. Fresh weights (FW) were measured after different organs were washed with distilled water. Shoots and roots were then dried in a drying oven (C-DF, Changshin Scientific Co., Korea) at 70 ºC until they reached a constant weight in order to measure their respective dry weights (DW). To evaluate salinity tolerance, relative dry weight (RDW) was calculated as a ratio of average values for each accession of seedlings (Chen et al., 2013): DW (salt treatment)/DW (control) × 100 (%), a trait commonly used to measure salinity tolerance.

Chlorophyll was extracted following the method outlined by Baruah et al. (2014). Briefly, collected leaves of experimental plants were washed properly and 100 mg of

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fresh leaves from those plants was weighed and cut into small pieces with a razor. Chlorophyll pigment was then extracted by grinding these cut leaves with a mortar and pestle for 5 min in about 8 ml of 95 % (v/v) acetone. The extract was filtered with Whatman number 1 filter paper. The filtrate was transferred to a 100-ml volumetric flask. The volume of the filtrate was increased to 10 ml by addition of 95 % acetone. After that, optical density (OD) of the extract was measured using a spectrophotometer (Biochrom Libra S22, Biochrom, England) at wavelength of 645 nm and 663 nm using 95% acetone as a blank. The wavelengths chosen the maximum absorption wavelengths for total chlorophyll and chlorophyll a/b, respectively. The amount of chlorophyll a and b and total chlorophyll content of leaf tissues (in mg/g) were calculated using the following equations:

$$\text{Chlorophyll a} = [12.7(\text{OD663 nm}) - 2.69(\text{OD645 nm})] \times (\text{V}/1000\text{W}) \tag{Eq.1}$$

$$\text{Chlorophyll b} = [22.9(\text{OD645 nm}) - 4.68(\text{OD663 nm})] \times (\text{V}/1000\text{W}) \tag{Eq.2}$$

$$\text{Total chlorophyll} = [20.2(\text{OD645nm}) + 8.02(\text{OD663 nm})] \times (\text{V}/1000\text{W}) \tag{Eq.3}$$

Proline content was analyzed with the modified procedure of Kanawapee et al. (2012). Briefly, the third and fourth leaves from the apical shoot of three plants per treatment were frozen immediately in liquid nitrogen at harvest. Approximately 0.1 g of leaf was homogenized with 5 ml of 3% aqueous sulfosalicylic acid. Two ml of the extract was then reacted with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid followed by boiling in a water bath at 100°C for 1 h. The reaction was stopped by placing the tubes on ice. The solution was then extracted with 4 ml of toluene and the absorbance of the toluene fraction was measured by spectrophotometry (Biochrom Libra S22, Biochrom, England) at wavelength of 520 nm. The amount of free proline was evaluated using a standard curve and expressed as μg/g tissue fresh weight.

Statistical analysis

Data are presented as mean ± standard deviation of nine replicates (N = 9). Statistical analysis was performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance (ANOVA). Treatment means were separated with Duncan's Multiple Range Test ($P \leq 0.05$).

Results

The survival rate of *Aster sphathulifolius* showed no significant difference between treatments in the initial stage. However, at the end of 5 months under salt stress conditions, survival rates of plants were reduced sharply in the following order: $CaCl_2$ 0 (Control; 100%) > $CaCl_2$ 1 (C1; 100%) > $CaCl_2$ 2 (C2; 80%) > $CaCl_2$ 5 (C5; 70%) > $CaCl_2$ 10 (C10; 0%) > $CaCl_2$ 15 g/L (C15; 0%). The survival rate of *Aster sphathulifolius* were significantly decreased compared to those of control as well as 1 g/L of $CaCl_2$ treated plants. Increasing $CaCl_2$ concentration in the substrates resulted in decreasing survival rate, especially when its concentration was higher than 10 g/L (*Figure 1*).

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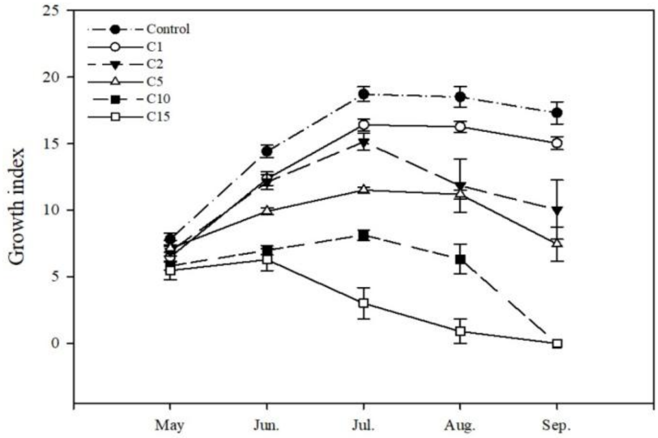
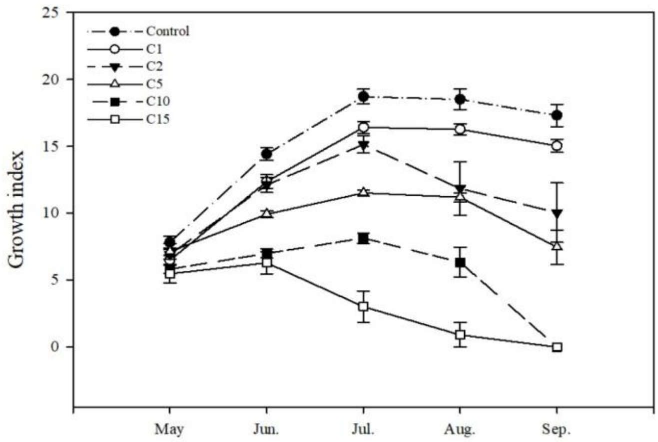


Figure 1. *Survival rates of Aster sphathulifolius after treated with $CaCl_2$ at different concentrations(0, 1, 2, 5, 10, and 15 g/L as Control, C1, C2, C5, C10, and C15, respectively) for 5 months. Values are presented as means ± SE of nine replications.*

The growth index of *Aster sphathulifolius* showed various degrees of growth retardation, although some differences existed between treatments with different $CaCl_2$ concentrations in June. However, the average growth indices of Control, C1, C2, C5, C10, and C15 were significantly decreased ($P \leq 0.05$) by 17.3, 15.2, 15.2, 11.8, 8.2, 6.7 cm, respectively in July, and plants grown in C10 and C15 treatment groups did not survive in September. The average growth indices of C1, C2, and C5 treatment groups were 20, 22, and 47%, respectively, compared to the control. For all treatements, the number of leaves showed a increase resulting in a range from 14.4 (C15) to 66.5% (Control) deicing salt stress in June, but then gradually decreased by 3.1 (Control) and 82.6% (C15) in response to salinity stress. The number of leaves of plants in C1, C2, and C5 treatment groups was significantly lower ($P \leq 0.05$) by 9, 18, and 57%, respectively, compared to that in the control at 5 months after treatment. However, there was no significant difference in the number of leaves between the Control and C1 treatment. Exposure to increasing $CaCl_2$ concentrations resulted in dramatic decreases in leaf width and leaf length with $CaCl_2$ at 1, 2, 5, and 10 $g \cdot L^{-1}$, leaf widths were 32, 33, 22, and 19 mm, respectively, while leaf lengths were 89, 92, 84, and 62 mm, respectively. There was no significant difference in leaf width or length between plants under Control and those with C1 treatment (*Figure 2*).



(A) : Growth index

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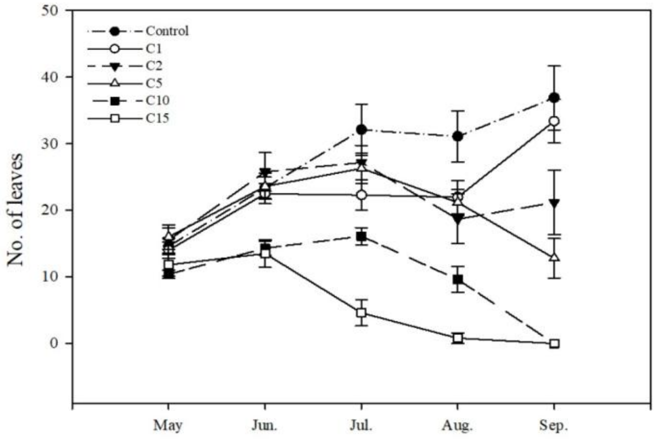
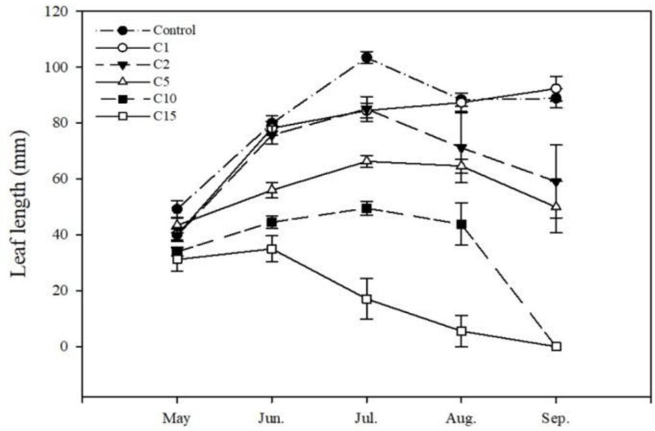
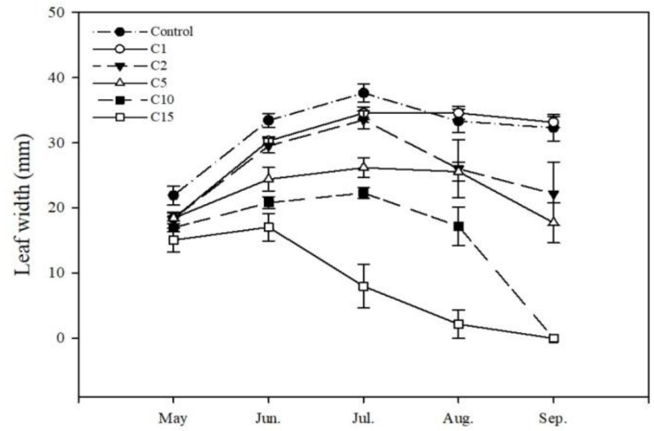


(B): Number of leaves



(C) : Leaf length



(D) : Leaf width

Figure 2. *Growth index(A), number of leaves(B), leaf width(C), and leaf length(D) of Aster sphathulifolius grown under $CaCl_2$ stress at various concentrations (0, 1, 2, 5, 10, and 15 g/L as Control, C1, C2, C5, C10, and C15, respectively) for 5 months. Values are presented as means ± SE of nine replications.*

As shown in *Figure 3*, exposure to increasing concentrations of calcium chloride resulted in dramatic decrease ($P \leq 0.05$) in biomass of *Aster sphathulifolius* compared to non-salt stress. Shoot showed more reduction than root fresh weight of *Aster*

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sphathulifolius. Average shoot fresh weights of plants in C1, C2, C5, C10, and C15 treatment groups were reduced by 3, 36, 56, 89, and 92%, respectively, while root fresh weights were reduced by 41, 50, 66, 83, and 88%, respectively in comparison with those in the Control ($CaCl_2$ 0 g/L). Shoot dry weights of salt stressed plants in C1, C2, C5, C10, and C15 treatment groups were also reduced by 24, 31, 52, 75, and 81%, respectively, while dry weights were reduced by 39, 46, 59, 67 and 74%, respectively, compared to those of Control (*Figure 4*). Relative dry weight (RDW), a trait commonly used to measure salinity tolerance, was also substantially reduced by 76.4, 69.5, 48.5, 25.4, 19.4% relative to Control (data not referred).

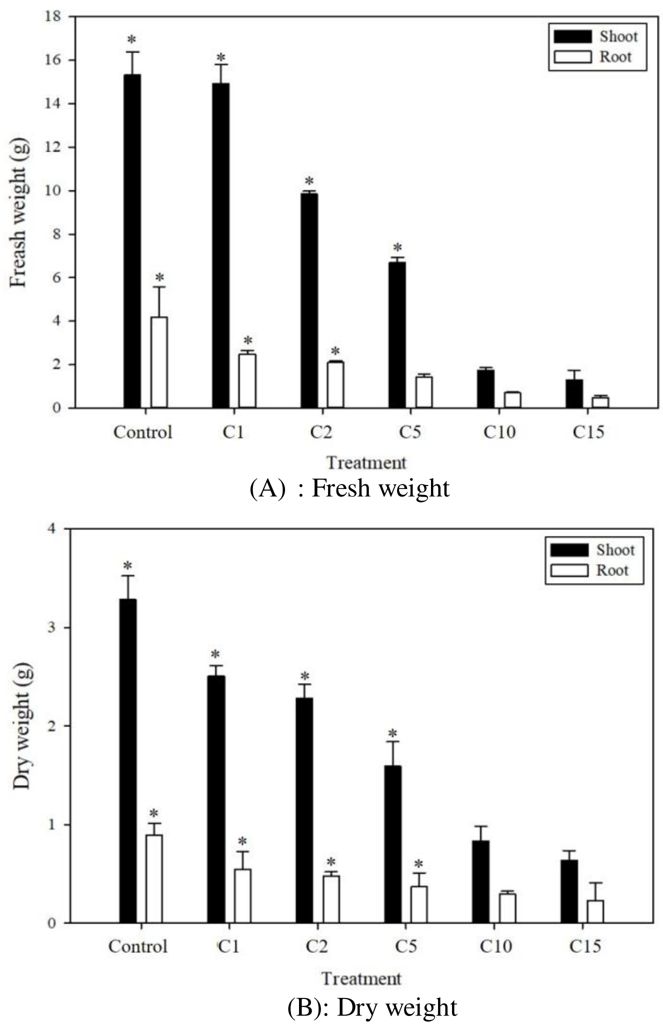

Figure 3 *Fresh(A) and dry weight(B) of Aster sphathulifolius grown under $CaCl_2$ stress with at various concentrations (0, 1, 2, 5, 10, and 15 g/L as Control, C1, C2, C5, C10, and C15, respectively) measured after hravest. Values are presented as means ± SE of nine replications (*P value ≤ 0.05).*

Salt stress for 5 months affected chlorophyll contents of *Aster sphathulifolius*. Interestingly, salt stress resulted in significant increase of chlorophyll *a* content, whereas decrease of chlorophyll *b* content. The mean chlorophyll *a* content was increased from 8.2 to 14.97 mg/g FW. For chlorophyll *b*, the mean was decreased from 21.18 to 11.17 mg/g FW respectively. Total chlorophyll contents in C1, C2, C5, and

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C10 treatments were decreased significantly by 98.6, 95.3, 92.9, and 89.5%, respectively, compared to those of Control. When *Aster sphathulifolius* plants were exposed to salt stress, the mean proline contents in the leaves were increased significantly from 23.0 in the control to 37.3 µg/g FW in C10 treatment (an increase of 62.2%). Salinity stress caused a significant increase in proline contents in C2, C5, and C10 treatment groups when compared with the control and C1 treatment group (*Table 1*).

Table 1. *Differences of chlorophyll and proline contents of Aster sphathulifolius leaves grown under $CaCl_2$ stress at various concentrations (0, 1, 2, 5, 10, and 15 g/L as Control, C1, C2, C5, C10, and C15, respectively) measured after harvest.*

Treatments	**Chlorophyll contents a (mg/g FW)**	**Chlorophyll contents b (mg/g FW)**	**Total chlorophyll contents (mg/g FW)**	**Proline contents (µg/g FW)**
Control	8.29 d[z]	21.18 a	29.46 a	23.00 c
C1	12.92 b	16.18 b	29.09 ab	23.33 c
C2	12.64 c	15.49 b	28.12 abc	26.00 bc
C5	12.88 b	14.56 b	27.43 bc	29.67 b
C10	14.96 a	11.47 c	26.44 c	37.33 a
C15	-	-	-	-

[z] Different letters in the same column indicate significant difference according to Duncan's multiple range test at *P* ≤ 0.05 (n=9).

Discussion

Plant survival and productivity is crucial for seedling establishment under saline conditions (Ponte et al., 2014). In this study, no significant correlation between plant suvival and $CaCl_2$ concentrations were found initially, however, after treatment for 5 months, a significant negative correlation was observed between the survival rate and the dose of $CaCl_2$. Higher $CaCl_2$ concentrations were associated with lower survival rate for a long term exposure, although *Aster sphathulifolius* is a halophyte that has grown and evolved under saline conditions. Soil salinity can inhibit plant growth by a number of mechanisms including low external water potential, toxicity of absorbed Cl^- ions, inhibition of various enzymatic activities and different cellular processes, and interference with the uptake of essential nutrients (Taffoue et al., 2014). The fundamental mechanisms of salt tolerance in salt tolerant plants seem to be mostly dependent on their capacities to sequestrate toxic ions in vacuoles and accumulate compatible osmotic pressure in the cytoplasm as previously suggested (Munns, 2002). Salt tolerance of herbaceous perennial species including *Achemilla mollis*, *Nepeta × faassenii*, *Sedum acre*, *Tymus praecox*, *Phlox subulata*, and *Solidago cutleri*, to aqueous solution of sodium chloride at 0~400 mM over a 21 day period has been evaluated by measuring their growth. These plants were grouped into three tolerance categories : highly sensitive to salt treatment (*Sedum acre*), those with intermediate sensitivity (*A. mollis*, *N. × faassenii*, *T. praecox*, and *P. subulata*), and those with salt tolerance (*S.*

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cutleri) (Eom et al., 2007). It has been reported that *Alternanthera bettzickiana* can grow even at a salinity level of 40 dS/m (Ali et al., 2012). In addition, it has been found that the critical level of species sensitive to chloride ions is 4 ~ 7 mg/g and that of species tolerant to chloride ions is 15 mg/g (Xu et al., 2000; White and Broadley, 2001). Considering this, it is generally accepted that *Aster sphathulifolius* could be able to grow at salt levels less than 1 g/L (Cl^- agent 0.094 g/L day^{-1}) for 5 months.

Biomass of seedlings is a trait commonly used to measure salinity tolerance (Chen et al., 2013). *Eupatorium greggii*, *Viguiera stenoloba*, and *Santolina chamaecyparissus* were the most salt-tolerance species with less redutions in shoot dry weight (Wu et al., 2016). It has been reported that *Alternanthera bettzickiana* plants at salinitiy level of 20 dS/m will produce 30.3% less biomass than controls. Further increasing salinity will lead to lower biomass in response to higher salt stress (Ali et al., 2012). Similarly, Semiz et al. (2012) have also reported that the biomass of *Foeniculum vulgare* is also affected negatively by chloride ions. Meanwhile, chloride ions (Cl^-) in soil is recognized as one key contributor to the decrease in the production of plants. The critical concentration of chloride ions is found to be 0.49 g/L. At this concentration, the biomass of plants is decreased by 10% (Dang et al., 2008). Considering the actual concentrations of chloride ions (Cl^-) in C1, C2, C5, C10, and C15 treatments are 0.047, 0.094, 0.236, 0.472, and 0.708 g/L day^{-1}, respectively, *Aster sphathulifolius* should be able to grow in roadside or urban soils contaminated by deicing salt because relatively low Cl^- concentrations are present in these field conditions.

The chlorophyll meter is a simple tool used to measure relative chlorophyll content or greenness. It is an efficient indicator of stress in plants (Netto et al., 2005). Changes in chlorophyll content of plants under salt stress dependeds on stress rate and plant species (Eom et al., 2007; Younes et al., 2016). Nadeem et al. (2006) have reported that salt stress can decrease chlorophyll pigments (a, b, and carotenoids contents) of plant. However, another study has shown that chlorophyll content cannot be used as an indicator of salt tolerance ability (Kanawapee et al., 2012). Although plant species can differ considerably in total amount of chlorophyll content under salt stress, results from this study suggest that chlorophyll content might be considered as an additional trait useful for screening salt tolerance.

Relative abundance of compatible solutes including proline is an important protective factor for plants under salt stress (Norastehnia et al., 2014; Abbas et al., 2014). Salinity stress has caused a significant increase in proline concentration in shoots of plants compared to that in the control (non-stressed seedlings) (Kamawapee et al., 2012). In response to salt stress, proline accumulation in plants has been implicated to play adaptive roles in osmoregulation and salt stress signaling (Szabados and Savouré, 2009; García-Caparrós et al., 2016). Our results revealed that proline content in plants under salt stress were apparently higher than those in plants under non- salt stress condition. Plants showed significantly higher levels of proline content compared to plants without $CaCl_2$ stress. These results suggest that proline accumulation might be considered as an additional trait useful for screening plants for salt tolerance ability.

Results of this study showed that increasing $CaCl_2$ concentrations dramatically reduced survival rate and plant growth of *Aster sphathulifolius*. Biomass was also affected negatively by increasing salt stress of $CaCl_2$, with shoots having more reduction in mass than root fresh weight. Calcium chloride salt stress significantly increased chlorophyll *a* and proline contents in leaves, but decreased chlorophyll *b*.

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Conclusion

Salt tolerant ornamental groundcover plants can be a very effective strategy to utilizing deicer salt affected soils. The morphological and pysiological responses of *Aster sphathulifolius* indicated that the one gram dosage of $CaCl_2$ for 5 months had no significant difference in survival rates and growth parameters as compared to those of control. However there was a significant negative effect exist by the cumulative dose of deicing $CaCl_2$ salt(>1 g/L). These results suggest that planting *Aster sphathulifolius* could be highly beneficial for sites with salinity soils such as roadside or urban areas since relatively low Cl^- concentrations are present in these fields. In the future, soil-chloride-plant continuum study warrants to determine the effect of deicers on diverse ecological characteristics of roadside groundcover plants.

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THE EFFECT OF PHYSICAL PRIMING OF SEED ON TRAITS AND YIELD OF CORN (*ZEA MAYS* L.) UNDER WATER DEFICIT CONDITIONS IN IRAN

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Abstract. In order to study the effect of physical priming of seed on traits and yield of corn under water deficit conditions, a two years experiment was conducted during 2014- 2015 as a split plot with three replications in Iran. The main factor includes two levels of irrigation (70 and 110 mm evaporation from pan class A as control and water deficit, respectively) after 8-9 leaves stage of corn and sub-factor includes corn seed treatment with gamma and beta rays, laser wave, magnetic field and ultrasonic waves, all in time intervals of 5 and 10 min. along with a non–primed control. Results indicated that maize seed priming under magnetic field of 5 and 10 min. and gamma and laser rays for 5 min. in normal irrigation regime were the best treatments with 20%, 18%, 16% and 13% increase value, respectively, while seed priming with gamma and laser rays for 10 min. under water deficit condition had the lowest grain yield. To increase corn grain yield in both normal and water deficit conditions, seed priming with the magnetic field in both time intervals, gamma and laser rays only in a short time is recommended.
Keywords: *evaporation, gamma ray, grain, laser wave, magnetic field*

Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops in the world therefore its cultivation is economically important and also due to its agricultural importance many fundamental researches have been conducted in a century on this plant (Marcu et al., 2013). According to Khan et al. (2008) seed priming is a pre-sowing strategy to improve germination and seedling establishment through priority of metabolic activities before emergence of radicle that often leads to more moisture, nutrients, and sunlight absorption and ultimately lead to increased yield.

According to Aladjadjiyan (2014), use of some physical elements are considered as a modern method for achieving higher yields in agricultural systems. Interest for use of ecological factors influencing plant products such as laser radiation, ultra violet, magnetic and electric fields has been increased (Faqenabi et al., 2009). Paolo and Rinaldi (2008) stated that the adverse effects of water stress on growth and development and yield of corn depends on the time of stress, stress tension, plant developmental stage, and its genotype. In a research study, the lowest grain yield of corn was obtained from those plants that under water deficit in flowering stage (Rabbani and Imam, 2011). Despite existence of limited information on physical methods of seed treatment, interest and willingness to use of these methods for seed priming crop plants has increased in recent years (Hernández et al., 2010).

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Gamma ray is the most important physical treatment that increases production in crop plants such as rice (*Oryza sativa*), corn, bean (*Phaseolus vulgaris*), and potato (*Solanum tuberosum*) (Mokobia and Anomohanran, 2005). Potato tubers pre-treated with gamma ray before sowing improved plant growth characteristics (Hamideldin and Hussin, 2014). Increase in the height and fresh weight of corn and followed by increase in the performance has been reported by seed treatment with magnetic fields (Racuciu et al., 2008). In an experiment conducted by Pietruszewski and Kania (2010) use of some physical treatments including magnetic field and laser radiation before sowing the seed increased grain yield of wheat. Exposure of sunflower (*Helianthus annuus*) seeds to magnetic fields gave rise to positive effects on plant growth and increased yield (Vashisth and Nagarajan, 2013). Rochalska (2008) reported that sugar beet (*Beta vulgaris*) seed priming with magnetic field leads to more rapid plant growth, due to the intensity of metabolic processes, and finally yield improvement.

Since the physical priming is one of the effective methods in improving the crop yield in preserving the natural environment and biological safety, then the specific aims of this study were:

- Evaluation effect of the studied treatments on morpho-physiological traits of corn
- Response of biomass and grain yields of corn crop to biophysical studied treatments

Materials and Methods

Maize is a tall, monecious annual plant of *Poaceae* family. It has staminate spikelets in long spike-like racemes that form large spreading terminal tassels and pistillate inflorescences in the leaf axils (Anonymous, 2015).

Site description

This research was carried out in two years (2014- 2015) at the Agricultural Research Station of Islamic Azad University, Tabriz Branch, Iran. The regional climate is semi-arid and cold. The location of this experiment was 1560 m above sea level by 38°5′ N and 46°17′ E geographical latitude and longitude, respectively.

Experimental design and variables

The experiments were carried out as split plot based on the randomized complete blocks design with three replications on the corn SC260. The main factor includes irrigation time (70 mm as normal treatment, and 110 mm evaporation from pan class A as water deficit treatment after 8-9 leaves stage of corn) and sub-factor was in 11 levels of seed treatment with gamma (cobalt 60) (Farahvash et al., 2007) and beta rays (strontium 90) (Bradford, 1995), both with constant intensity of two microcurie, continuous wave helium-neon laser with a wavelength of 6328 angstroms (Chen et al., 2002), magnetic field of 40 mT (Iqbal et al., 2012), and ultrasonic waves with a maximum of three Wcm^{-1} (Yaldagard and Mortazavi, 2008) all in 5 and 10 min. intervals along with control.

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Equipment's used and seed treatment

Before applying the treatments, corn seeds were put in hypochlorite sodium 5% for two minutes for disinfection and then were washed three times with distilled water. The seeds were kept in dark place for 24 h in distilled water at a temperature of 25°C (Artola et al., 2003) and then physical treatments were applied on the seeds inside petri dishes in Physics Laboratory. In order to pre-treatment of seeds with gamma and beta rays, laser wave, magnetic field, and ultrasonic wave were used lead syringe source of LEYBOLD (Germany), laser device IR2000 (Iran), transformer 2086 (Iran) and ultrasonic device of Sa-Iran company, respectively.

Land preparation

The experimental area was ploughed in the fall and then disked in early spring. After preparing the experimental plots, treated seeds were sown immediately in the field. Sowing was conducted every two years on May 10 at a distance of 75 cm between the rows on the rows of 20 cm at density of 67000 P ha^{-1} and a depth of 3-4 cm. Based on the results of soil analysis, ammonium phosphate and potassium sulfate fertilizers were used as 110 and 100 kg ha^{-1}, respectively before sowing and nitrogenous fertilizer was added as 80 kg $ha^{-1}$from urea source in two equal parts at each stage of sowing and on the corn stem elongation (4-5 leaves stage) as strips to the soil. Weeds control was carried out manually as needed two times during the growing season. Irrigation was conducted from the time of sowing the seeds until the beginning of water deficit treatment equally in all treatments based on 70 mm evaporation from pan and then continued to the end of the growing season depending on the type of treatment based on the 70 and 110 mm of evaporation.

Studied traits

At the time of ripening, plant height, time of tasseling, leaf area index (LAI), stem dry weight, leaf proline concentration, biomass and grain yield were measured based on standard procedures. Also, LAI was measured by the method of described by Acquaah (2002). Leaf proline concentration was determined using a spectrophotometer at a wavelength of 520 nm (Bates et al., 1973).

Statistical analysis

Variance analysis of data was achieved using MSTAT-C software (Alizadeh and Tarinejad, 2010) and Duncan Multiple Range Test at 5% was used for mean comparisons. Excel software was used to drawing the figures.

Results

Combined variance analysis on the effects of irrigation levels and physical seed treatments on the studied traits in corn revealed that the year effect on all traits, the effect of irrigation level on stem dry weight and grain yield, interaction of year and irrigation level on the plant height and leaf proline concentration, the effect of physical pre-treatment on all traits, interaction of irrigation level in physical pre-treatment on the biomass and grain yield per unit area was significance at $p<0.05$ (*Table 1*).

***Table 1.** Combined variance analysis of irrigation levels and seed physical pre-treatments on studied traits of corn in two years of experiment.*

(S.O.V)	Degree of freedom (df)	Plant height	Time of tasseling	LAI	Stem dry weight	Leaf proline concentration	Biomass per unit area	Grain yield
Y	1	15356.54**	1749.09**	1.94*	8754.73**	33.83*	1368843.66**	191496.71**
R/Y	4	139.93	80.01	0.19	119.93	2.85	13289.17	2750.50
A	1	6119.33 ns	643.72 ns	17.03 ns	7500.18*	54.49 ns	1191680.03 ns	517338.92*
A×Y	1	264.20*	4.18 ns	0.63 ns	9.28 ns	4.22**	10945.48 ns	279.27 ns
A/Y×R	4	14.61	38.62	0.13	2.60	0.04	6795.96	5733.10
B	10	4107.17**	424.19**	4.16**	1969.54**	5.39**	162520.51**	64373.34**
B×A	10	51.35 ns	5.96 ns	0.03 ns	55 ns	0.76 ns	3505.61*	422.40*
B×Y	10	112.29 ns	16.12 ns	0.09 ns	28.82 ns	0.46 ns	2167.48 ns	539.69 ns
B×A×Y	10	31.40 ns	2.76 ns	0.04 ns	30.76 ns	0.43 ns	1146.40 ns	140.32 ns
B/YA×R	80	164.45	29.86	0.13	79.15	0.95	14430.58	5296.97
C.V. (%)	-	6.03	7.80	10.69	12.01	21.26	9.50	11.26

ns, *, and ** are non-significant, significant at 5% and 1% probability levels, respectively.
Y, R, A, and B mean year, replication, irrigation levels and physical pre-treatments, respectively.

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Plant height

Based on the comparing the means, seed treatment with magnetic fields for 5 and 10 min. and laser radiations and gamma for 5 min. had the highest plant height while the lowest value from 10 min. treatment with gamma radiation (*Table 2*).

Table 2. *Mean comparisons of seed physical pre-treatments on studied traits of corn in two years.*

Seed pre-treatment	Plant height (cm)	Time of tasseling (day)	LAI	Stem dry weight (g)	Leaf proline concentration (µmol/g)
Control	202.68 cd	69.70 bc	3.16 d	66.66 e	4.30 cd
Gamma 5 min.	227.45 b	66.43 cd	3.85 b	84.27 b	5.73 a
Gamma 10 min.	180.64 e	80.06 a	2.37 e	51.97 f	3.85 de
Beta 5 min.	204.18 c	71.58 b	3.38 cd	72.50 cd	4.31 cd
Beta 10 min.	209.97 c	70.22 bc	3.52 c	76.25 c	4.98 bc
Laser 5 min.	229.66 ab	63.56 de	4 ab	82.50 b	4.98 bc
Laser 10 min.	193.35 d	78.52 a	2.59 e	56.45 f	3.48 e
Magnetic 5 min.	238.87 a	62.25 e	4.16 a	91.77 a	4.94 bc
Magnetic 5 min.	236.18 ab	62.77 de	4.10 ab	90.52 a	5.32 ab
Ultrasonic 5 min.	202.81 cd	72.14 b	3.18 d	70.10 de	4.50 cd
Ultrasonic 10 min.	211.70 c	72.43 b	3.36 cd	71.56 cde	4.04 de
LSD %5	9.64	3.652	0.280	4.884	0.622

Means in each column with similar letters have not significant difference based on DMRT at 5% probability level.

Time of tasseling

Minimum tasseling time obtained from those seeds treated with magnetic fields for 5 and 10 min., laser radiations and gamma for 5 min., while the maximum time to tasseling was measured in gamma and laser rays for 10 min., so that the use of them leads to happening 6 days earlier and 10 days delay in the adventure of tasseling, respectively compared to the control (*Table 2*).

LAI

LAI increased by corn seed priming with magnetic field in both time intervals and laser and gamma rays for 5 min., as 22.27%, and 32%, respectively compared to the control. Extending of duration of seed priming with both gamma and laser rays from 5 to 10 min. led to reduction of corn leaf area compared to the control as 25% and 18%, respectively (*Table 2*).

Stem dry weight

Stem dry weight of corn decreased by 15% due to water deficit compared to the normal irrigation (*Figure 1*). The highest dry weight obtained from those seeds under magnetic field treatments of 5 and 10 min. followed by gamma and laser rays for 5 min. The reducing effect of gamma and laser for 10 min. on dry weight was significant. So that, seed treatment with the above radiations led to 22% and 16% reduction on dry

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weight. Seed treatment with beta radiation in both time intervals revealed better efficacy compared with ultrasonic wave and 9.59 g and 5.84 g increase in stem dry weight compared to the control (*Table 2*).

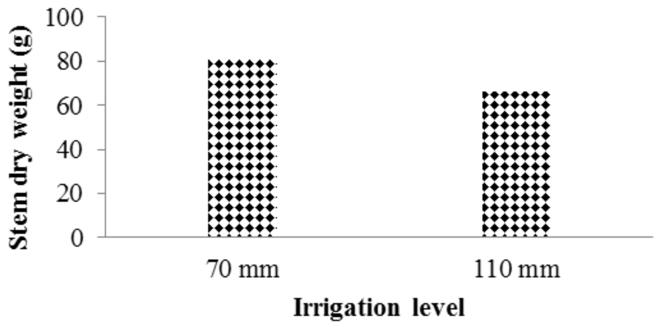

Figure 1. *Effect of irrigation levels on stem dry weight. 70 and 110 mm evaporation from pan are as normal and water deficit conditions, respectively.*

Leaf proline concentration

Seed priming with gamma radiation for 5 min. and magnetic field for 10 min. led to increase in the concentrations of proline by 1.43 and 1.02 µmol g^{-1} of fresh weight compared to the control. Additionally, the laser radiation for 10 min. was more destructive than other treatments, so that the leaf proline concentration decreased by 19% compared with control and there was no statistically significant difference between other treatments and the control (*Table 2*).

Biomass

Interaction of irrigation and physical treatments during two years of experiment demonstrated that the maximum biomass per unit area was obtained from normal irrigation and seed priming with magnetic field in both intervals of 10 and 5 min., and laser and gamma rays of 5 min., respectively while the minimum amount was obtained from water deficit and seeds primed under gamma and laser rays for 10 min. (*Figure 2*).

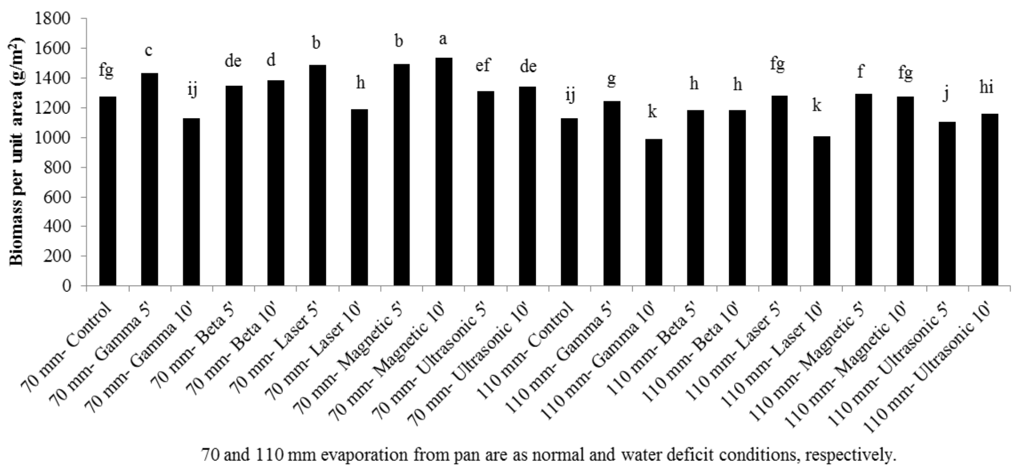

Figure 2. *Mean comparisons of physical pre-treatments × irrigation levels on corn biomass. Means with similar letters in each column are not significant with together by DMRT at 5% probability level.*

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Water deficit led to reduction of biomass compared to normal irrigation in all treatments. In normal irrigation and water deficit conditions primed seeds under magnetic field for 10 and 5 min., laser and gamma rays for 5 min. crop biomass increased as 17% and 13% compared to the control, while in those seeds primed with gamma and laser rays for 10 min. biomass reduced 9% and 12% compared to the control in both irrigation levels (*Figure 2*).

Based on means comparison (*Figure 2*) interaction of irrigation level with priming of gamma and laser rays for 10 min. had major reducing effect on corn biomass.

Grain yield

The highest grain yield was obtained in normal irrigation and seed priming with magnetic fields for 10 and 5 min. and gamma and laser rays for 5 min. by 808, 790.9, 778.2, and 758.4 g/m^2 and the lowest value was obtained from water deficit and priming with gamma and laser rays for 10 min. by 458.4 and 473.1 g/m^2, respectively. Also, seed priming with magnetic field for 10 and 5 min. and gamma and laser rays for 5 min. along with the implementation of normal irrigation increased grain yield by 20, 18, 16 and 13%, respectively and was considered as the best treatments while priming with gamma ray and laser wave for 10 min. with water deficit showed 32 and 30% decrease and was considered as the most harmful treatments in grain yield compared with the control. Additionally, water deficit and irrigation implementation based on 110 mm evaporation reduced yield in all treatments compared with the normal irrigation (*Figure 3*).

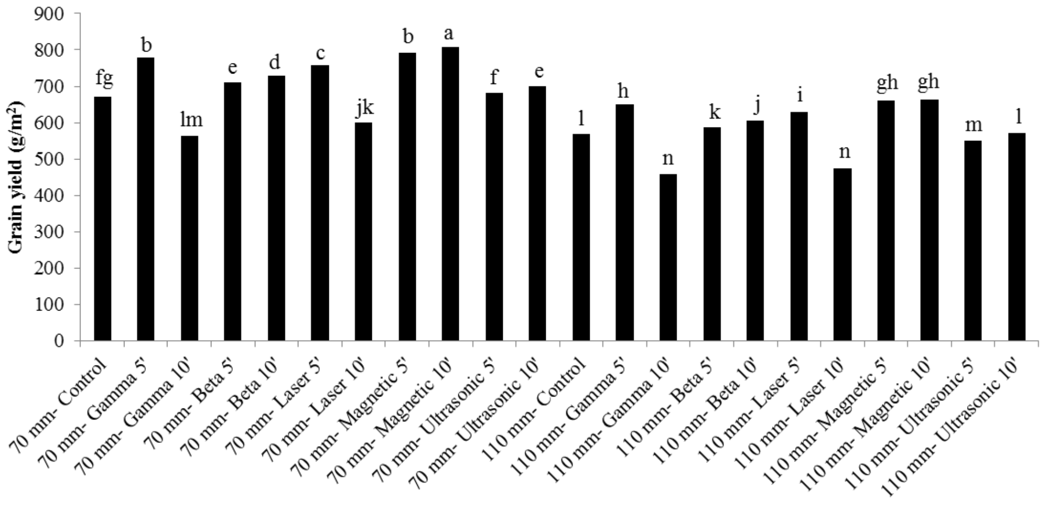

Figure 3. *Mean comparisons of physical pre-treatments × irrigation levels on corn grain yield. Means with similar letters in each column are not significant with together by DMRT at 5% probability level.*

Orthogonal comparison

Based on the orthogonal comparison results there is a significant difference between 5 and 10 min. physical treatments except to leaf proline concentration. In this study 5 min. interval in all studied traits was better than 10 min. (*Table 3*).

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Table 3. *Orthogonal comparison between 5 and 10 minutes physical pre-treatments, drought and no drought condition of 5 minute magnetic treatment, and finally magnetic and non-magnetic treatment.*

	Traits							
	5 and 10 minutes	**Plant height (cm)**	**Time of tasseling (day)**	**LAI**	**Stem dry weight (g)**	**Leaf proline concen-tration (μmol/g)**	**Biomass per unit area (g/m^2)**	**Grain yield (g/m^2)**
Physical pre-treatments	5 min.	220.60 a	67.29 a	3.72 a	80.23 a	4.05 a	1319.2 a	679.76 a
	10 min.	206.38 b	72.8 b	3.19 b	69.35 b	3.58 a	1220.5 b	617.38 b
a1	5 min. magnetic	225.86 a	69.31a	4.09 a	89.08 a	3.48 b	1416.3 a	744.3 a
a2		215.34 a	65.08 b	3.34 b	71.37 b	4.62 a	1222.9 b	615.3 b
All data	no-magnetic	202.7 b	69.71 a	3.16 b	66.67 b	3.49 a	1201.9 b	620.54 b
	magnetic	237.5 a	62.51 b	4.14 a	91.15 a	4.23 a	1401 a	730.94 a

a1=normal condition (70 mm evaporation from pan)
a2=drought stress condition (110 mm evaporation from pan)

Discussion

Plant height

In this research, plant height increased by magnetic field and gamma ray which is in agreement with those reported by Racuciu et al. (2008) on corn and Pasangka (2013) on soybean (*Glycine max*). Increasing plant height is probably related to stimulating phytohormones like auxin, cytokinin and gibberellin in plant.

Time of tasseling

In order to premature emergence of radical and plumule of plantlets in field, priming by gamma ray resulted in earlier tasseling. In the Pasangka (2013) experiment flowering stage happened 18 day earlier under gamma ray treatment.

LAI

The result of this research on corn LAI are in good agreement with findings of Hoseini et al. (2013) on lemon (*Melissa officinalis)* and Rochalska et al. (2008) on sugar beet (*Beta vulgaris*). This may a probably result of reported by magnetic field probably since the optimum growth of root in the end of nutrient absorption from soil and finally resulted nutrition transport from stem to leaves as described by Rochalska et al. (2008).

Stem dry weight

Stem dry weight decline of corn by water deficit like this study, reported by Nesmith and Ritchi (1992) and Singh and Usha (2003). As well increasing of stem dry weight enhanced by seed priming by magnetic field in advance was stated by Florez (2007).

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Biomass

Reduction in crop biomass under water deficit conditions reported by Rabbani and Imam (2011) and Pirevatlou et al. (2010) on corn and wheat (*Triticum aestivum*), which is probably resulted from decline in photosynthesis area and assimilate production. Seed priming by magnetic field and laser radiation resulted in higher biomass due to its positive and significant effect on corn LAI. Similar results have reported by Iqbal et al. (2012) in pea (*Cicer aritinum*) and Hernandez et al. (2010) on corn using magnetic field and laser radiation.

Grain yield

Based on the results of this study, due to the water deficit from increased irrigation duration from 70 to 110 mm evaporation, corn grain yield reduced in all treatments, and increasing of duration of seed priming with gamma and laser rays from 5 to 10 min. and accompanying that treatments with water deficit led to significant grain yield loss. Therefore corn seeds priming with magnetic field in both time intervals or under gamma and laser rays only for 5 min. can be used to improvement of grain yield in normal and stress irrigations. Seed priming by magnetic field and laser radiation caused yield improvement via enhancement of growth and LAI followed by increasing of photosynthesis rate. Grain yield increasing reported by Racuciu et al. (2008); Iqbal et al. (2012); Faqenabi et al. (2009) on corn, chickpea and safflower, respectively; using seed priming with magnetic field. According to Pasangka (2013), average grain yield of soybean increased from 2.5 to 4.5 ton/ha via seed priming by gamma ray.

Comparison of physical treatment on groups for 5 min. then 10 min. under both normal and drought conditions demonstrated that for most agronomic traits such as grain yield physical treatments for 5 min. was better than 10 min. and on the other hand comparison of seeds priming with magnetic field with non-treated seeds revealed increases of some important agronomic traits such as grain yield and the biomass.

Conclusions

As a conclusion from this study, to increase corn grain yield in both normal and water deficit conditions, seed priming with the magnetic field in time intervals of 5 and 10 min., gamma and laser rays only in a short time is recommended. The future studies should be extended to evaluation the response of corn yield when seed exposed under lower intervals of the biophysical treatments.

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THE RELATION BETWEEN THE LANDSCAPE DESIGN AND BRAND IMAGE IN PURCHASE PREFERENCES OF TOURISTS: THE CASE OF SAFRANBOLU AND NEVŞEHIR, IN TURKEY

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Abstract. With the developments in the tourism sector, the preferences and demands of tourists are handled with more care. Parallel to this, the tourism businesses that host tourists are in constant search for innovation. The aim of this study is to investigate the relation between the landscape design works and the brand image in the purchase preferences of the tourists in accommodation businesses; and to reveal the relation between these two factors by obtaining the demographical information of the tourists visiting Safranbolu and Nevşehir destinations. The Questionnaire Technique was made use of in this study. The percentage and frequency distributions are given. The Pearson Correlation Analysis was used in order to determine the relation between the landscape design works and the brand image in the preferences of tourists in accommodation businesses. As a result, it was determined that as the role of personal area regulations increase in the purchase preferences of tourists, so do the brand awareness and associations, brand image, quality perceptions, and brand trust.
Keywords: *brand, image, landscape, accommodation businesses, purchase*

Introduction

Many factors including demographical, economic, and socio-cultural ones, and the facilities provided by tourism businesses in the tourism sector play important roles in the purchase preferences of the tourists. In addition, some attractive characteristics of the destination also have important effects on the selection of the destinations by tourists (Correia and Pimpao, 2008; Demir, 2010). For this reason, the attractive factors of a destination are important because they provide advantage for that specific destination to be preferred among the other destinations. The brand image, physical structures of the tourism businesses and the services they provide are important in making the destinations be perceived as different from the other ones.

The spatial properties of touristic areas are influential on the purchase preferences of tourists, which are considered as being important by tourism businesses. Purchase decision-making process is a study field that is emphasized and studied much in tourism sector. The purchase decision of tourists, their decisions before the travel, their experiences, their evaluations on their experiences and their intentions after the purchase are important for tourism businesses. The purchase decision in tourism services means the starting point of a complex process. While the decision given to purchase a product is the sole factor in purchase decision-making process, the purchase

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decision of a tourist includes many other factors related with their travels. One single decision of the tourists on their travels will trigger their other decisions. In other words, tourists have to make many decisions on the products and services they will purchase in their travels. In general, it is accepted that the decision-making process of a consumer consists of 5 basic steps (Filiatrault and Ritchie, 1980; Solomon, 2004; Chen and Tsai, 2007; Hyde, 2008; Smallman and Moore, 2010; Yüncü and Kozak, 2010). These steps are as follows;

- Determining the problem,
- Searching for information,
- Evaluation of the alternatives,
- Purchase decision / preference
- The behavior after the purchase

Although the brand value refers to the whole of the assets owned by a brand, the components of a brand consist of *the awareness on the brand*, *being influenced by the brand*, *trust in the brand*, *the associations about the brand*, *the brand image*, and *the devotion to the brand* elements. The reason of testing the effect of all the components of brand value on the devotion to the brand is that this devotion is considered as one of the most important components in establishing and sustaining long-term relations with the customers. Although brand image was recognized as the driving force of brand asset and brand performance, few studies have elaborated on the relationship between brand image and brand equity (Morrison, 1989; Torlak et al., 2014; Zhang, 2015).

The brand image consists of the whole of the positive or negative, emotional or aesthetical impressions created by a product in the target market. In other words, it may be considered as the whole of the emotional and rational evaluations of a person on a product or service. Based on these definitions, it is possible to claim that the Brand Image is created in the minds as a result of the perceptions of the consumers about a product or a service and as a result of the marketing efforts of the brand. In other words, consumers may have an image in their minds with the help of the associations about a brand. In one sense, the attitude of the consumers towards a product may create the Brand Image. A positive brand image affects the brand loyalty and ensures the loyalty of the consumers (Ker, 1998; Peltekoğlu, 2007; Özdemir, 2009; Başgöze and Kazancı, 2014; Baloglu et al., 2014).

There have been some changes in the purchase preferences of tourists in recent years, and the importance of the outdoor areas has come to the forefront. The design concepts in outdoor areas and relevant projects and practices that change every year are presented for the liking of the tourists. The landscape design works by landscape architects are among the first and most important factors in the preferences of tourists (Avan, 2010; Dönmez and Türkmen, 2015).

The purpose of this study is to investigate the relation between the Landscape Design and Brand Image in the Purchase Preferences of Tourists. Questionnaires were applied to the tourists visiting important tourism destinations like Safranbolu and Nevşehir.

Material and Methods

In this study, in which the viewpoints of tourists on the effects of landscape design on purchase preferences in accommodation businesses were analyzed; an answer was sought for the question “Is there a relation between the landscape design and brand image in the preferences of the tourists for accommodation?” The study population consisted of the tourists who visited the hotels in Nevşehir and Safranbolu (*Fig. 1*) between June and April, 2016-2017. Safranbolu and Nevşehir cities are important tourist destinations of Turkey because of their natural and cultural features, and they are visited by more than one million local and foreign tourists a year.

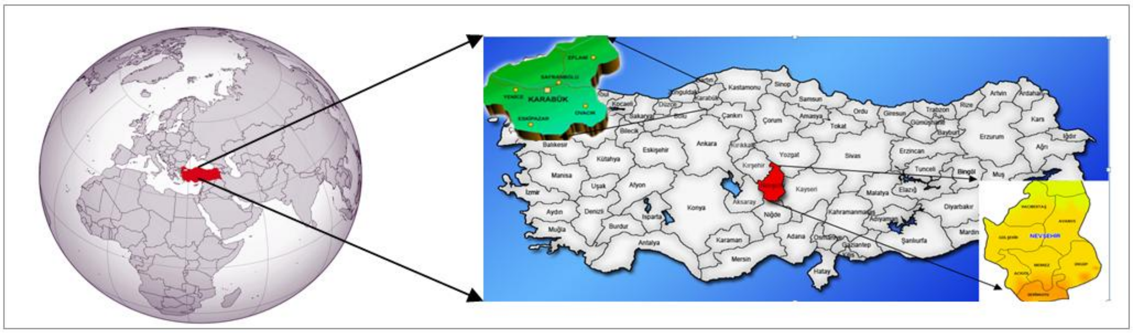

Figure 1. *Location of Research Areas in the World and Turkey*

In determining the sampling that represent the features of the study population, the Convenient Sampling Method was used. The total number of the tourists coming to

Nevşehir and Safranbolu was determined as 2,250,000, and the n= NPq/(N-1) B^2+Pq/ Z^2 Model, which was developed by Ryan (1995) was used to determine the sampling size.

$$n = NPq/(N-1)B^2 + Pq/Z \qquad \text{(Eq.1)}$$

According to the formula:
n= Sampling number,
N= The study population,
P= The rate or estimation of the population,
q= 1-P,
B= Bearable error rate,
Z= Desired confidence interval.

In this respect; since N= 2,250,000 tourists, P= 0.5, q= 0.5, B= 0.05, Z= 1.96, n= 2,250,000 (0.5) (0.5) / (2,250,000 - 1) $(0.05)^2$ + (0.5) (0.5) / $(1.96)^2$; n= 384 tourists were determined.

The Questionnaire Technique was used in the study. The Questionnaire form consisted of three parts. In the first part, seven questions were asked to determine the demographical properties of the participants. There were seventeen questions, which were prepared by receiving specialist viewpoints and with a literature review on landscape design in the second part. In the last part, there were 42 statements about the Brand Image. The statements in the second and third parts were assessed with 5-Point Likert-type scale ranging through “I do not agree et all” and “I completely agree”. This Questionnaire Form was copied as 750 forms and applied in the target population. After the Questionnaires with missing or invalid parts were eliminated, 511 Questionnaires were included in the study.

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The data were analyzed in the SPSS 23 (Statistical Package for the Social Sciences 23) software. The Factor Analysis was applied to the data by using the Varimax Rotation and Principal Components Methods. The applicability of the Factor Analysis was checked with the Bartlett Test, and the adequacy of the sampling size was checked with the Kaiser-MePlace-Olkin (KMO) value. The Cronbach's Alpha values were calculated for the internal consistency of the scale and for Reliability Analysis. After the percentage and frequency values were given about the demographical values of the participants, the Pearson Correlation Analysis was made use of to determine the relation between the landscape design and brand image in the preferences of the tourists for accommodation businesses.

Results

The Factor Analysis results on the viewpoints of the tourists about the role of the landscape design works in purchase preferences are given in *Table 1*. According to these results, the Kaiser-MePlace-Olkin value gave the sampling size adequacy as KMO=0.833; and the Bartlett Test gave the applicability of the Factor Analysis as χ^2 =3407.132; p<0.001. In addition, out of the 4 factors, which explained 23.204% of the total variance, the "Entertaining Area Organizations" sub-dimension was the first factor, the "Compulsory Area Organizations" sub-dimension was the second factor explaining 18.135% of the total variance; "Green Area Organizations" sub-dimension was the third factor explaining 13.916% of the total variance; and as the last item, the "Personal Area Organizations" sub-dimension was the fourth factor explaining 10.342% of the total variance. Again, according to the findings in *Table 1*, the General Reliability Coefficient (Cronbach's Alpha) of the scale, which involved the viewpoints of the tourists on the landscape design works in their purchase preferences, was calculated as α=0.869. Kayış, 2009 considered the ranges where Reliability (Cronbach's Alpha) Coefficient values would be and accepted that if the reliability was "0.6≤α<0.80", the Scale would be extremely reliable; and if it was "0.80≤α<l.00", the scale would be highly reliable. For this reason, it is possible to say that the scale is highly reliable.

Table 1. *The Factor Analysis Made on the Statements of the Tourists about the Role of Landscape Design on the Purchase Preferences*

Statements	**Factor 1:** Entertaining Area Organizations	**Factor 2** Compulsory Area Organizations	**Factor 3** Green Area Organizations	**Factor 4** Personal Area Organizations
1. There must be a swimming pool.	0.860			
2. There must be swimming pools designed specifically for children.	0.720			
3. There must be playgrounds for children.		0.806		
4. There must be recreational areas.		0.543		
5. There must be Open/Closed car parks.		0.786		
6. There must be a Botanical Garden.			0.810	
7. There must be trekking areas.			0.682	
8. There must be vegetation design in different forms.			0.852	
9. There must be designs with plenty of flowers.			0.853	

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10. The grass areas must be bigger.			0.783	
11. There must be different designs in accordance with the structure of the area.				0.615
12. There must be recreational water elements like ornamental pool and waterfall.				0.687
13. There must be sales stand for regional products.				0.596
14. There must be amphitheaters for concerts or other activities.				0.675
15. The night lighting in the garden must be in different color and design.				0.703
$\overline{X}$/sd	**4.18/1.00**	**4.20/0.87**	**3.75/1.02**	**3.99/0.77**
Eigenvalues	**3.481**	**2.720**	**2.087**	**1.551**
Variance Explanation Rate	**23.204**	**18.135**	**13.916**	**10.342**
Cumulative Variance	**23.204**	**41.340**	**55.256**	**65.597**
Sub-Dimension Reliability (Cronbach's Alpha)	**0.701**	**0.675**	**0.893**	**0.757**
General Scale Reliability (Cronbach's Alpha)		**0.869**		

Kaiser-MePlace-Olkin (KMO)=0.833
Bartlett test: χ^2 =3407.132; p=0.000

When the reliability coefficients of the sub-dimensions of the landscape design were analyzed, similarly, it was seen thet all the dimensions had Reliability Coefficients (Cronbach's Alpha) higher than 0.6. The Reliability Coefficient of the Entertaining Area Organizations dimension was found to be 0.70; the Reliability Coefficient of the Compulsory Area Organizations dimension was found to be 0.68; the Reliability Coefficient of the Green Area Organizations dimension was found to be 0.89; and the Reliability Coefficient of the Personal Area Organizations dimension was found to be 0.76.

The Factor Analysis results on the statements that reveal the viewpoints of tourists about the effects of the brand image on purchase preferences are given in *Table 2*. According to these results, the Kaiser-MePlace-Olkin value gave the adequacy of the sampling volume as KMO=0.906; the Bartlett Test gave the applicability of the Factor Analysis as χ^2 =14816.096; p<0.001. In addition, the "Brand Awareness and Associations" sub-dimension, which explains 12.541% of the total variance out of the 8 Factors that constitute the scale, is the first factor; the "Brand Image" sub-dimension is the second factor that explains 11.760% of the total variance; the "Perceived Quality" sub-dimension is the third factor that explains 10.378% of the total variance; the "Brand Trust" sub-dimension is the fourth factor explaining 9.212% of the total variance; the "Brand Loyalty" sub-dimension is the fifth factor explaining 8.147% of the total variance; the "Brand Purchase" sub-dimension is the sixth factor explaining 7.123% of the total variance; the "Brand Attitude" sub-dimension is the seventh factor explaining 5.009% of the total variance; and as the last item, the "Perceived Risk of the Brand" sub-dimension is the eighth factor explaining 2.807% of the total variance. Again, according to the findings given in *Table 2*, the General Reliability Coefficient (Cronbach's Alpha) of the scale that investigated the role of the brand image in purchase preferences of the tourists was calculated as α=0.95. For this reason, it is possible to claim that the scale is highly reliable.

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Table 2. *The Factor Analysis on the Statements of the Tourists on Purchase Preferences and Brand Image*

Statements	**Factor 1:** Brand Awareness and Associations	**Factor 2:** Brand Image	**Factor 3** Perceived Quality	**Factor 4** Brand Trust	**Factor 5** Brand Loyalty	**Factor 6** Brand Purchase	**Factor 7:** Brand Attitude	**Factor 8** Perceived Risk of the Brand
1. I know the architectural structure of this hotel.	0.593							
2. I am aware of the brand name of this hotel.	0.735							
3. I differentiate this hotel from the other brand hotels.	0.733							
4. Some characteristics of this hotel cross my mind immediately when I hear its name.	0.651							
5. I immediately remember the symbol or logo of this hotel.	0.710							
6. When I want to make a reservation at a hotel business, I firstly remember the name of this hotel.	0.604							
7. This hotel is comfortable.		0.521						
8. The service presentation is at a high level in this hotel.		0.531						
9. This hotel has a very good image.		0.644						
10. This hotel is a luxurious one.		0.642						
11. This hotel is suitable for people from upper class.		0.589						
12. I feel special when I arrive at this hotel.		0.480						
13. The service in this hotel is beyond my expectations.		0.572						
14. This hotel has a long history.		0.666						
15. This hotel belongs to a good brand.		0.513						

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16. The employees of this hotel make me feel that I am a special and precious guest.			0.639					
17. This hotel has modern equipment.			0.495					
18. The employees of this hotel are clean and elegant.			0.551					
19. The employees who are specialists in their fields work in this hotel.			0.788					
20. This hotel provides the correct service at the correct time.			0.806					
21. The employees of this hotel solve the problems of the guests in a fast manner.			0.680					
22. The employees of this hotel establish efficient communication with guests.			0.449					
23. This food and beverage of this hotel are quality.			0.680					
24. The brand of this hotel gives me trust.				0.640				
25. This hotel provides the service quality it promises.				0.520				
26. This hotel is consistent and continuous in its service quality.				0.480				
27. Everything the brand of this hotel refers is in agreement with each other (price. quality, advertisement, variety, etc.).				0.689				
28. I am happy to have been accommodated in this hotel.					0.543			
29. When I compare this hotel with the others this is my first choice.					0.517			
30. I consider myself as a loyal guest of this hotel.					0.638			
31. I recommend this hotel to my friends.					0.724			
32. I do not change this hotel even if the other hotels make promotions.					0.744			
33. I have the intention of coming to this hotel again.						0.819		
34. I will come to this hotel again in the future.						0.747		

35. I find this hotel attractive.							0.646	
36. I like it when I stay in this hotel.							0.599	
37. I miss staying in this hotel.							0.576	
38. My preference for this hotel is a waste of money.								0.789
39. My preference for this hotel is a waste of time.								0.884
40. The goods and services of this hotel do not cover my expectations.								0.856
41. Staying in this hotel makes me feel unease in psychological terms.								0.864
42. When I prefer this hotel, people around me think negative about me.								0.836
$\overline{X}$ **/ sd**	**3.38/0.95**	**3.54/0.79**	**3.76/0.81**	**3.71/0.88**	**3.44/0.90**	**3.42/1.04**	**3.38/1.00**	**2.40/1.23**
Eigenvalues	**5.267**	**4.939**	**4.359**	**3.869**	**3.422**	**2.992**	**2.104**	**1.179**
Rate of Explaining the Variance	**12.541**	**11.760**	**10.378**	**9.212**	**8.147**	**7.123**	**5.009**	**2.807**
Cumulative Variance	**12.541**	**24.301**	**34.679**	**43.891**	**52.038**	**59.160**	**64.170**	**66.976**
Sub-Dimension Reliability(Cronbach's Alpha)	**0.861**	**0.871**	**0.893**	**0.789**	**0.819**	**0.818**	**0.775**	**0.906**
General Scale Reliability(Cronbach's Alpha)	**0.945**							

Kaiser-MePlace-Olkin (KMO)=0.906
Bartlett test: χ^2 *=14816.096; p=0.000*

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Similarly, when the reliability coefficients in the sub-dimensions of the Brand Image were analyzed it was seen thet all the Reliability Coefficients (Cronbach's Alpha) were bigger than 0.6 and highly reliable. The Reliability Coefficient of the Brand Awareness and Associations dimension was 0.86; the Reliability Coefficient of the Brand Image Sub-Dimension was 0.87; the Reliability Coefficient of the Perceived Quality dimension was 0.89; the Reliability Coefficient of the Brand Trust Sub-dimension was 0.79; the Reliability Coefficient of the Brand Loyalty Sub-dimension was 0.82; the Reliability Coefficient of the Brand Purchase dimension was 0.82; the Reliability Coefficient of the Brand Attitude dimension was 0.78; and the Reliability Coefficient of the Perceived Risk of the Brand dimension was 0.91.

The demographical and personal properties of 511 tourists who participated in the study are given in *Table 3*. In this context, 59.5% (f=304) of the tourists who participated in the study had touristic activities in Nevşehir, 40.5% (f=207) of the tourists who participated in the study had touristic activities in Safranbolu. It was determined that 45% (f=230) of the participants were local tourists, and 55% (f=281) of them were foreign tourists.

Table 3. *Frequency and Percentage Distributions of the Demographical Properties of the Participants (n=511)*

Variables	Groups	f	%
Place	Nevşehir	304	59.5
	Safranbolu	207	40.5
Gender	Women	300	58.7
	Men	211	41.3
Age	20 ≤	67	13.1
	21-30	91	17.8
	31-40	234	45.8
	41-50	76	14.9
	51 ≥	43	8.4
Marital Status	Married	339	66.3
	Single	172	33.7
Education	Primary School	36	7.0
	High School	81	15.9
	2-Year Degree	35	6.8
	Undergraduate Degree	268	52.4
	Postgraduate Degree	91	17.8
Nationality	Local	230	45.0
	Foreign	281	55.0
Profession	Self-Employed	129	25.2
	Public Employee	130	25.4
	Retired	60	11.7
	Private Sector Employee	192	37.6
Income (Monthly)	1000TL ≤	17	3.3
	1001-2000TL	18	3.5
	2001-3000TL	72	14.1
	3001-4000TL	187	36.6
	4001-5000TL	150	29.4
	5001TL ≥	67	13.1

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When the statistical values of the demographical properties of the participants were analyzed it was seen that 58.7% (f=300) were women, 41.3% (f=211) were men. When the age distributions of the participants were analyzed it was seen that 45.8% (f=234) of the tourists were between 31-40 years of age. Those between 21-30 years of age followed this with 17.8% (f=91). The rate of the participants between 41-50 years of age was 14.9% (f=76); and the rate of those at and below the age of 20 was 13.1% (f=67); and the rate of those who were at and above the age of 51 was 8.4% (f=43). In addition, the majority of the tourists who were included in the study (66.3%; f=339) were married, while 33.7% (f=172) were single.

When the distribution of the participants in term of educational status was analyzed it was determined that the majority had Undergraduate Degrees (52.4%; f=268) and 17.8% (f=91) of them had postgraduate degrees. This situation is the proof that the culture level of the participants is very high. High School graduates follow these rates with 15.9% (f=81); Primary School graduates with 7% (f=36) and 2-Year Degree graduates with a rate of 6.8% (f=35).

When the professional groups of the tourists who were included in the study were analyzed it was determined that 37.6% (f=192) worked at private sector, 25.4% (f=130) worked at public sector; 25.2% (f=129) were self-employed; and 11.7% (f=60) were retired. As the last item, the income levels of the participants were analyzed and it was determined that the majority of them had a monthly income of 3001 and 5000 TL (Total 66%; f=337). The percentage of those with 2000 TL and below income was determined as 6.8% (f=35). Only 13.1% (f=67) of the participants had a monthly income at and over 5001 TL.

In calculating the income levels of the foreign tourists, the participants were asked to respond with a currency of the country where they lived, and then this amount was converted into Turkish Liras over the exchange rate of then-present conditions.

As it is seen in *Table 4*, the level of the relation between the sub-dimensions of the Role of Landscape Design Works and Brand Image in the Purchase Preferences of the tourists was tested by applying Correlation Analysis. In this context, the level of the relation between the variables was defined as “Very Weak” when the Pearson Correlation Coefficient was below 0.19; “Weak” when the Coefficient was 0.20-0.39; “Medium Level” when Coefficient was 0.40-0.59; “Strong” when Coefficient was 0.60-0.79; and “Very Strong” when Coefficient was 0.80-1.00 (Ural and Kılıç, 2005).

When the sub-dimensions of the Entertaining Area Organizations, which is one of the sub-dimensions of Landscape Design Works, and the Brand Image were compared, it was seen that there was a weak and positive relation between the four dimensions that constituted the Brand Image and Purchase Preferences on Entertaining Area Organizations. In this context, as the role of the Entertaining Area Organizations increase in the Purchase Preferences of the tourists, the Brand Awareness and Associations (r=0.252), Brand Image (r=0.175), Quality Perceptions (r=0.129) and Brand Trust (r=0.105) increase in a linear manner. No significant relations were detected between the role of Preferences Entertaining Area Organizations in Purchase Preferences of the tourists and the other four dimensions that constituted the Brand Image.

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Table 4. The Comparison of the Relation between the Role of Landscape Design Works in Purchase Preferences of the Tourists and the Sub-dimensions of the Brand Image (Correlation Analysis)

Brand Image / Role of Landscape Design Works		Entertainment Area	Compulsory Area	Green Area	Personal Area	Brand Awareness and Associations	Brand Image	Perceived Quality	Brand Trust	Brand Loyalty	Brand Purchase	Brand Attitude	Perceived Risk of the Brand
Entertainment Area	r		0.465	0.215	0.343	0.252	0.175	0.129	0.105	0.065	0.030	0.004	-0.030
	p	1	0.000**	0.000**	0.000**	0.000**	0.000**	0.003**	0.018*	0.142	0.501	0.930	0.498
Compulsory Area	r	0.465		0.326	0.433	0.160	0.118	0.070	0.102	-0.027	-0.024	-0.042	-0.133
	p	0.000**	1	0.000**	0.000**	0.000**	0.008**	0.115	0.021*	0.539	0.596	0.344	0.003**
Green Area	r	0.215	0.326		0.522	0.128	0.060	-0.056	0.041	-0.040	-0.017	-0.029	0.035
	p	0.000**	0.000**	1	0.000**	0.004**	0.179	0.203	0.355	0.363	0.696	0.520	0.436
Personal Area	r	0.343	0.433	0.522		0.323	0.175	0.180	0.165	0.016	-0.026	-0.054	-0.156
	p	0.000**	0.000**	0.000**	1	0.000**	0.000**	0.000**	0.000**	0.713	0.559	0.222	0.000**
Brand Awareness and Associations	r	0.252	0.160	0.128	0.323		0.739	0.628	0.527	0.444	0.410	0.358	0.005
	p	0.000**	0.000**	0.004**	0.000**	1	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.907
Brand Image	r	0.175	0.118	0.060	0.175	0.739		0.772	0.644	0.606	0.552	0.558	0.058
	p	0.000**	0.008**	0.179	0.000**	0.000**	1	0.000**	0.000**	0.000**	0.000**	0.000**	0.189
Perceived Quality	r	0.129	0.070	-0.056	0.180	0.628	0.772		0.704	0.515	0.451	0.462	-0.023
	p	0.003**	0.115	0.203	0.000**	0.000**	0.000**	1	0.000**	0.000**	0.000**	0.000**	0.597
Brand Trust	r	0.105	0.102	0.041	0.165	0.527	0.644	0.704		0.565	0.446	0.512	0.018
	p	0.018*	0.021*	0.355	0.000**	0.000**	0.000**	0.000**	1	0.000**	0.000**	0.000**	0.679
Brand Loyalty	r	0.065	-0.027	-0.040	0.016	0.444	0.606	0.515	0.565		0.713	0.695	0.184
	p	0.142	0.539	0.363	0.713	0.000**	0.000**	0.000**	0.000**	1	0.000**	0.000**	0.000**
Brand Purchase	r	0.030	-0.024	-0.017	-0.026	0.410	0.552	0.451	0.446	0.713		0.744	0.176
	p	0.501	0.596	0.696	0.559	0.000**	0.000**	0.000**	0.000**	0.000**	1	0.000**	0.000**
Brand Attitude	r	0.004	-0.042	-0.029	-0.054	0.358	0.558	0.462	0.512	0.695	0.744		0.204
	p	0.930	0.344	0.520	0.222	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	1	0.000**
Perceived Risk of the Brand	r	-0.030	-0.133	0.035	-0.156	0.005	0.058	-0.023	0.018	0.184	0.176	0.204	
	p	0.498	0.000**	0.436	0.000**	0.907	0.189	0.597	0.679	0.000**	0.000**	0.000**	1

**p<0.01 *p<0.05

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When the Compulsory Area Organizations and the sub-dimensions of Brand Image are compared, a weak and negative relation was detected between the three dimensions that constituted the Compulsory Area Organizations and Brand Image; and a negative and opposite relation was detected with one dimension. In this context, as the role of the Compulsory Area Organizations increase in Purchase Preferences of the tourists, the perceptions on Brand Awareness and Associations (r=0.160), Brand Image (r=0.118) and Brand Trust (r=0.102) increase. A negative and weak relation was detected between the Compulsory Area Organizations and Perceived Risk of the Brand (r= -0.133). No significant relations were detected between the other four dimensions that constitute the Brand Image and the Compulsory Area Organizations sub-dimension.

When the relation between the Green Area Organizations and the sub-dimensions that constitute the Brand Image were analyzed, a weak and positive relation was detected in terms of only one dimension that constituted the Brand Image. As the Green Area Organizations in the Purchase Preferences of the tourists increase, the Brand Awareness and Associations (r=0.128) also increase parallel to this. No significant relations were detected as a result of the Correlation Analysis between the Green Area Organizations and the other seven dimensions that constituted the Brand Image.

When the relation between the Personal Area Organizations, which is the last dimension of the Landscape design works, and the sub-dimensions that constitute the Brand Image was analyzed, it was determined that there was a positive and weak relation between the four dimensions that constitute the Brand Image, and a negative relation with only one dimension. In this context, as the role of the Personal Area Organizations in Purchase Preferences of the tourists increased, the Brand Awareness and Associations (r=0.323), Brand Image (r=0.175), Quality Perceptions (r=0.180) and Brand Trust (r=0.165) increased. A negative and weak relation was detected between the Personal Area Organizations and Perceived Risk of the Brand (r= -0.156). When the coefficient obtained as a result of the Correlation Analysis and the Sigma-p values were assessed, no significant relations were detected between the Personal Area Organizations and the other three dimensions that constitute the Brand Image.

Discussion

A substantial number of studies have focused on tourist destination image, its components, and how they relate to behavioral intentions. The findings suggest that a positive destination image is a favorable competitive advantage for attracting first-time visitors as well as generating greater likelihood to revisit and recommendation after the visit. Earlier studies have tested the adaptability of the image and personality concepts, which were originally developed in other fields and disciplines, into the context of tourism (e.g. Baloglu and Brinberg, 1997; Ekinci and Hosany, 2006; Hosany et al., 2006; Baloglu et al., 2014).

Given their explicit importance to brand loyalty, positioning and destination selection (Ekinci, Sirakaya-Turk, and Baloglu, 2007; Keller, 1998; Baloglu et al., 2014), this is somewhat surprising. In addition, a recent study indicated that visitors do not use brand personality descriptors when asked to provide free-elicited responses to describe tourist destinations (Kneesel et al., 2010; Baloglu et al., 2014).

When the sub-dimensions of the Brand Image and the Entertaining Area Organizations, which is one of the sub-dimensions of Landscape design works, were compared, a positive and weak relation was detected between the Purchase Preferences

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in Entertaining Area Organizations and the four dimensions that constitute the Brand Image. In this context, it is seen that as the role of the Entertaining Area Organizations increased in the Purchase Preferences of the tourists, the Brand Awareness and Associations (r=0.252), Brand Image (r=0.175), Quality Perceptions (r=0.129) and Brand Trust (r=0.105) increased in a linear manner. No significant differences were detected between the role of the Entertaining Area Organizations in the Purchase Preferences of the tourists and the other four dimensions that constitute the Brand Image.

When the sub-dimensions of the Compulsory Area Organizations and Brand Image were compared it was determined that there was a positive and weak relation between the Compulsory Area Organizations and the three dimensions that constitute the Brand Image, and a negative and weak relation was detected with only one dimension. In this context, as the role of Compulsory Area Organizations in Purchase Preferences in the tourists increased, the Brand Awareness and Associations (r=0.160), Brand Image (r=0.118) and Brand Trust perceptions (r=0.102) increased. A negative and weak relation was detected between the Compulsory Area Organizations and Perceived Risk of the Brand (r= -0.133). No significant relations were detected between the Compulsory Area Organizations sub-dimension and the other four dimensions that constitute the Brand Image.

When the relation between the Green Area Organizations and the sub-dimensions that constitute the Brand Image was analyzed, a positive and weak relation was detected with only one single dimension that constituted the Brand Image. As the role of the Green Area Organizations in Purchase Preferences of the tourists increased, the Brand Awareness and Associations (r=0.128) also increased parallel to this. No significant relations were detected between the Green Area Organizations and the other seven dimensions that constitute the Brand Image.

When the relation between the Personal Area Organizations and the sub-dimensions that constitute the Brand Image, which is the last dimension of the Landscape design works, was analyzed, a positive and weak relation was detected between the four dimensions that constitute the Brand Image; and a negative and weak relation was detected with only one dimension. In this context, as the role of the Personal Area Organizations increased in the Purchase Preferences of the tourists, the Brand Awareness and Associations (r=0.323), Brand Image (r=0.175), Quality Perceptions (r=0.180) and Brand Trust (r=0.165) also increased. A negative and weak relation was detected between the Personal Area Organizations and Perceived Risk of the Brand (r=-0.156). When the coefficients obtained as a result of the Correlation Analysis and the sigma-p values were analyzed, no significant relations were detected between the Personal Area Organizations and the three dimensions that constituted the Brand Image. Right at this point, the quality perceptions of the tourists increase in businesses which care for the Landscape Area design works. However, no trust perceptions were detected for the business organizations that do not have landscape design works.

Conclusions

Countries seek ways to increase their tourism incomes by applying various encouragements in different fields. The tourism businesses, which have become a brand name in the sector, apply different regulations and organizations according to the purchase behaviors of the tourists, and try to attract the attention. For this reason,

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landscape design works are made in agreement with the desires and demands of the tourists who spend their times in outdoor environments. As it is seen in the present study, there is a significant relation between the factors that constitute the Brand Image and the landscape design works. The size of the outdoor environments owned by tourism businesses, activity areas, entertainment units, and the organizations for children are important for the Purchase Preferences of tourists.

As a conclusion, the landscape design works that will be performed in outdoor areas by tourism businesses that have become a brand name in the sector will affect the preferences of the tourists in terms of covering the targets and expectations. In this way, the occupancy rates of the tourism businesses may be increased and the stay of the tourists may be extended; and the satisfaction of the tourists will increase, and they will prefer the same business again. Thus, the tourism business, the destination and the tourists will be affected in a positive way.

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AGROFORESTRY STATUS AND ITS ROLE TO SEQUESTER ATMOSPHERIC CO_2 UNDER SEMI-ARID CLIMATIC CONDITIONS IN PAKISTAN

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Abstract. Carbon dioxide (CO_2) is the major cause of global warming. Many countries including Pakistan have signed the Kyoto Protocol agreement under the United Nations Framework Convention on Climate Change (UNFCC) and agreed to control the release of CO_2 and to increase the CO_2 sequestration. Agroforestry sector can contribute efficiently in carbon sequestration. This study was carried out to determine the status and potential of agroforestry and its role in carbon sequestration under semi-arid conditions. Data was collected through a multi-objective and pre-tested questionnaire from 250 village farmers in tehsil Sumandri, Pakistan. Height and girth of trees were measured from 250 randomly selected 0.405 ha farm plots. Soil samples from each cropping pattern were collected and analyzed. The whole study area has 2069.19 Mt of above ground carbon stocks and has sequestered a total of 7579.46 Mt of carbon dioxide at the rate of 186201.85 t CO_2 yr^{-1}. Furthermore, the study area has the potential of stocking 3607.61 Mt and sequestering 13214.67 Mt of CO_2 at the rate of 327232.46 t CO_2 per year. According to calculations, increasing the number of farm trees/ha, average CO_2 sequestration rate of the study area can be increased from 2.05 t CO_2 $ha^{-1}yr^{-1}$ to 3.59 t CO_2 ha^{-1} yr^{-1}. The role of agroforestry as C sink in not negligible and it should be given a dire consideration in policies, especially, in low forest countries like Pakistan to meets the melinium goals of atmospheric C reduction.
Keywords: *climate change, global warming, carbon cycle, envrionment, forestry*

Introduction

Changes in climate are happening which are unparalleled. If we continue on our present course, life on the Earth will be modified to such an extent that it will be very difficult to fix (IPCC, 2000; Mahowald et al., 2017). Due to several activities like combustion of fossil fuels and deforestation during the last 100 years, chemical makeup of this flimsy layer of the atmosphere has been extremely altered (Pold et al., 2017). Such kind of modifications in chemical composition has a wide range of significant harmful results on the long term weather conditions of the planet, the ecological systems which are being supported by the climate of the Earth, and the welfare of human beings and economy (CDIAC, 2000; Harsch et al., 2017).

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Global warming is among the greatest terrible horrors of the modern times (Salvatore et al., 2017). It is believed that carbon is among the most significant casual factors which cause global warming (Kerr, 2007; Franzluebbers et al., 2017; Jones et al., 2017). So, enhanced carbon discharge in the atmosphere is one of today's major interests and is significantly referred in Kyoto Protocol (Stewart and Hessami, 2005). Globally, transportation and industrial sources are the cause of more than 80% CO_2 anthropogenic emissions. The rest 20% comes mainly from deforestation and biomass combustion (CDIAC, 2000). Moreover, the rate of accumulation to the atmosphere from these sources surpasses the rate of loss to major CO_2 sinks by about 3.3 GtC /year (Schroeder, 1994). Therefore, the concentration of CO_2 in the atmosphere is increasing continuously and it has extended up to 400 ppm in certain regions (Bala, 2013). These current atmospheric CO_2 concentrations are much higher than pre-industrial levels (180-280 ppm) and it goes on increasing exponentially at about 0.5% per year (IPCC, 2000).

Among various carbon storage options, oceanic storage is not a cost-effective method to sequester CO_2 and likely other geological choices of atmospheric CO_2 storage are also vulnerable to leak out and not eco-friendly (Stewart and Hessami, 2005; Khatiwala et al., 2013). However, biotic sequestration overcomes all those environmental concerns which are linked along with geological and ocean storage (Lal, 2004; Weissert et al., 2017). Plants capture atmospheric CO_2 via natural process of photosynthesis. Each tree has a considerable capacity to store CO_2 and the C storage rate varies with the age of trees and it is species dependant under a set of ecological conditions (Nair et al., 2009). Plants store this CO_2 into their leaves, roots, bark, branches and stem (Nair et al., 2009). About 50% of tree's biomass is composed of carbon and its significant amount is stored into trees in the form of cellulose and lignin (De Villiers et al., 2014).

Agroforestry plays a very significant role and it is a very vital part of daily life of population of Pakistan, particularly, population of rural areas (Qureshi, 2005; Nawaz et al., 2016). Agroforestry system has a great potential to capture both above and below-ground carbon (Kotto-Same et al., 1997; Nair et al., 2009). The practices of agroforestry have been approved as a scheme for capturing of soil carbon under reforestation and aforestation programs and Clean Development Mechanisms of the Kyoto Protocol. Agroforestry systems (AFS) not only help in the sequestration of carbon but also responsible for providing a great number of other services to the communities of rural areas (Masera et al., 2001). It has been estimated that up to 12-228 Mg ha^{-1} carbon can be stored through AFS, with an average of 95 Mg ha^{-1} (Schroeder, 1994; de Jong et al., 1995; Nair et al., 2009). The C sequestration capacity by AFS in the most of cases is less than the pure forests but still comparable and more important in the areas with ecological hindrances for forest establishment (Jones et al., 2017). It has been reported that quantity of carbon stored in any agroforestry system depends on number of different factors like age of the system, composition and purpose, management practices of silviculture, climatic conditions of soil like texture and other properties and history of the land-use (Albrecht and Kandji, 2003).

The prospective role of agroforestry systems to serve as a C sink and to be incorporated into a global C trading system has not been given the deserved importance. Thus, incorporation of agroforestry practices and systems into C sequestration and C trading projects can be very useful to meet up the targets of CO_2 emissions anticipated at Kyoto while at the same time maintaining sustainable agricultural production and preventing further deforestation. It is very necessary to know about the accurate

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information about agroforestry practives and the spatial distribution of carbon in both vegetation and soil. As there was no any significant study in Pakistan relevant to the carbon stocks of farm trees, so, the first objective of this study was to assess agroforestry status and peoples's interaction with agroforestry in the study area. The second major obective of this study was to assess the carbon stocks and analyse the potential of carbon sequestration by farm trees in study area under semi-arid climatic conditiosn. Tehsil Sumandri has been selected due to presence of diversified nature of agroforestry systems there.

Materials and methods

Description of Study Area

Tehsil Samundri of district Faisalabad was selected for our study. Samundri was on a major trade route during the reign of Sher Shaha Suri. Samundri is located at 30°48'30N 71°52'15E, with an altitude of 130 metres (429 ft), and is 45 km from Faisalabad. The driest month is November, with 3 mm of rain. In July, the precipitation reaches its peak, with an average of 107 mm (*Fig. 1* and *Fig. 2*).

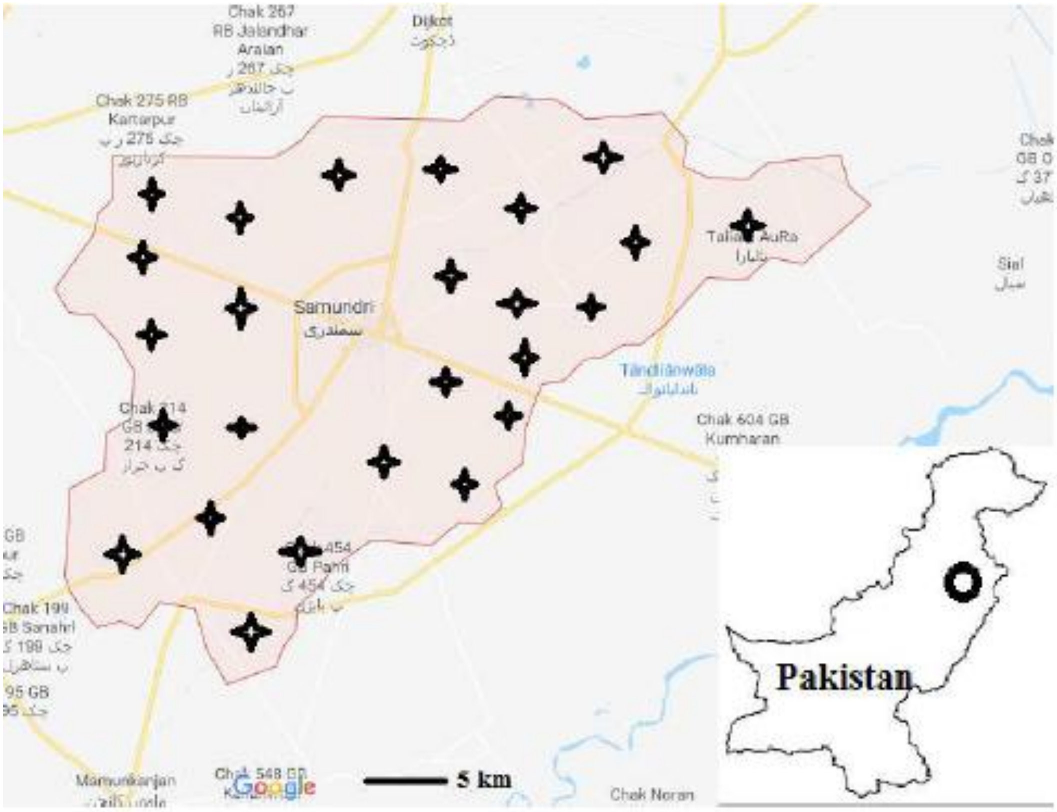

Figure 1. *Location of study area with sampled locations*

Agroforestry Status and Potential

To analyze the current status of agroforestry and peoples interaction with agroforestry, a questionnaire was developed through a proper and consultative process, keeping in view the goals and objective of study. Keeping in view the administration

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distribution of Samundri tehsil, it had 28 union councils. Out of which 3 were urban and 25 were rural. Data from 2 villages of each rural union council was collected. Five farmers were interviewed from each village. Thus, total numbers of interviewed farmers were 250 from 25 rural union councils. The attributes which were being studied were: income, education, land holding, income source, annual income from trees, annual trees products used domestically, maximum number of trees per acre, agroforestry pattern, trees services, tree products sold per anum, distribution of trees Species.

10 plots of 0.405 ha (one acre) were being selected from each union council randomly. All the trees were counted at each plot. Their girth, height and age was calculated of each tree and carbon was calculated. Total number of trees per plot was calculated. And then the average number of trees per ha were calculated. The total number of trees in a union council were calculated by multiplying the average number of trees per ha with total area of each union council in ha.

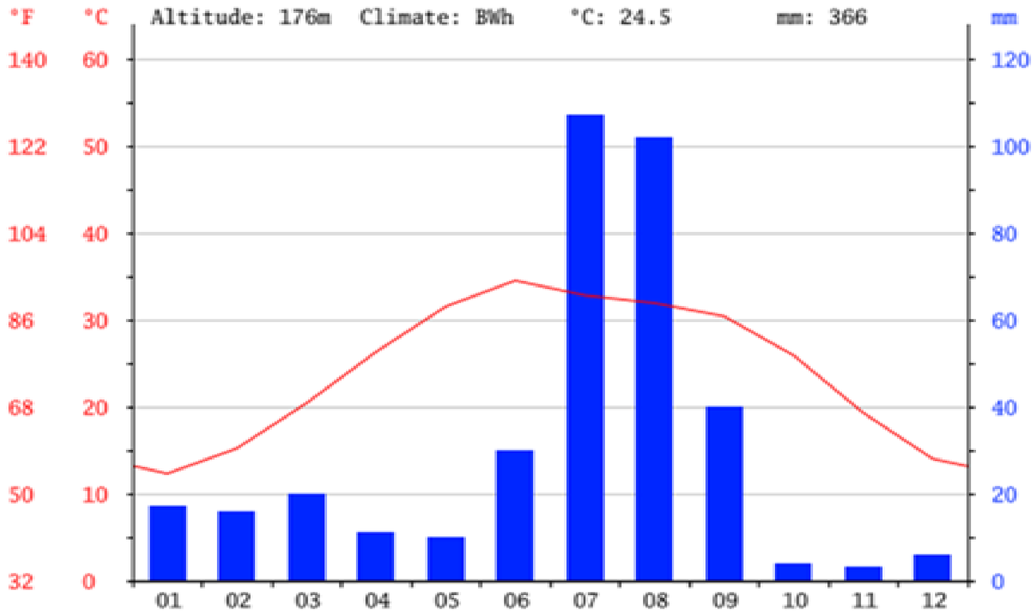

***Figure 2.** Climat of Tehsil Sumandri*

Carbon Status and Potential of Carbon Sequestration

Carbon stock and CO_2 sequestration rate was determined by using the standard methods in the literature by calculating the true tree volume and biomass with the available equations and wood density in literature (Juwarkar et al., 2011; Kumar and Nair, 2011). The below ground root mass was supposed 20% of the above ground biomass, so, it was added in above ground biomass and dry biomass was calculated on the supposition of 27% moisture. Carbon was estimated as 50% of the biomass of the tree (Afzal and Aqeela, 2013; Nawaz et al., 2017a).

The potential of carbon sequestration refers to the ability or capacity of the amount of carbon and CO_2 which can be stored by farm trees of tehsil Samundri. To determine this, first of all the maximum number of trees grown at a single plot of each union council were determined by using the statistical tool of Range. Then the average was taken of theses maximum number of trees grown at one ha. The average of the

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maximum number of trees was 54ha^{-1}. It means that 54 trees can be grown at one ha. 54 trees were taken as potential trees and the potential of each union council to capture carbon and carbon dioxide was calculated by using statistical tools (Nawaz et al., 2017b).

Collection of Soil Samples

Soil samples from each agroforestry combination from Tehsil Samundri were collected from two depths (0-15 cm) and (15-30 cm) by using Soil Auger. And carbon concentration of soil for each cropping pattern was calculated by using Walkely Black method (Savoy, 2013). The other characteristics of soils which were being studied are soil EC, pH, Organic Matter (%), Saturation percentage, N, P, K and C/N according to standard protocols (Nawaz et al., 2017b).

Statistical Analysis

To calculate the average, range, frequency and standard deviation for different sets of data, the statistical tools in MS-Excel were employed.

Results

Current C status

Table 1 shows the current status of carbon stocks and CO_2 sequestered by farm trees. The average number of trees were found 31 trees ha^{-1} with the maximum 39 trees ha^{-1} in union council number 105 and the minum 28 treesha^{-1} in few unin councils. For the whole study area, it was estimated to be the 2069.19 and 7579.46 Mt respectively with an average of 0.02 Mt C ha^{-1}and 0.08 Mt of CO_2 ha^{-1}. The average rate of sequestration of CO_2 was estimated to be the 2.05 t CO_2 ha^{-1}yr^{-1}. The total amount of rate of CO_2 sequestered in one year was estimated to be 186201.85 t CO_2 yr^{-1}. The C storage and sequestration capacity varied among union coucils (UC) and some times it was independt of number of trees. Due to rounding off the figures, the variation in C stock/ha was less visible among UCs as compared to CO_2 sequstration rate/ha/year. It can be seen that despite less tree/ha in UC-111 (28) as compared to UC-101 (38) the CO_2 sequstration rate/ha/year wascomparable in both of them. Similarly, Despite more number of trees in UC-103 (33) it has less CO_2 sequstration rate/ha/year (1.87) as compared to UC-110 (2.01) with 28 trees ha^{-1}. However, the differences among UCs for total C stocks, total CO_2 sequestration rate and total CO_2 sequestration rate per year were not only dependant on number of trees to some extent but on the total area of that union council as well. The area of UC-125 was the highest, so, all the above metnioned area dependant parameters were high in the UC-125.

C Sequestration Potential

In *Table 2*, the average trees/ha for each UC were calculated from radomly sampled 10 plots. However, the maximum number of trees/ha is for one plot in that UC with the highest number of trees (without compact plantation). The maxumimum number of trees/ha were the highest (62) in UC-101 and the lowest (49) in UC-106 and UC-118. The average of maximum number of treees/ha in all Ucs was found 54 trees/ha. Considering the 54 trees/ha as practicable AFS in the study area, it was set as potetial

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number of trees that can be grown in this area. The average potential of farm trees for carbon stocks and CO_2 were estimated to be the 0.04 Mt ha^{-1} and 0.08 Mt ha^{-1} respectively (*Table 2*). The total amount of carbon which can be stored by farm trees of tehsil Samundri was estimated to be the 3607.61 Mt of carbon and 13214.67 Mt CO_2. It was estimated that farm trees have the potential to store 327232.46 t of CO_2 per year at an average rate of 3.59 CO_2 t ha^{-1} yr^{-1} (*Table 2*). Likewise to current status, the area dependat parameters such as total C stocks, total CO_2 sequestration rate and total CO_2 sequestration rate per year were highest in UC125.

Table 1. *Current Status of Agroforestry, Carbon Stocks and CO2 sequestration rate by farm trees in Tehsil Samundri*

UC#	Trees per ha	C Stock/ha (Mt)	CO_2 Sequestered /ha (Mt)	CO_2 Sequestration rate /ha/year (t)	Total Area (ha)	Total C Stock (Mt)	Total CO_2 Sequestered (Mt)	Total CO_2 Sequest. rate/year (t)
101	38	0.03	0.11	2.35	3148.47	90.47	331.38	7398.90
102	37	0.03	0.10	2.33	3142.40	88.80	325.28	7334.35
103	33	0.03	0.09	1.87	4653.45	119.75	438.63	8701.95
104	38	0.03	0.11	1.95	3519.05	102.05	373.81	6862.14
105	39	0.03	0.11	2.24	3345.30	96.76	354.44	7493.47
106	30	0.02	0.08	2.35	3061.40	69.07	252.99	7194.28
107	29	0.02	0.08	1.89	3070.31	66.59	243.91	5802.88
108	29	0.02	0.08	1.81	3971.43	85.66	313.76	7188.29
109	31	0.02	0.08	1.92	2973.11	67.01	245.46	5708.36
110	28	0.02	0.08	2.01	4586.22	95.70	350.57	9218.30
111	28	0.02	0.08	2.34	3428.33	71.19	260.77	8022.28
112	30	0.02	0.08	2.05	3179.25	68.92	252.46	6517.46
113	30	0.02	0.08	2.11	4093.34	88.37	323.69	8616.47
114	28	0.02	0.07	1.85	3814.70	77.55	284.07	7057.19
115	29	0.02	0.08	1.82	2757.65	57.08	209.09	5018.91
116	28	0.02	0.08	1.87	2534.49	52.69	193.00	4747.10
117	30	0.02	0.08	2.06	2208.47	50.35	184.44	4540.60
118	31	0.02	0.09	2.13	3669.30	85.86	314.50	7815.61
122	36	0.03	0.09	2.26	3289.41	84.83	310.73	7420.91
123	28	0.02	0.07	1.93	2913.17	58.64	214.80	5634.06
124	32	0.02	0.08	2.12	3798.09	85.66	313.79	8063.35
125	30	0.02	0.08	2.35	7533.41	163.02	597.12	17680.90
126	28	0.02	0.08	1.90	3881.52	79.76	292.16	7355.48
127	28	0.02	0.08	1.94	4846.23	102.48	375.37	9377.46
128	28	0.02	0.08	1.83	2967.84	60.95	223.26	5431.15
Total	25	--------	-------	-------	90386.28	2069.19	7579.46	186201.8
Av.	-----	0.02	0.08	2.05	-------	--------	---------	--------

Soil Carbon

It was found that farmers are mostly planting diverse trees species (not mono-species per plot) alongwith all crops. However, four types of tree-crop combinations were frequent as compared to all other types. Farmers liked to plant trees with wheat (*Triticum aestivum*), sugarcane (*Saccharum officinarum*), berseem (*Trifolium alexandrinum*) and sarsoun or mastard (*Brassica rapa*). So, soil C was measured for these four combinations (*Fig. 3*). At the soil depth of 0-15 cm, it was estimated that maximum amount of soil carbon was present under the combination of trees plus berseem crop (0.45%). It was followed by sugarcane plus trees (0.43%) as shown in *Fig. 3*. Results showed that trees grown in combination with wheat crop captures less

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soil carbon than tree plus sugarcane but more carbon than the sarsoun plus trees. At 15-30 cm soil depth, the minimu carbon (%) was observed for sarsoun plus trees with no significant difference for other three combinations. The overall carbon percentage in the soils was less than 0.5 signifying that studied soils were carbon deficient and have poor organic matter contents.

Table 2. *Potential of Agroforestry, Carbon Stocks and CO_2 Sequestration rate of farm trees in Tehsil Samundri*

Union Council #	Average trees/ha	Max No. of Trees/ha	Potential trees	Potential Carbon Stock (Mt)	Potential CO_2 sequestered (Mt)	Potential CO_2 sequestration rate/ha/year(t)	Potential Co2 Sequestration rate per year(t)
101	38	62	54	128.56	470.90	3.34	10514.23
102	37	50	54	129.60	474.73	3.41	10704.19
103	33	52	54	195.95	717.75	3.06	14239.56
104	38	55	54	145.02	531.21	2.77	9751.46
105	39	53	54	133.98	490.77	3.10	10375.58
106	30	49	54	124.32	455.38	4.23	12949.70
107	29	51	54	123.99	454.18	3.52	10805.36
108	29	56	54	159.50	584.25	3.37	13385.09
109	31	58	54	116.73	427.57	3.34	9943.60
110	28	60	54	184.57	676.09	3.88	17778.15
111	28	59	54	137.30	502.92	4.51	15471.54
112	30	53	54	124.06	454.42	3.69	11731.43
113	30	55	54	159.06	582.64	3.79	15509.65
114	28	56	54	149.56	547.85	3.57	13610.29
115	29	51	54	106.29	389.34	3.39	9345.56
116	28	57	54	101.61	372.21	3.61	9155.12
117	30	52	54	90.63	331.99	3.70	8173.09
118	31	49	54	149.56	547.84	3.71	13614.29
122	36	50	54	127.24	466.09	3.38	11131.36
123	28	52	54	113.09	414.26	3.73	10865.69
124	32	55	54	144.56	529.52	3.58	13606.89
125	30	57	54	293.43	1074.82	4.22	31825.62
126	28	61	54	153.82	563.44	3.65	14185.57
127	28	55	54	197.63	723.93	3.73	18085.09
128	28	57	54	117.54	430.56	3.53	10474.36
Total=25	Av. =31	Av.=54	54	Total=3607	Total=13214	Av.= 3.59	Total=327232

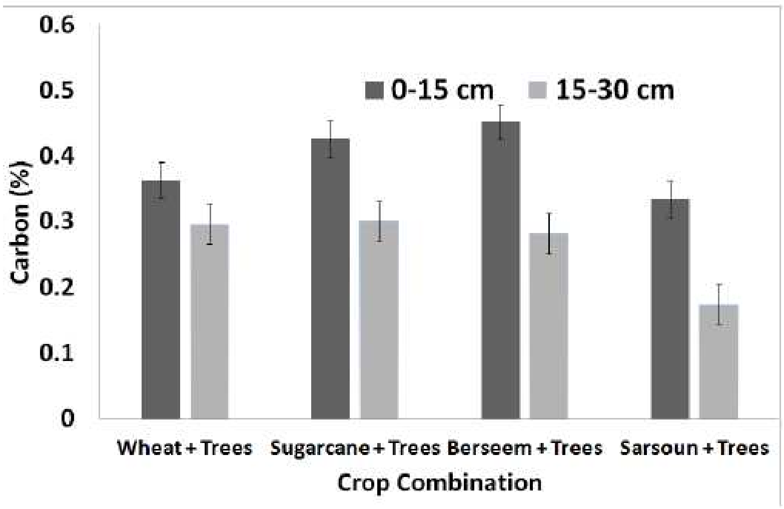

Figure 3. *Measurement of carbon for different cropping combinations*

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Socioeconomic Conditions of Farmers

Fig. 4 shows that maximum number of farmers have the age between 31-40 years followed by 41-50 years and then 51-60 years. The majority of the respondents have the annual income in between 4-8 lac followed by the respondents which have income less than 4 lac.

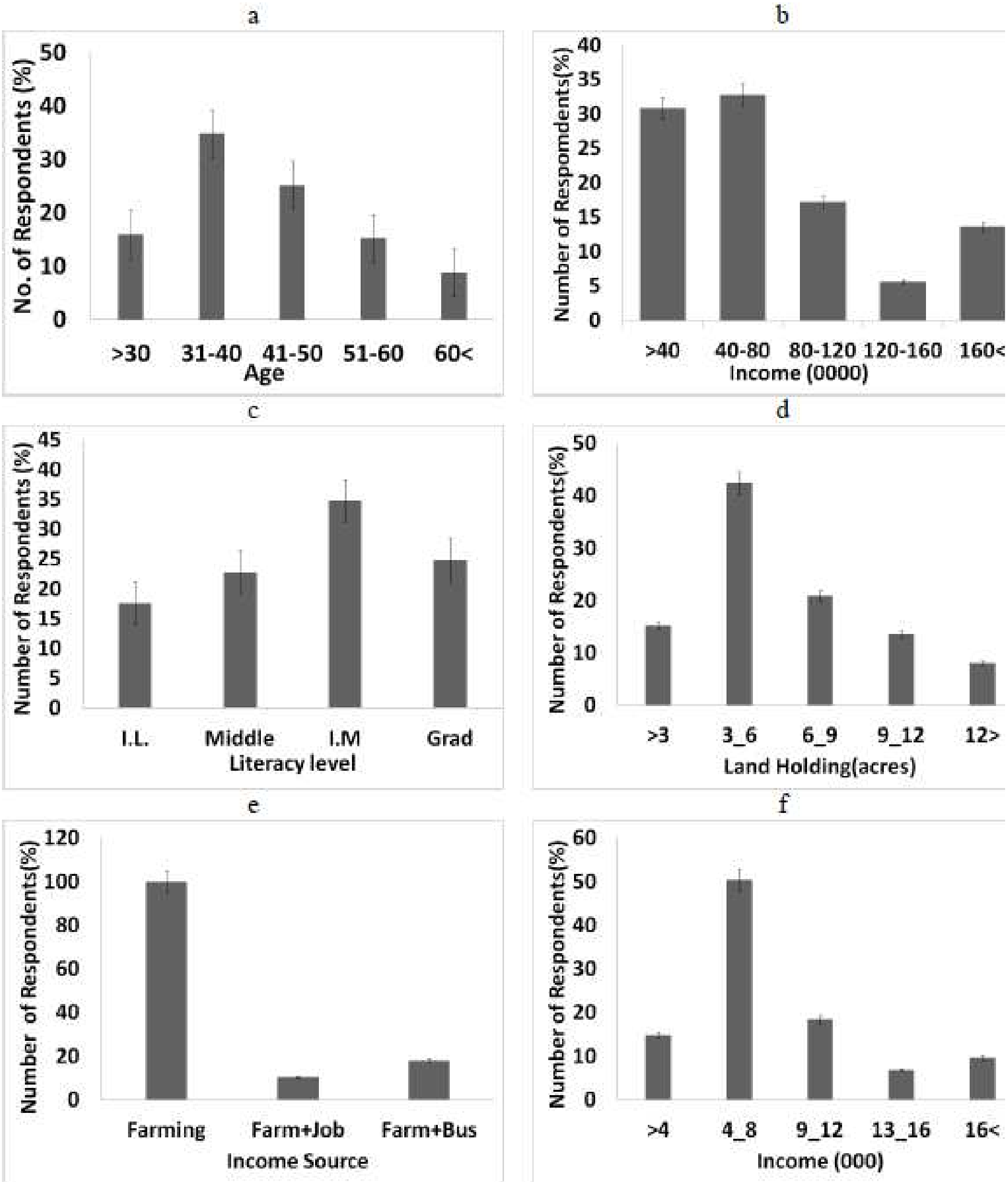

Figure 4. *Socioeconomic conditions of respondents. a; age of respondants. b; income of respondents. c; literacy level. d; land holding of respondents. e; income source of the respondents. f; income from trees per anum*

Then comes the category which has income in between 8-12 lac followed by those respondents which have income more than Rs. 16 lac. Mostly respondents were from middle to intermediate level of education. The maximum number of respondents has

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land holding of 3-6 acres. Mostly farmers are small land holders followed by the category of farmers which possess the land holding of 6-9 acres. Very few farmers have more than 12 Acres of land. But those who were having more than 12 acres were financially stable and were daring for new adoptions. Most of the respondents in our study were farmers but there were some respondents who were doing their own business or jobs as well as farming. Mostly respondents earn 4-8 thousand rupees per year from trees followed by 9-12 thousand followed by the category of less than 4000, which is being followed by the category of more than 16 thousand. Minimum number of farmers responded that they earn in between 13-16 thousand (*Fig. 4f*). Annual income from trees is usually earned by selling of timber. Or farmers sell fruit of trees to earn income on annual basis. The timber of the trees doesn't give income on annual basis. So, average was calculated for the period of five or ten years.

Agroforestry Status and Trends

Fig. 5a shows that the maximum number of respondents replied that they mostly use trees for fuel wood purposes. Almost equal number of respondents uses the farm trees to obtain fruit or fodder for their animals. Very less number of farmers obtains timber on yearly basis. The *Fig. 5* shows that almost all farmers (250) use trees for fuel wood (F.W), followed by farmers (50) use trees for fruit and fodder purposes, whereas (> 50) respondents obtains timber on annual basis.

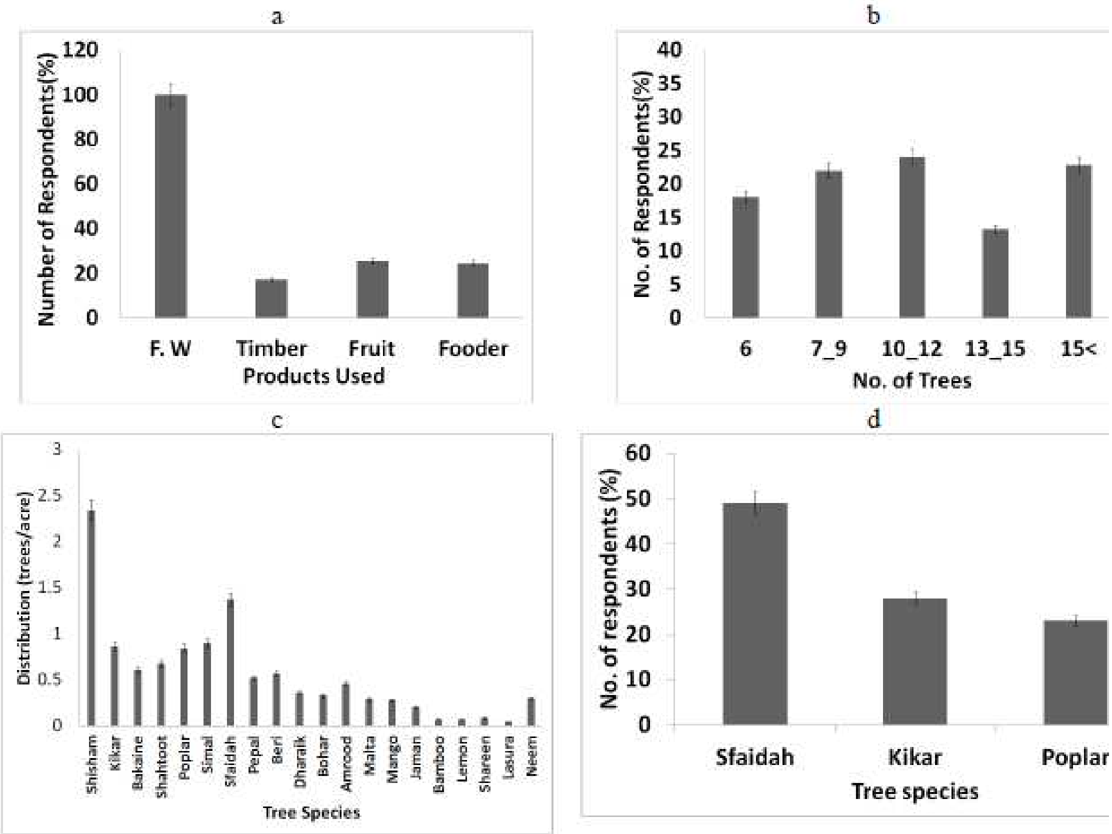

Figure 5. *Agroforestry status and trends. a; products used. b; no. of trees per acre c;distribution of trees. d;trends of tree planting (previous five years)*

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Fig. 5b shows that maximum number of respondents tells that there should be 10-12 trees at one acre followed by the category of more than 15 trees per acre. Minimum number of respondents said that there should be 13-15 trees at one acre. The farmers were reluctant in growing of trees due to several reasons. The maximum number of shisham trees were grown on the farmers' fields but they were not grown during last five years. Sufaidah is followed by shisham in which is being followed by kikar and so on. The distribution is shown in *Fig. 5c*. The maximum number of farmers planted *Eucalyptus* during the period of last five years. Then people planted kikar and poplar. These tree species are replacing shisham (*Fig. 5d)*.

Allometric relations for farm trees

Allometric relations for two most common farm trees: Shisham (*Dalbergia sissoo*) and Sufaida (*Eucalyptus camaldulensis*), are presented in *Fig. 6*. These relations were derived for other farm trees as well but not presented in this manuscript due to similarity among findings and trends. At the age of 10 years, height and diameter at breast height (DBH) for both shisham and sufaida were about 10 m and 15 cm respectively. It was found that height and DBH of both tree species increased with the increment of age but after 20 years, the height and DBH of sufaida was more than shisham trees.

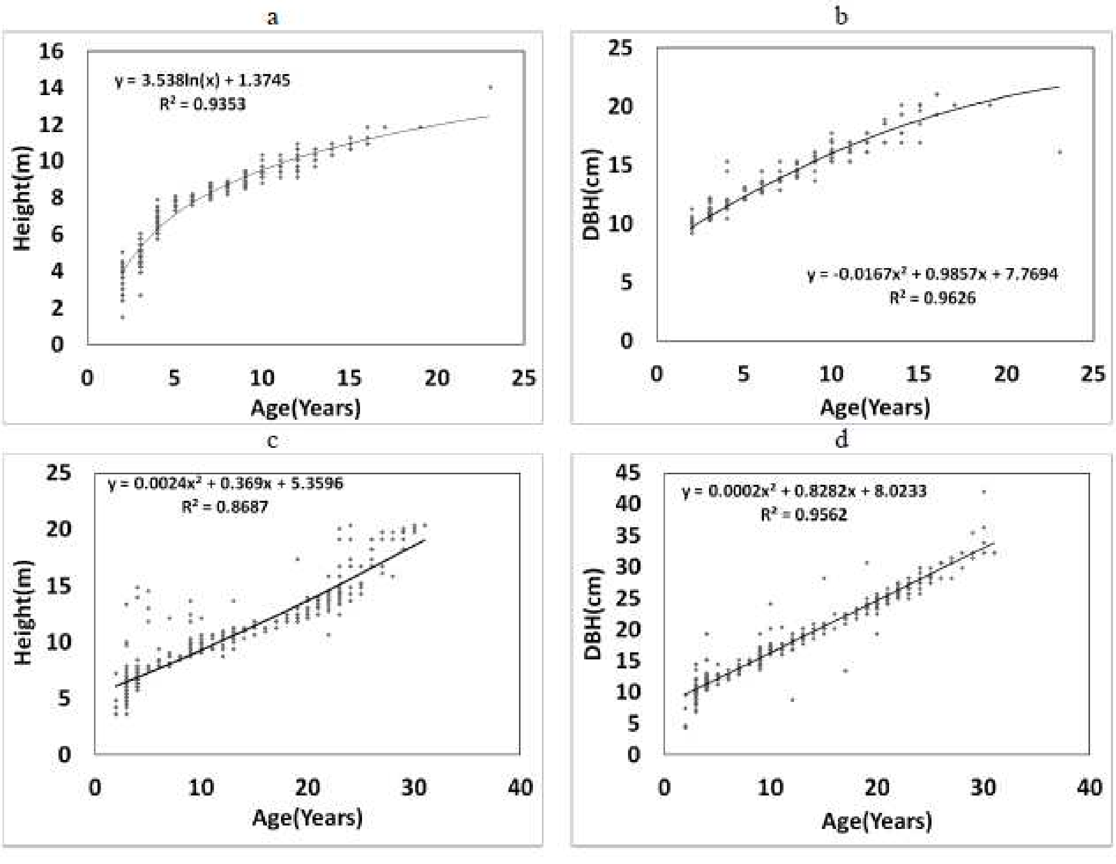

Figure 6. *Alometric relations for Dalbergia Sissoo (a and b) and Eucalyptus Camaldulensis (c and d)*

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Soil Parameters

Fig. 7a shows that the electrical conductivity is more at the depth of 0-15cm rather than 15-30cm. Sarsoun plus trees has the maximum value of electrical conductivity(2.26 mS/cm) followed by sugarcane plus trees (2.10 mS/cm) and then wheat plus trees (2.09 mS/cm). The *Fig. 7b* showed that maximum pH is shown sarsoun plus trees (8.38) followed by wheat plus trees (8.2) followed by sugarcane plus trees (8.16).

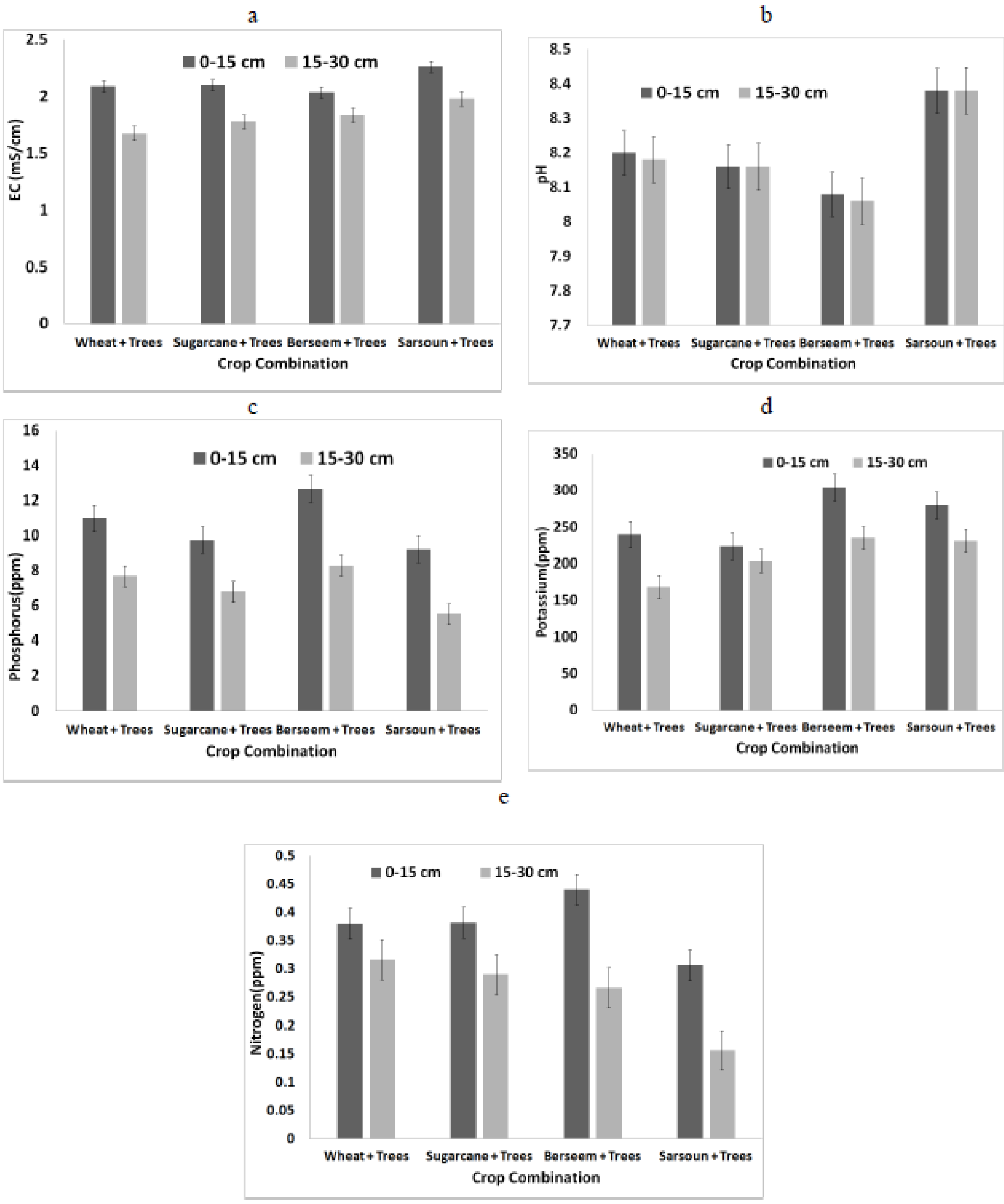

Figure 7. *Measurement of soil parameters for different cropping combinations a; EC. b; pH. c; Phosphorus d; Potassium. e; nitrogen*

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The minimum pH is shown by berseem plus trees (8.08). The data in the *Fig. 7c* represents that maximum amount of phosphorus was present in the combination of berseem plus trees followed by wheat plus trees which was being followed by sugarcane plus trees. The minimum amount of phosphorus was found in the sarsoun plus trees combination at both depths.

The data in the *Fig. 7d* shows that berseem plus trees have higher amount of potassium in their soil which is being followed by sarsoun plus trees. And then comes the areas where trees are grown along with wheat. Minimum amount of potassium is shown by the combination of sugarcane plus trees. The data in the *Fig. 7e* shows that the maximum amount of nitrogen is fixed by berseem plus trees followed by sugarcane plus trees. Almost equal amount of nitrogen was present in the soils for the combinations of wheat plus trees and sarsoun plus trees. This trend was observed at the depth of 0-15 cm, whereas at the depth of 15-30 cm the trend was wheat plus trees followed by sugarcane plus trees followed by berseem plus trees which was being followed by sarsoun plus trees.

Discussion

Current Status and Potential

The amount of carbon sequestered in each union council differs from each other slightly. The climatic conditions of the tehsil Samundri are almost same. But species distribution also determines the amount of carbon sequestered in each union council. Moreover the area of each union council is different. So the statistical tools which are being used also depend on the area of each union council. It was also observed that if the average number of trees per hectare is greater than the amount of carbon sequestered by the trees is also in huge amount. The carbon storage capacity in agroforestry differs across species and geography. Furthermore, the amount of carbon in any agroforestry system depends on the structure and function of different components within the systems (Schroeder, 1994; Albrecht and Kandji, 2003; Nawaz et al., 2017a).

It has been estimated that the croplands of the whole world have potential to sequester about 0.75-1 Pg yr^{-1} of carbon. It is important to mention that removal of forests and other agricultural activities are responsible for removal of about 1.6-1.8 Pg C yr^{-1} (Lal and Bruce, 1999). The rate of storing carbon in silvopastoral systems was 6.72tC$ha^{-1}yr^{-1}$ while the rate at which carbon accumulated in grass lands was only 3.14 tC $ha^{-1}yr^{-1}$ (NRCAF, 2007).

Similary to current status, the potential of each union council is different. At the full potential, additional 1.54 CO_2 t ha^{-1} yr^{-1} can be sequestered by increasing and properly managing the farm trees. In the literature it is reported that the tree species which were grown on the agricultural lands have the potential to capture 3.9 tC $ha^{-1}yr^{-1}$. And if tree species are grown on the forest lands which are considered to be degraded then they can sequester 1.79 tC$ha^{-1}yr^{-1}$ (Maikhuri et al., 2000; Oelbermann, 2004). So, our findings are well coherent with the previous studies and it shows that even under semi-arid climatic conditions, if these agroforestry areas are well managed they have the great potential to sequester and stock the atmospheric CO_2.

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Soil Carbon

For soil carbon, the results are agreed with Ariapour and Asgari (2012) and Hughes et al. (2006), who showed the higher C values under the trees canopies. In the literature, the enhancement of soil carbon and soil organic matter after afforestation practices is well documented fact. A study was performed to calculate the increment in the soil organic carbon (SOC) by growing crop of *Cymbogon sp.* In combination with two types of trees' species. The first one was *Populus deltoides* and the other one was *Eucalyptus*. The results were very astonishing and it was observed that about 33.3% - 83.3% carbon can be enhanced in soil by introducing tree species. It was also concluded that SOC was more where *Populus deltoides* was grown as compared to *Eucalyptus* (Singh et al., 1989).

Socioeconomic Conditions

Similar to other developing countries, mostly farmers of the study area were young with low earnings and less education. Furthermore, small land holdings and lack of advanced technologies were major problems of the atudy area. For poor farmers, farm trees are considred as bank deposits and planted on each farmland, so that, they could be sold at the time of need or emergency (Zubair and Garforth, 2005). Although the additional income from farms trees per year was not too much but its provision timing worths great. So, farmers with small land holdings plant more number of trees as compared to progressive farmers (Nawaz et al., 2017b) . In forest deficient developing countries like Pakistan, farmers can be well motiviated to increase the farm trees through proper policies and provision of financial and technical support (Nawaz et al., 2016).

Agroforestry Status and Trends

Farmers grow trees on their fertile lands for two major benefits: fuels wood and timberwood (Nawaz et al., 2016). Pakistan is an energy deficient country and fuelwood is the major source of energy/fire in the most of the villages and rural areas of Pakistan (Zubair and Garforth, 2005). So, farmers of the study area mostly grow trees for feulwool while a small fraction of fuelwood is directly consumed by farmers to fulfil the domestic needs and remaining trees are sold in the market at weight based prices (Masera et al., 2001). Although trees grown for timberwood provide more income than fuelwood trees but they have to be retained for long periods on farmlands, however, financial conditions of the farmers do not allow them to wait for delayed incomes. So, even timber tree species are oftenly sold out as fuelwood to avail quick incomes (Nawaz et al., 2016). Other additional non-wood benefits like medicine, climatic moderation, climate change mitigation, soil improvement etc. are not given well deserved consideration due to lack of awareness and bad financail conditions (Albrecht and Kandji, 2003). Selecting multipurpose trees such as fruit trees and fodder trees provide additional benefits and more popular when farmer has livestock as well (Githae et al., 2011). 10-12 trees/acre or 25-30 trees/ha can be planted on farmlands under irrigated conditions without severe damage to cultivated crops (Nair, 2011). The results are in agreement as well with previous studies (Qureshi, 2005). Field trends are changing for planting farm trees due dieback disease in shisham (*D. Sissoo*) (Nawaz et al., 2016). Shisham was a very popular tree among farmers and was intensively planted with crops due to its growing characteristics (nitrogen fixing timber tree species) and value in market. However, recently *E. camaldulensis* (Sufaida) is replacing the Shisham only due to fast growing nature and better profitabiltiy. Other than sufaida, widly

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planted trees are Kikar (*Acacia nilotica*) and Poplar (*Populus deltoides*). Similar findings are reported in Nawaz et al. (2016; 2017b).

Allometric Relations and Soil Parameters

On irrigated farmlands, being fast growing trees, both *D. sissoo* and *E. camaldulensis* show almost similar primary and secondary growth under semi-arid climatic conditions. However, contrary to forest or plantations, secondary growths are more linked with tree ages.Our results are in agreement with Kumar (2010) and Maiknuri et al. (2000).

Physico-chemical properties of soils under tree canopies are different from fallow or cultivated areas and these differences are also significant for different agroforestry combinations (Nair, 2011). Noureen et al. (2007) reported relatively, higher level of EC in under canopy soils while checking the effects of litter of *Calligonum polygonoides* in cholistan desert. Abd El-Fattah and Dahmash (2002) examined lower values of pH while studying the effect of *Alhagi maurorum* (Medio), *Tamarix aphylla* (Ehrenb) *Ege*, *Zygophyllum coccineum* (L), *Halocmmum strobilaceum* (M. Bieb) and also, *Parkinsonia aculata* (L) on soil conditions. The above mentioned results of this study have also been supported by the findings of Rehman et al. (2010).

Xu et al. (2006) studied the soil Physico-chemical properties under the canopy of *Tamarix ramosissima* and higher K content under the canopy of that tree. Same findings were quoted by Githae et al. (2011) while studying the effect of *Acacia Senegal* on soil physico-chemical properties in sub Saharan desert of Africa. The results for soil nitrogen have been supported by Tirpathi et al. (2009). He examined higher N values under the canopy of trees of different agro-forestry systems in India. Similar results were also quoted by Imoro et al. (2014) while studying soil plant improvement as influence by planting *Voandzeia subterranea* and *Arachis hypogea*.

Conclusions

In the forest deficient countries like Pakistan where agroforestry is fulfilling the major demands of wood, farm trees can serve as front lines against the global challenges of climate change by sequestering huge amount of CO_2. Althrough the farm trees are not planted to reduce the atmosheric CO_2 but they are currently sequestring huge amounts of carbon and they have great potential to mitigate climate change. However, there is immediate need for acceptance of farm trees for accrediting the carbon credits to promote the agroforestry in Pakistan and to encourage afforestation on farmlands.

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EARTHWORM POSITIVELY INFLUENCES LARGE MACROPORES UNDER EXTREME DROUGHT CONDITIONS AND CONSERVATION TILLAGE IN A CHINESE MOLLISOL

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Abstract. It is often claimed that earthworms exert a huge influence on soil macroporosity. Nevertheless, gaps still exist in our knowledge of rangeability in soil macroporosity caused by earthworm activity. Earthworms are generally recognized as ecosystem engineers vital for soil ecosystem function and services, but the potential mechanism of earthworm response to the combination of extreme drought and conservation tillage has not yet been identified. To improve understanding of the effect of the earthworm on soil macroporosity and how earthworms, through soil macroporosity, respond to extreme drought and conservation tillage, a study was conducted to compare soil macroporosity under different tillage treatments with the same number of earthworms in incubation conditions and soil structural properties (soil penetration resistance, infiltration rate, saturated hydraulic conductivity) associated with macroporosity, together with crop yields under different tillage systems and drought stress in field conditions. The results show that earthworms only increase the volume of large macropores (>100 μm) rather than small macropores (30-100 μm) under different tillage systems. Earthworms played a positive role in the development of large macropores, as evidenced by the formation of paths of least resistance under high penetration resistance, higher infiltration rate and saturated hydraulic conductivity and no obvious yield loss under extreme drought and conservation tillage in a Chinese Mollisol.
Keywords: *soil macroporosity, soil penetration resistance, infiltration rate, saturated hydraulic conductivity, yield*

Introduction

Soil macroporosity plays an important role in the exchange of gases and water in soil (Francetto et al., 2016). One of the major ecological factors influencing soil porosity, especially macroporosity, is the burrowing activity of the earthworm (Maboeta et al., 2008). Numerous studies have emphasized that earthworms can greatly affect soil macroporosity (Bertrand et al., 2015). However, the extent of this effect on soil macroporosity is still somewhat vague, and very little is known.

Although earthworms are known to be affected by climate change (Eggleton et al., 2009), the impact of intense rainfall on earthworm behavior has up to now been given more attention than that of drought. Earthworms can increase the soil's ability to absorb water by creating macropores against intense rainfall disturbance and sustaining plant

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growth (Andriuzzi et al., 2015). During drought, current research indicates that earthworms become less active, and different species use different strategies to survive dry periods during the dry summer months (Bohlen et al., 1995) or when drought has a strong effect on earthworm communities (Mariotte et al., 2016). However, earthworm behavior under drought conditions is still unclear. In addition, conservation tillage has been recommended to improve resistance of crops to drought (Franzluebbers and Stuedemann, 2014), and many previous studies attribute the improved resistance to a heightened water retention capacity (Brouder and Gomez-Macpherson, 2014), whereas no further research has been evaluated. Thus, there is a need to explore the potential mechanisms of drought resistance under conservation tillage.

Modification of soil physical structure, especially macroporosity, by earthworms is well documented (Amossé et al., 2015), but most research has focused on studying the qualitative relationship between earthworm activity and macroporosity. In addition, the above qualitative relationship was obtained without considering the earthworm population. Earthworm population is largely influenced by tillage managements in different agricultural systems (van Schaik et al., 2016). Conservation tillage generally resulted in higher earthworm populations than conventional tillage (Johnston et al., 2015). Hence, in order to more accurately obtain the rangeability in soil macroporosity caused by earthworm activity, it is necessary to study the soil macroporosity changes caused by the same number of earthworms under different tillage practices. Nevertheless, it is difficult to uniformly distribute the same number of earthworms in field conditions. Sampled in situ soil columns, used with the addition of the same number of earthworms, can solve this problem, because sampled in situ soil columns are closest to and can simulate real field conditions. The question studied is thus how earthworms affect crop growth under drought and conservation tillage practices through soil macroporosity. Therefore, we hypothesized that (1) different tillage practices would lead to different effects on soil macroporosity under the same number of earthworms (sampled in situ soil columns with the addition of the same number of earthworms) in incubation conditions, and (2) soil structural properties (soil penetration resistance, infiltration rate, saturated hydraulic conductivity) associated with macroporosity would positively affect crop yields. Accordingly, the objectives of this study were to (1) compare the soil macroporosity under different tillage treatments and the same number of earthworms (sampled in situ soil column with the addition of the same number of earthworms) in incubation conditions, and (2) identify the soil structural properties (soil penetration resistance, infiltration rate, saturated hydraulic conductivity) associated with macroporosity and crop yields, to determine the influence of earthworms on macroporosity under extreme drought and tillage systems. The present study may not only improve understanding of the effect of earthworms on macroporosity and plant growth under conservation tillage and drought conditions but also deepen the comprehensive understanding of how earthworms affect soil macroporosity.

Materials and methods

Study area

The study area was established in 2001 at the Mollisols Experiment and Demonstration Base (44°12′ N, 125°33′ E) of the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, in Dehui County, Jilin Province, northeast China (*Fig. 1)*. It is located in the north temperature zone with semi-humid temperate

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continental monsoon conditions. Annual average rainfall was 520 mm over the past 30 years with more than 70% occurring from June to August. Average annual temperature is 4.4 °C. The soil type in the current study is Typic Hapludoll with clay loam texture. Prior to this tillage experiment, the field had undergone more than ten years of conventional tillage management for continuous corn cultivation. The selected properties of the soil are reported in the references listed in *Table 1* (Liang et al., 2011).

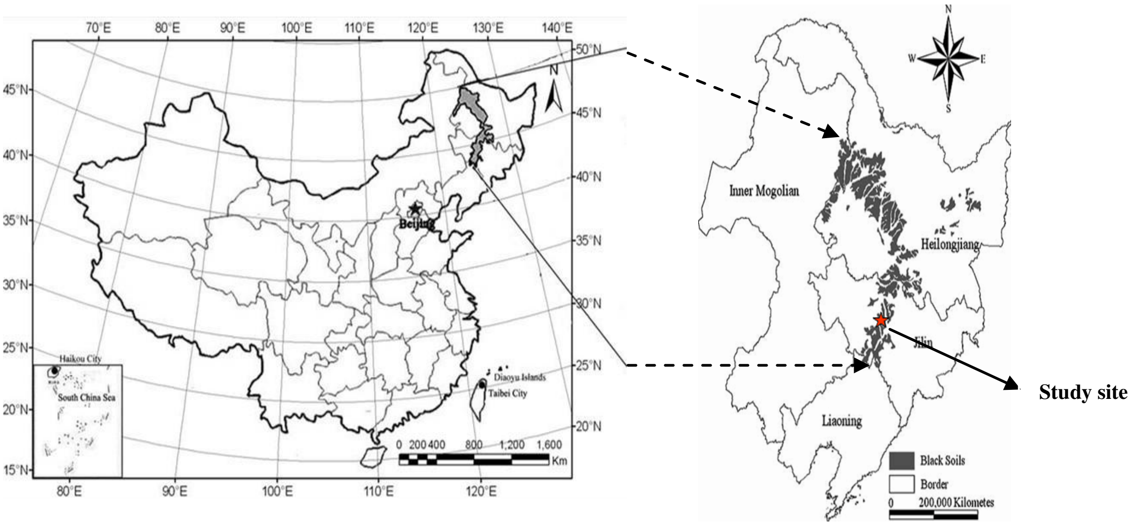

Figure 1. *Location of study area (Left is map of China, right is Mollisols region of northeast China and study site)*

Table 1. *Selected soil physical and chemical properties in 2001 prior to initiation of tillage treatments (Liang et al., 2011)*

Depth (cm)	pH	Clay (%) (<2 μm)	Silt (%) (2-20 μm)	Sand (%) (20-200 μm)	Bulk density (g/cm^3)	Soil organic carbon (g/kg)	Total soil nitrogen (g/kg)
0-5	6.48	36.03	24.00	39.97	1.24	16.48	1.42
5-10	6.45	35.83	23.78	40.39	1.38	16.29	1.39
10-20	6.51	35.68	24.35	39.98	1.36	16.08	1.37
20-30	7.03	36.56	25.00	38.72	1.38	14.22	1.16

Experimental design

The tillage treatments, including moldboard plow (MP), ridge tillage (RT) and no tillage (NT), were arranged in a completely randomized block design with four replicates. Each tillage plot was split into two sub-plots (5.2 m x 20 m). Corn-soybean rotation, with both crops present each year, was applied at the sub-plot level. Tillage for the MP treatment included one fall moldboard plowing (approximately 20 cm in depth) after harvest, one spring disking (7.5 to 10 cm in depth) and field cultivation. The RT treatment included ridging in June for corn and soybeans, chopping the crop stalk/roots in the fall (approximately 1/3 row width) and spring planting with an NT planter. For NT, no soil was disturbed except for spring planting by a KINZE-3000 NT planter (Kinze Manufacturing Inc., USA). Each year, 100 kg/ha of nitrogen (N), 45.5 kg/ha of

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phosphorus (P) and 78 kg/ha of potassium (K) were applied as starter fertilizer and an additional 50 kg/ha of N was applied as top dressing at the six-leaf (V6) stage for corn. All fertilizers including 40 kg/ha of N, 60 kg/ha of P and 80 kg/ha of K were used as starter fertilizer for soybeans.

Soil sampling and analysis

Laboratory experiment

On 22 April 2015 (before spring planting), 6 replications of in situ soil columns from each NT, MP and RT plot were collected down to a 10 cm depth using polyvinyl chloride (PVC) tubes (10 cm diameter, 15 cm height); the total number of PVC tubes was 36. These PVC tubes were then divided into two groups of 18 PVC tubes: one with added earthworms (3 specimens of *Eisenia fetida*) and the other without earthworms. Gas-permeable parafilm covered the tops and bottoms of PVC tubes. A MEMMERT HPP750 Constant Climate Chamber (Memmert Inc., Schwabach, Germany) was used to incubate the samples. Incubation time was from 15 June 2015 to 16 July 2015. On 30 June 2015 (15 days after incubation), 3 replications of soil samples from each NT, MP and RT treatment were collected using 5-cm-diameter cylinders from the above PVC tubes (including with and without earthworms, thus 18 PVC tubes were used to sample at this stage) to determine soil pore size distribution. The same procedure was used for sampling on 16 July 2015 (30 days after incubation). These 36 soil samples, from groups with and without earthworms at two stages, were saturated and submitted to a tension table within a pressure potential range of 0 to 1500 kPa (Soil Moisture Equipment Inc, USA). The relationship between capillary water retention and pore size was used to identify soil pore size distribution (Bhattacharyya et al., 2006). According to Bhattacharyya et al. (2006), at a given matric pressure (h), soil water potential (kPa), and equivalent pore diameter (EPD), the diameter of the smaller pores drained (μm) can be computed from the following equation:

$$EPD = \frac{300}{h} \quad \text{(Eq.1)}$$

Soil pore volume occurring within a given size interval per unit soil (total) volume was used to present soil pore size distribution (Hayashi et al., 2006). According to the classification criteria defined by Chen et al. (2015), soil pore size distribution was classified as large macropores (>100 μm), small macropores (30-100 μm), mesopores (0.2-30 μm) and micropores (<0.2 μm) in this study.

Field experiment

On 29 July 2015, soil penetration resistance was determined in situ at 2.5 cm intervals down to 30 cm using a SC-900 handheld digital penetrometer (Spectrum Technologies Inc., USA) from each NT, MP and RT plot.

From 20 August 2015 to 22 August 2015, a Hood Infiltrometer IL 2700 (diameter 17.6 cm) (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany) was used to determine the infiltration rate, saturated hydraulic conductivity, and macropore flow for each NT, MP and RT plot. Detailed information on the methods for Hood Infiltrometer IL 2700 measurement was given in Schwärzel and Punzel (2007).

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2015 extreme drought in northeast China

Because of El Nino, our field site in 2015 experienced an extreme drought. Monthly precipitation during the growing season (April to September) decreased 48.82%, 52.53%, 27.79%, 170.31%, 57.23% and 134.88%, respectively compared to the 40-year (1975-2014) mean corresponding monthly precipitation (*Table 2*). Total precipitation decreased 80.91% compared to the 40-year mean value (*Table 2*). Precipitation in 2015 was also the lowest since the field's establishment in 2001 compared to the results obtained by Fan et al. (2012) and Zhang et al. (2015) in our field site. In addition, according to the evaluation standards for the variability and predictability of the northeast China climate through 1948-2012 in Gao et al. (2014), 2015 is still an extreme drought year. Thus, to better understand the effect of earthworms on macroporosity under drought and tillage systems, we selected 2015 as the studied year.

Table 2. *Monthly precipitation (mm) during the growing season in 2015 and 40-year mean monthly precipitation (mm) from 1975 to 2014 in Dehui County, Jilin Province, northeast China*

	April	May	June	July	August	September	Total
2015	12.7	31.6	68.0	57.6	83.7	32.4	286
40-yr mean (1975-2014)	18.9	48.2	86.9	155.7	131.6	76.1	517.4

Note: Data were taken from the National Climatic Center of China Meteorological Administration.

Statistical analysis

All statistical analysis was done by SPSS 13.0 software (SPSS Inc., USA). Since the main objectives of the present study were to compare the effects of tillage practices on soil pore size distribution, soil penetration resistance, infiltration rate, saturated hydraulic conductivity and crop yields, one-way analysis of variance (ANOVA) was conducted to test the treatments' main effects on soil pore size distribution, soil penetration resistance, infiltration rate, saturated hydraulic conductivity and crop yields. Means of soil pore size distribution, soil penetration resistance, infiltration rate, saturated hydraulic conductivity and crop yields among the treatments were compared using the least significant difference test at a 0.05 significance level.

Results

Soil macroporosity with and without earthworms under different tillage systems in incubation conditions

Earthworms greatly affected soil macroporosity (*Fig. 2*). NT, MP and RT had no significant ($p > 0.05$) effect on the volume of large macropores (>100 μm) after 15 and 30 days incubation without earthworms (*Fig. 2 a* and *Fig. 2 c*). However, with earthworms, NT and RT had significantly ($p < 0.05$) greater volume of large macropores (>100 μm) than MP after 15 and 30 day incubations (*Fig. 2 b* and *Fig. 2 d*). Concerning small macropores (30-100 μm), NT and RT led to significant ($p < 0.05$) volume reduction in contrast to MP after 15 and 30 day incubations both with and without earthworms (*Fig. 2*). There were no significant differences in the volume of mesopores (0.2-30 μm) and micropores (<0.2 μm) among NT, MP and RT after 15 and

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30 days incubation either with and without earthworms (*Fig. 2*). Regarding incubation time, the volume of large macropores (>100 μm) in the NT, MP and RT plots with earthworms increased 16.73%, 1.69%, and 15.47%, respectively compared to those without earthworms after 15 days incubation (*Fig. 2 a* and *Fig. 2 b*). A similar result was found after 30 days incubation and the corresponding increasing ranges were 33.22%, 17.05%, and 31.35%, respectively (*Fig. 2 c* and *Fig. 2 d*).

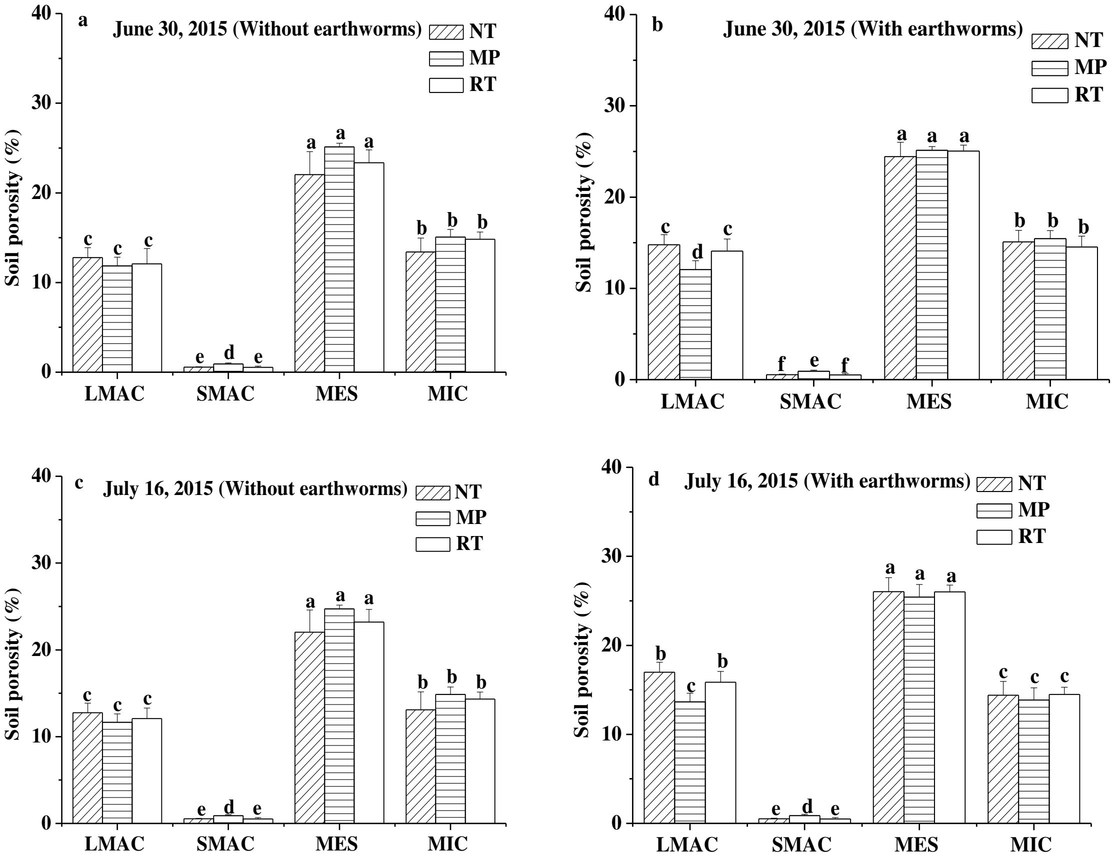

Figure 2. *Soil pore size distribution with and without earthworms on 30 June 2015 (15 days after incubation) and 16 July 2015 (30 days after incubation) under different tillage practices. NT: no tillage; MP: moldboard plow; RT: ridge tillage; LMAC: large macropores (>100 μm); SMAC: small macropores (30–100 μm); MES: mesopores (0.2–30 μm); MIC: micropores (<0.2 μm)*

Soil structural properties associated with macroporosity under extreme drought and different tillage systems in field conditions

Similar to 2013 (normal year), soil penetration resistance in NT and RT increased significantly ($p < 0.05$) compared to that of MP at 2.5-17.5 cm depth in 2015 (extreme drought year) (*Fig. 3*). Soil penetration resistance in all treatments reached the critical value of 2000 kPa at as shallow a depth as 12.5 cm in 2015 (extreme drought year), while the soil depth for this critical value in 2013 (normal year) was 20 cm (*Fig. 3*). Compared with 2013 (normal year), soil penetration resistance in NT, RT and MP soils increased an average of 31.99%, 66.05%, and 30.95%, respectively in 2015 (extreme drought year) (*Fig. 3*).

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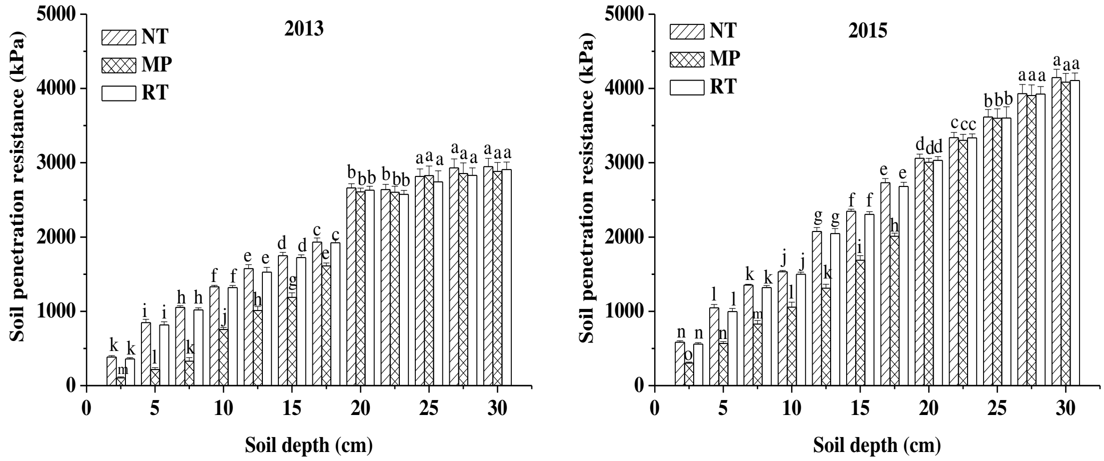

Figure 3. *Soil penetration resistance under no tillage (NT), moldboard plow (MP) and ridge tillage (RT) systems in 2013 (normal year) and 2015 (extreme drought year)*

In NT, MP and RT soils, variation trends of infiltration rates were high at first, gradually dropped, and then became close to a stable stage (*Fig. 4*). 20 min was the time to reach the stable stage for each tillage treatment, and the corresponding infiltration rates for NT, MP and RT plots were 5.2, 2.5 and 4.8 mm/min, respectively (*Fig. 4*).

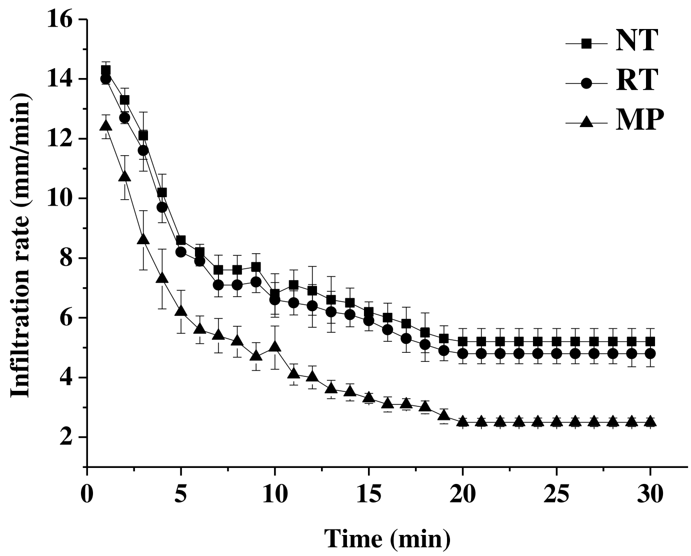

Figure 4. *Infiltration rate under no tillage (NT), moldboard plow (MP) and ridge tillage (RT) systems*

A significantly ($p < 0.05$) greater saturated hydraulic conductivity was found in NT and RT than in MP soils, and this was similar to the observations of contributions of macropores to total flow (*Table 3*).

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Table 3. *Saturated hydraulic conductivity and contribution of macropores to total flow under different tillage practices*

	Saturated hydraulic conductivity (cm/min)	Contribution of macropores to total flow (%)
NT	1.92a	69.93a
MP	1.60b	41.48b
RT	1.83a	66.37a

Note: Contribution of macropores to total flow was calculated from the proportion of hydraulic conductivity corresponding to equivalent pore diameter for macroporosity of saturated hydraulic conductivity. NT: no tillage; MP: moldboard plow; RT: ridge tillage; values followed by the same letter within a column indicate no significant difference at the 0.05 level.

Comparison of crop yield in extreme drought year and long-term average

As in long-term average yields (2002-2014), MP had a significantly ($p < 0.05$) lower yield than NT and RT in the extreme drought year (2015) (*Table 4*). Although crop yield for NT and RT in the extreme drought year (2015) decreased 8.85% and 9.55%, respectively, compared with the long-term average (2002-2014), no significant differences in crop yield were found ($p > 0.05$) (*Table 4*). However, crop yield for MP in the extreme drought year (2015) significantly ($p < 0.05$) decreased by 51.74% compared to the long-term average (2002-2014) (*Table 4*).

Table 4. *Average crop yield from 2002 to 2014 and crop yield in 2015 (extreme drought year) under different tillage practices*

Crop yield (kg/ha)	Tillage practices		
	NT	MP	RT
2002-2014 average data	10769a(a)	10241b(a)	10822a(a)
2015 (extreme drought year)	9816a(a)	4942b(b)	9788a(a)

Note: NT: no tillage; MP: moldboard plow; RT: ridge tillage; values followed by the same letter inside and outside the brackets indicate no significant difference at the 0.05 level for vertical and horizontal comparisons, respectively.

Discussion

Soil macroporosity with and without earthworms under different tillage systems in incubation conditions

Earthworms resulted in a significant change in the volume of large macropores (>100 μm). On the surface, this is mostly due to the lower level of compression offered by NT, which protects the large macropores and keeps them intact. In addition, disturbance of soil by primary or secondary tillage would be expected to result in loosening of soil and thus an increase in macroporosity of the tilled zone (Kay and van den Bygaart, 2002). Moreover, traffic-induced compaction in MP intensified extrusion to large macropores. However, the essential reason was that earthworm activity, through bioturbation and egestion, influences soil porosity extrusion (Mariotte et al., 2016). This was agreed upon by previous research (Peigné et al., 2009; Amossé et al., 2015). While previous

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studies simply state earthworms can increase soil macroporosity (Blouin et al., 2007) or accelerate soil macroporosity turnover (Bottinelli et al., 2010), they do not clearly articulate the range of soil macroporosity for this change.

A significantly lower volume of small macropores was observed in NT and RT compared to MP after 15 and 30 day incubations, with and without earthworms. This means that earthworm activity has no effect on the small macropores. The reason for this situation was the partial destruction of large macropores by soil disturbance, resulting in the formation of a greater volume of small macropores under MP (Chen et al., 2016). Kay and van den Bygaart (2002) found that shifts from conventional to conservation tillage usually led to a reduction in the volume of small macropores. Considering the nonsignificant results concerning mesopores and micropores, our results again indicate that earthworms only affect large macropores (>100 μm) under different tillage systems. Greater differences between the large macropore volumes among the NT, MP and RT plots with and without earthworms under different incubation times indicate that the increasing range in the volume of large macropores (>100 μm) increased with time.

Soil structural properties associated with macroporosity under extreme drought and different tillage systems in field conditions

Both NT and RT increased soil penetration resistance in the 2.5-17.5 cm layer compared to MP ($p < 0.05$). This was mainly related to the fact that the accumulated soil consolidation with time occurred in NT and RT soils, in contrast to the tillage compaction in fall and spring of MP soil (Alvarez and Steinback, 2009). The results of different depths reaching 2000 kPa, which is the critical value for relatively unimpeded root growth (Reynolds et al., 2007), in 2015 (extreme drought year) and 2013 (normal year), together with the greater increment of soil penetration resistance in 2015 (extreme drought year) compared to 2013 (normal year), indicate that the actual resistance of the soil to root penetration is generally less than the average resistance measured by the penetrometer; this is because roots seek the path of least resistance during growth rather than penetrating straight through the soil (Olibone et al., 2010). The path of least resistance was associated with the large macropores burrowed by earthworms (Bengough, 2012; Bottinelli et al., 2015). In this study, it also means that earthworm activity, through the volume of large macropores, made a positive impact on soil penetration resistance under extreme drought conditions.

Higher infiltration rates for NT and RT than in MP soils in this study contrasted with the study of Lipiec et al. (2006) but were parallel to the results of other research (Abid and Lal, 2009; Kahlon et al., 2013). Previous studies have attributed this infiltration rate difference among tillage practices to soil texture, macropore quantity and continuity, soil organic matter content, soil moisture and soil bulk density (Ginting et al., 2003; Gajda et al., 2016; Zaibon et al., 2017), but all of these vary case by case. Ultimately, earthworm burrowing and root decomposition played an important role in increasing macropore quantity and thus made for preferential transport (Emmerling et al., 2015). This important role can also be verified by the results of saturated hydraulic conductivity values and contribution of macropores to total flow. These hydraulic property results indicate that the effect of earthworms on large macropores plays an important role in water infiltration and conservation under extreme drought conditions.

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Comparison of crop yield in the extreme drought year to the long-term average

From the view of tillage effect on crop yield under long-term averages *versus* an extreme drought year (2015), these results were consistent with those reported by He et al. (2011), who found that conservation tillage practices were beneficial to crop yield. Crop yield variation under conservation tillage and extreme drought indicates that conservation tillage practices can make production highly resistant to climatic variables (Thierfelder et al., 2015). It also means that NT and RT can function as a buffer against the poor performance of crops in low-yield years, leading to better crop performance to counteract extreme adverse environmental effects compared to that of MP (Šíp et al., 2013). In other words, this could be explained by the fact that earthworm can buffer the soil-plant system against the effects of extreme drought events (Johnson et al., 2011). Bertrand et al. (2015) also noted that aboveground biomass generally increases in the presence of earthworms in extreme weather events.

No obvious yield losses were found under NT and RT in the extreme drought year (2015). This confirms that earthworms, through macroporosity and its related soil structural properties, affect crop growth positively under conservation tillage systems. Our results are in accordance with Spurgeon et al. (2013), who found positive effects of earthworms on soil macroporosity together with structural properties and plant growth. The possible explanation is that earthworm-induced changes and modification of soil macroporosity results in changes of water and oxygen availability to plants, and better soil structure conditions, which are beneficial for improving the carrying capacity of nutrients (van Groenigen et al., 2014).

Previous studies have emphasized the great effects of earthworms on soil macroporosity, but neglected the rangeability in soil macroporosity caused by earthworm activity and what range of soil macroporosity accounts for this change. On the other hand, earthworms' response to drought and different tillage is still inadequately characterized. To solve these problems, in the current study, research was conducted to create an in depth evaluation of the effect of earthworms on macroporosity under drought conditions and different tillage treatments, based on the comparison of soil macroporosity under different tillage treatments and the same number of earthworms in incubation conditions, soil structural properties (soil penetration resistance, infiltration rate, saturated hydraulic conductivity) associated with macroporosity under different tillage systems and drought in field conditions, and crop yields.

In summary, our study found that not only would earthworms increase the volume of large macropores (>100 µm), though not small macropores (30-100 µm), under different tillage systems, but that earthworm activity through large macropores also formed the path of least resistance under high penetration resistance, leading to higher infiltration rate and saturated hydraulic conductivity and affecting crop yield positively under conservation tillage and extreme drought conditions.

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APPENDIX

Appendix 1. *Soil bulk density (BD), soil water content (SWC) and soil air-filled porosity (AFP) under no tillage (NT), moldboard plow (MP) and ridge tillage (RT) systems in this study site*

Soil properties	**Soil depth (cm)**	**Treatments** NT	MP	RT
BD (g/cm^3)	0-5	1.15a	1.01b	1.10a
	5-10	1.36a	1.21b	1.30a
	10-20	1.38a	1.23b	1.32a
	20-30	1.36a	1.35a	1.34a
SWC (weight, %)	0-5	30.20a	26.78b	31.65a
	5-10	24.78a	20.23b	25.99a
	10-20	16.12b	18.90a	16.87b
	20-30	12.36a	12.50a	12.47a
AFP (cm^3/cm^3)	0-5	1.24c	1.55a	1.35b
	5-10	0.90c	1.13a	0.98b
	10-20	0.87c	1.10a	0.95b
	20-30	0.90a	0.91a	0.93a

Values followed by the same letter within a row indicate no significant difference at the 0.05 level.

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DIVERSITY OF TEA ENDOPHYTIC FUNGI: CULTIVAR- AND TISSUE PREFERENCES

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Abstract. Endophytic fungi were isolated from four healthy tissues (new leaf, old leaf, bark, and xylem) of three Japanese tea cultivars (Hokumei, Sayamakaori, and Yabukita) at the Saitama Tea Research Institute, Japan in July 2015. A total of 520 isolates was obtained from 600 segments and were classified into 44 fungal taxa; the majority (93.2%) belonged to the phylum Ascomycota. The lowest infection rate was found in the xylem tissue of all cultivars. The total infection rate did not differ significantly among the cultivars. *Colletotrichum gloeosporioides* f. sp. *camelliae* and *Pleosporales* sp. were the predominant endophytes in all tissue types and cultivars. Most of the dominant endophytes showed obvious cultivar and tissue preferences. Tissue type played a more important role in shaping community structure than did cultivar. *Colletotrichum gloeosporioides* f. sp. *camelliae* preferred bark and old leaf tissue while *Pleosporales* sp. preferred new leaf tissue. The colonization frequency of *C. gloeosporioides* f. sp. *camelliae* was significantly lower in the Yabukita cultivar. Stem tissues harbored more diverse endophytes than did leaf tissues.
Keywords: *fungal community, healthy tissues, Japanese tea cultivars, leaf, stem*

Introduction

Endophytic fungi are mycosymbionts that colonize the internal tissues of plants without causing damage (Petrini, 1991). They are diverse, ubiquitous, and can be isolated from flora worldwide, from flowerless plants such as mosses (Davey and Currah, 2006) to urban forest trees (Matsumura and Fukuda, 2013). These fungi can improve plant survival under adverse environmental conditions (Cheplick et al., 2000). Endophytes have been a focus of research as substitutes for agrochemicals (Rabha et al., 2014; Nath et al., 2015).

Endophytic fungal composition and diversity can be affected by several factors like host species or cultivars, location, soil types, plant physiological status, seasons, geographic coordinates and tissues or organs of the host plants (Arnold and Lutzoni, 2007; Tian et al., 2004; Naik et al., 2009). Campisano et al. (2014) studied endophytic fungal composition in grapevines cultivated using organic production and Integrated Pest Management (IPM). They observed *Mesorhizobium*, *Caulobacter* and *Staphylococcus* genera as dominant endophytes in organic vineyards, while *Ralstonia*, *Burkholderia* and *Stenotrophomonas* were more abundant in grapevines from IPM vineyards.

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Tea (*Camellia sinensis*) is the most popular drink in the world. In the recent decades, endophytes gain attention from many researchers to be used as the substitutes for agrochemicals in the sustainable tea production (Rabha et al., 2014; Nath et al., 2015). Because of their benefits, there should be a better understanding of tea endophytes. To use endophytes for agricultural application, many researchers are trying to discover new stains. Studying their diversity, distribution and colonization pattern can give basic information for further advance studies for the commercial use of ecofriendly biofertilizers and biocontrol agents in sustainable tea production. In accordance with Fang et al. (2013), the systematic study of tea endophytes was started in the beginning of the 21st century. Therefore, research about tea endophytes is still limited if compared to any other economically important crops.

According to Yagi et al. (2010), 52 tea cultivars are registered with the Japan Ministry of Agriculture, Forestry, and Fisheries. Despite the importance of the crop, the diversity of endophytic fungi of Japanese tea cultivars remains unexplored. According to Osono (2014), 45 papers related to phyllosphere fungi, including endophytes and epiphytes, in Japan were published from 1990 to 2013; most focused on forest trees. Few endophytic studies of agricultural crops, including tea plants, have been performed in Japan. Use of endophytic fungi of tea plants as substitutes for agrochemicals in sustainable tea production requires an enhanced understanding of tea endophytes. Studying their diversity, distribution, and colonization of different tissues and cultivars would provide basic information for commercial application of endophytes in the industrial, pharmaceutical, and agricultural sectors. This study aimed to elucidate the species composition and tissue and cultivar preferences of endophytic fungi isolated from three Japanese tea cultivars.

Materials and Methods

Study plant

Tea (*Camellia sinensis*) was discovered about around 2700BC and so it can be assumed that it is one of the oldest beverages in the world (Chang, 2015). Numerous pathogens including fungi, bacteria and virus can attack tea plants and lead to serious crop losses. In Assam, India, about 14–50 % of crop loss is caused by pests and diseases. Among the diseases, grey blight and brown blight caused by *Pestalotiopsis theae* and *Colletotrichum camelliae*, respectively, are the most destructive and economically important ones (Rabha et al., 2013). Keith et al. (2006) described that blister blight, horse-hair blight, and twig dieback/stem canker are very serious diseases in major tea-producing area of the world. Crane and Balerdi (2013) listed blister blight (*Exobasidium vexans*), net blister blight (*E. reticulum*), anthracnose (*Colletotrichum theasinensis*), and red rust (*Cephaleuros parasiticus*) as tea plant pathogens. According to these authors, we can easily realize that tea plants are threatened by several different pathogens. Moreover, new diseases which can cause economic losses in various crops emerge recurrently due to the drastic changes in temperature and precipitation pattern. Various chemical pesticides (insecticides and fungicides) containing different chemical compounds with different mode of actions are now being used by tea growers to control tea diseases. These chemical residues could cause harmful effects to plants, human beings, environment and biodiversity including endophytes.

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Sample collection

Plant samples were collected in July 2015 from the Saitama Tea Research Institute (35°48'32"N, 139°20'47"E), Saitama Prefecture, 40 km northwest of Tokyo, Japan. Branches bearing four tissue types (new leaf, old leaf, bark and xylem of the stem) were cut from three cultivars; Hokumei, Sayamakaori, and Yabukita (*Table 1*). New and old leaf tissues were on average 1 month and 1 year old, respectively. All cultivars were grown in lines in the same plot; each line was 50 m long and 1 m wide. One branch per 2 m was cut from two lines of each cultivar. From each branch, five segments of each tissue type (*Fig. 1*) were used. A total of 600 segments (3 cultivars × 4 tissues × 2 lines × 5 branches × 5 segments = 600 segments) were used for isolation of endophytic fungi.

Table 1. *Characteristics of the tested cultivars*

	Yabukita *1	**Sayamakaori** *2	**Hokumei** *3
Year registered	1953	1971	1992
Cultivar number	6	31	43
Parents	Native Shizuoka species	'Yabukita' plants	'Sayamakaori' x 5507
Leaf	Oval, long, straight and intensely green	Oval, bigger and thicker than 'Yabukita'	Oval, bigger and thicker than 'Yabukita'
Harvest time	April – mid May	2 to 3 days earlier than 'Yabukita'	3 to 5 days later than 'Yabukita'
Vigor	High	High	More vigorous than 'Yabukita'
Yield	High	Higher than 'Yabukita'	Same as 'Sayamakaori'
Disease resistance	Susceptible to anthracnose and gray blight	Susceptible to anthracnose, slightly more resistant to gray blight than 'Yabukita'	Highly resistant to anthracnose, susceptible to gray blight

Note: *1 Yagi et al. (2010); *2 Caidedo (2016) http://www.myjapanesegreentea.com/sayamakaori; *3 Caidedo (2016) http://www.myjapanesegreentea.com/hokumei

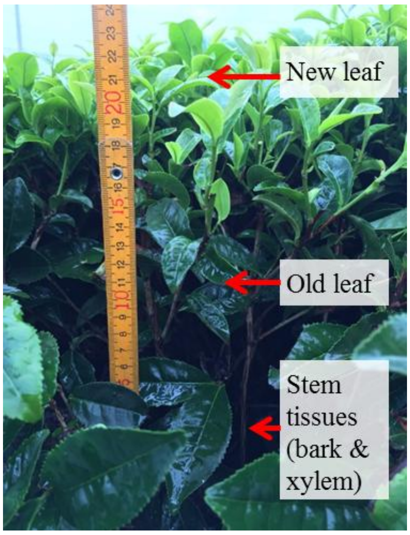

Figure 1. *Tissue types used for isolation of endophytic fungi*

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Isolation and identification of endophytic fungi

After collection, the plant materials were placed in clean polythene bags, labeled, and transported to the laboratory. Isolation from health tissues was carried out within 24 h. Surface sterilization and isolation were conducted as described by Matsumura and Fukuda (2013). First, the collected plant materials were placed under running tap water for ~ 12 h. Surface sterilization was performed by immersing the plant materials in 80% ethanol for 1 min, 1% sodium hypochlorite for 1 min, and 80% ethanol for 1 min. The disinfected materials were washed twice in sterilized distilled water for 1 min, and allowed to dry on sterilized blotting paper. For leaf tissues, 6-mm-diameter discs were removed using a sterilized cork borer. Stem tissues were debarked to separate bark and xylem using a sterilized razor blade and cut into 3 mm^2 segments. These segments were placed on half-strength potato dextrose agar (1/2 PDA) medium containing chloramphenicol (600 mg/L) in 9-cm-diameter plastic Petri dishes (five discs per Petri dish). The Petri dishes were incubated at 20°C in the dark for 1 week. Each fungal isolate was subcultured on PDA medium in 6 cm Petri dishes for 5 days and identified by morphological and molecular analyses. For morphological analysis, colony color, texture, shape, size, conidia, and mycelia color were evaluated. Fungal isolates with the same morphological characteristics were placed in the same group, and > 50% of the isolates underwent molecular identification.

DNA extraction and PCR amplification

DNA extraction was conducted according to Izumitsu et al. (2012). Mycelia (0.1–1 µg) were collected from the growing edges of 4–6-day-old cultures using sterilized toothpicks and dissolved in 100 µL TE buffer in a 1.5 mL tube. The tubes were microwaved at 600 W for 1 min, stored at room temperature for 30 s, microwaved for 1 min, and immediately cooled at −30°C for at least 10 min. Samples were finally centrifuged at 10,000 rpm for 5 min.

The internal transcribed spacer (ITS) region of the rDNA (ITS 1-5.8S rDNA-ITS2) of each isolate was amplified using the forward primer ITS5 (5’-GGAAGTAAAAGTCGTAACAAGG-3’) and the reverse primer ITS4 (5’-TCCTCCGCTTATTG ATATGC-3’) according to White et al. (1990). The reaction mixture comprised 8 µL sterilized Milli Q distilled water, 0.2 µL of each of the 20 M forward and reverse primers, 10 µL of 2× GoTaq Master Mix (Promega) and 1.6 µL of fungal DNA template. PCR was carried out in a reaction volume of 20 µL in 0.2 mL tubes in a thermocycler. The reaction began with initialization for 10 min at 94°C, then 30 cycles of initial denaturation at 94°C for 30 s, annealing at 51°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Sequencing was conducted using a 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequence alignment was performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 6. Isolates with > 97% similarity were identified as the same species. Sequence data were submitted to the DNA Data Bank of Japan (DDBJ) under the accession numbers LC168755–LC168797.

Data analysis

To determine the cultivar and tissue preferences of tea endophytic fungi, the infection rate (IR), colonization frequency (CF), and relative dominance (RD) were calculated according to Fang et al. (2013) and Nalini et al. (2014):

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$$IR = \frac{Number\ of\ tissue segments\ colonized by\ fungi}{Total\ number\ of\ tissue segments\ used\ for\ isolation} \times 100 \quad \text{(Eq.1)}$$

$$CF = \frac{Number\ of\ tissue segments\ colonized by\ a\ fungal species}{Total\ number\ of\ tissue segments\ used\ for\ isolation} \times 100 \quad \text{(Eq.2)}$$

$$RD = \frac{CF\ \%\ of\ each\ fungal species}{Total\ number\ of\ fungal isolates} \times 100 \quad \text{(Eq.3)}$$

IR and CF were subjected to two-way analysis of variance (ANOVA) with cultivar and tissue type as factors, but no significant interaction between cultivars and tissues was detected. The CFs of the four most common endophytes (RD > 7%) were analyzed statistically using the Tukey-Kramer honest significant difference (HSD) test to compare mean values with JMP Pro version 12.2 at the $\alpha = 0.05$ level.

The Shannon–Wiener diversity index (H') and evenness index (E5) were used to analyze the diversity of the endophytic fungal community (Ludwig and Reynolds, 1988).

$$H' = -\sum RD_i \left(\log_e \left(RD_i\right)\right) \quad \text{(Eq.4)}$$

$$\lambda = \sum RD_i^2 \quad \text{(Eq.5)}$$

$$E5 = \left(\frac{1}{\lambda} - 1\right) \Big/ \left(e^{H'} - 1\right) \quad \text{(Eq.6)}$$

Rarefaction curves were calculated to compare species richness among the tissue types and cultivars using EstimateS9 software.

A permutational multivariate analysis of variance (PERMANOVA) was used to determine the effects of cultivar and tissue type on endophytic fungal community structure using the vegan package in the R console. Non-metric multidimensional scaling (NMDS) ordinations were used to visualize the similarities of the endophytic fungal community among the cultivars and tissues.

Results

Infection rate

Fifty segments of each tissue type and 200 segments of each cultivar were used to isolate endophytic fungi. The IR differed significantly among the tissue types (*Fig. 2A*). The IRs of bark and old leaf tissues were 100% in all cultivars. New leaf tissues showed a significantly lower IR than old leaf tissues and a higher IR than xylem tissues. The lowest IR among the tissues (46, 42, and 30% in Sayamakaori, Hokumei, and Yabukita, respectively) was in the xylem (*Tables 2, 3; Fig. 2A*). IRs did not differ significantly among the cultivars (*Fig. 2B*).

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Table 2. *Colonization frequency (CF%), relative dominance (RD%), Shannon–Wiener diversity index (H'), and evenness (E5) values of endophytic fungi isolated from different tissues of tea cultivars (H = Hokumei, S = Sayamakaori, Y = Yabukita), July 2015*

Phylum / Order	Endophytic fungal species	Colonization frequency % (CF %)												RD %
		Bark			Xylem			New leaf			Old leaf			
		H	S	Y	H	S	Y	H	S	Y	H	S	Y	
Ascomycota														
Glomerellales	*Colletotrichum gloeosporioides* f. sp. *camelliae*	50	62	22	2	8	2	20	16	6	72	60	48	70.8
Pleosporales	Pleosporales sp.	8	8	14	12	14	8	48	38	44	20	32	26	52.3
Pleosporales	*Peyronellaea glomerata*	2	14	4		8	2	2	10	12		10	2	12.7
Botryosphaeriales	*Botryosphaeria dothidea*			24	6						2		2	7.7
Capnodiales	*Cladosporium asperulatum*			2	6	2	10	6	2	4			4	6.9
Diaporthales	*Diaporthe eres*		10	12			2		4				2	6.2
Pleosporales	*Alternaria mali*	4	2	8				10	2	4				5.8
Botryosphaeriales	*Guignardia mangiferae*							2	2	2		2	10	3.5
Pleosporales	*Setophoma chromolaena*							4	2	10		2		3.5
Diaporthales	*Diaporthe nobilis*	4		2		2			2	4				2.7
Diaporthales	*Phomopsis* sp. c1	4	6						2		2			2.7
Diaporthales	*Diaporthe* sp. c1	4			2	2				4				2.3
Diaporthales	*Phomopsis* sp. c2	4		6										1.9
Hypocreales	*Acremonium strictum*				2		2	2	2					1.5
Glomerellales	*Glomerella* sp.	2	2		2		2							1.5
Diaporthales	*Phomopsis* sp. c3		4	4										1.5
Xylariales	*Pestalotiopsiaaaas* sp.			6		2								1.5
Diaporthales	*Phomopsis* sp. c4	4												0.8
Diaporthales	*Phomopsis* sp. c5	2								2				0.8
Incerti ordinis	Dothideomycetes sp.				2	2								0.8
Pleosporales	*Epicoccum nigrum*	2		2										0.8
Pleosporales	*Paraphaeosphaeria neglecta*					4								0.8

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Xylariales	*Pestalotiopsis camelliae*		2							2				0.8
Xylariales	*Nemania* sp.							2					2	0.8
Xylariales	Xylariales sp.				2									0.4
Diaporthales	*Diaporthe pustulata*									2				0.4
Diaporthales	*Diaporthe sackstonii*	2												0.4
Diaporthales	*Diaporthe* sp. c2			2										0.4
Diaporthales	*Phomopsis amygdali*											2		0.4
Diaporthales	*Phomopsis* sp. c6			2										0.4
Diaporthales	*Phomopsis subordinaria*								2					0.4
Diaporthales	*Phomopsis* sp. c7								2					0.4
Diaporthales	*Melanconiella* sp.					2								0.4
Pleosporales	*Microdiplodia hawaiiensis*			2										0.4
Pleosporales	*Phoma herbarum*				2									0.4
Sordariales	*Phialemonium dimorphosporum*				2									0.4
Pleosporales	*Plenodomus* sp.				2									0.4
Capnodiales	*Pseudocercospora* sp.										2			0.4
Pleosporales	*Stagonosporopsis cucurbitacearum*								2					0.4
Hypocreales	*Trichoderma koningiopsis*			2										0.4
Incertae sedis	Fungal endophyte sp.												2	0.4
Basidiomycota														
Incerti ordinis	Uncultured Basidiomycota sp.								6	2		2	2	2.3
Russulales	*Peniophora incarnata*						2							0.4
	Total number of species	15	9	17	12	10	8	9	15	13	5	7	10	44
	Total number of isolates	50	55	58	21	23	15	48	47	49	49	55	50	520
	Infection Rate (%)	100	100	100	94	94	98	100	100	100	42	46	30	
	E5	0.43	0.53	0.67	0.71	0.8	0.7	0.61	0.58	0.54	0.6	0.64	0.61	
	H'	1.98	1.51	2.48	2.27	1.96	1.85	1.53	2.01	1.93	0.79	1.23	1.54	

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***Table 3.** Blast search results*

Fungal species	**Accession number**	**BLAST search result**	**Accession number**	**Score (%)**
Colletotrichum gloeosporioides f. sp. *camelliae*	LC168755	*Colletotrichum gloeosporioides* f. sp. *camelliae*	KP635401	557/559(99%)
Pleosporales sp.	LC168756	Pleosporales sp. LH70	HQ832808	570/576(99%)
Peyronellaea glomerata	LC168757	*Peyronellaea glomerate*	FJ481024	525/527(99%)
Botryosphaeria dothidea	LC168758	*Botryosphaeria dothidea*	KJ801792	568/572(99%)
Cladosporium asperulatum	LC168797	*Cladosporium asperulatum*	LN834357	518/518(100%)
Diaporthe eres	LC168760	*Diaporthe eres*	FJ478132	559/566(99%)
Alternaria mali	LC168759	*Alternaria mali*	AF314575	554/555(99%)
Guignardia mangiferae	LC168761	*Guignardia mangiferae*	KF381072	616/618(99%)
Setophoma chromolaena	LC168762	*Setophoma chromolaena*	KR093876	541/544(99%)
Diaporthe nobilis	LC168763	*Diaporthe nobilis*	KJ609006	552/555(99%)
Phomopsis sp. c1	LC168765	*Phomopsis* sp. LH223	HQ832822	557/576(97%)
Diaporthe sp. c1	LC168766	*Diaporthe* sp. M96	LC041048	528/542(97%)
Phomopsis sp. c2	LC168768	*Phomopsis* sp. EF06	JQ809664	558/562(99%)
Acremonium strictum	LC168769	*Acremonium strictum*	GU219464	558/562(99%)
Glomerella sp.	LC168770	*Glomerella* sp. JD08-18	JQ809667	534/536(99%)
Phomopsis sp. c3	LC168771	*Phomopsis* sp. BFM-L44	AB369483	541/549(99%)
Pestalotiopsis sp.	LC168772	*Pestalotiopsis* sp. 1 MJ-2014 i	KJ572189	513/515(99%)
Phomopsis sp. c4	LC168775	*Phomopsis* sp. 687	KC662228	544/550(99%)
Phomopsis sp. c5	LC168773	*Phomopsis* sp. 129SD/L	GU066691	553/558(99%)
Dothideomycetes sp.	LC168774	Dothideomycetes sp. Z4	JN198394	536/543(99%)
Epicoccum nigrum	LC168776	*Epicoccum* cf. *nigrum*	JQ676202	528/532(99%)

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Paraphaeosphaeria neglecta	LC168777	*Paraphaeosphaeria neglecta*	JX496038	401/407(99%)
Pestalotiopsis camelliae	LC168778	*Pestalotiopsis camelliae*	KM199336	567/569(99%)
Nemania sp.	LC168780	*Nemania* sp. AK-1	GQ906959	561/570(98%)
Xylariales sp.	LC168779	*Xylariales* 10A_co2	KC181931	592/600(99%)
Diaporthe pustulata	LC168781	*Diaporthe pustulata*	KC343186	529/547(97%)
Diaporthe sackstonii	LC168782	*Diaporthe sackstonii*	KJ197287	511/521(98%)
Diaporthe sp. c2	LC168783	*Diaporthe* sp. 3 PRJ-2013	KC145882	542/551(98%)
Phomopsis amygdali	LC168784	*Phomopsis amygdali*	AF102998	565/566(99%)
Phomopsis sp. c6	LC168764	*Phomopsis longicolla*	FJ755236	493/535(92%)
Phomopsis subordinaria	LC168785	*Phomopsis subordinaria*	GQ922519	555/560(99%)
Phomopsis sp. c7	LC168786	*Phomopsis vaccinia*	KJ739493	502/565(89%)
Melanconiella sp.	LC168788	*Melanconiella meridionalis*	JQ926293	337/403(84%)
Microdiplodia hawaiiensis	LC168789	*Microdiplodia hawaiiensis*	JN198395	558/559(99%)
Phoma herbarum	LC168791	*Phoma herbarum*	KP739881	519/523(99%)
Phialemonium dimorphosporum	LC168792	*Phialemonium* aff. *Dimorphosporum*	AY188371	529/537(99%)
Plenodomus sp.	LC168793	*Plenodomus* sp. SC5S1-1	KT235910	456/496(92%)
Pseudocercospora sp.	LC168794	*Pseudocercospora* sp. U02	JQ809678	512/515(99%)
Stagonosporopsis cucurbitacearum	LC168795	*Stagonosporopsis cucurbitacearum*	AB266846	533/537(99%)
Trichoderma koningiopsis	LC168796	*Trichoderma koningiopsis*	KP340235	568/568(100%)
Fungal endophyte sp.	LC168787	Fungal endophyte sp. JP33	AB255254	534/543(98%)
Uncultured Basidiomycota sp.	LC168767	Uncultured Basidiomycota clone R043SL3_C3	JX999054	558/562(99%)
Peniophora incarnata	LC168790	*Peniophora incarnate*	KC820949	599/603(99%)

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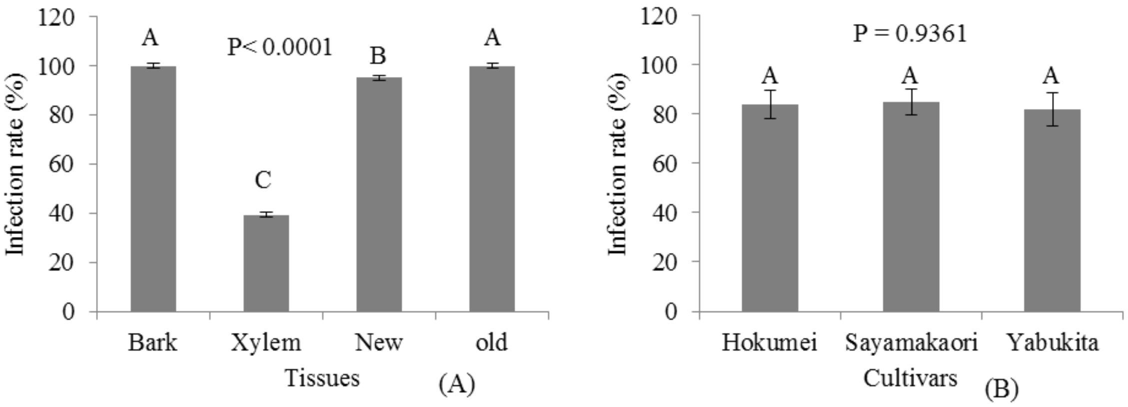

Figure 2. *Comparison of mean infection rates among tissue types (A) and cultivars (B) by Tukey-Kramer test at the α = 0.05 level (different letters show significant differences among tested cultivars and tissue types). Error bars = standard error (SE)*

Relative dominance

A total of 520 isolates was obtained from 600 segments (*Table 2*), and classified by morphological and molecular analyses into 44 species and operational taxonomic units (OTUs; defined by DNA sequences, OTUs are regarded as 'species' below). Forty-one species (93.2%) belonged to the phylum Ascomycota and two (4.5%) to Basidiomycota; one species could not be identified and was named Fungal endophyte sp. Glomerellales was the predominant order and Diaporthales and Pleosporales were the most diverse orders. Seventeen species (38.6%) belonged to Diaporthales and 10 species (22.7%) to Pleosporales. Other species were from the orders Botryosphaeriales, Capnodiales, Hypocreales, Sordariales, Xylariales, and Russulales.

The RD ranged from 70.8 to 0.4%. *Colletotrichum gloeosporioides* f. sp. *camelliae* and Pleosporales sp. predominated (70.8 and 52.3%, respectively). Other species with a D > 1% included *P. glomerata*, *Botryosphaeria dothidea*, *Cladosporium asperulatum*, *Diaporthe eres*, *Alternaria mali*, *Guignardia mangiferae*, *Setophoma chromolaena*, *Diaporthe nobilis*, *Phomopsis* spp., *Acremonium strictum*, *Glomerella* sp., uncultured *Basidiomycota* sp., and *Pestalotiopsis* sp. The 26 endophytes with a D of < 1% were regarded as rare species.

Cultivar preference

The CF (*Table 2; Fig. 3*) of the endophytic fungi differed significantly among the cultivars. The four most common endophytes, with the exception of *Pleosporales* sp., showed significant differences in host preference for *C. gloeosporioides* f. sp. *camelliae*, *P. glomerata*, and *B. dothidea* (*P* = 0.018, 0.0004, and 0.0251, respectively). The predominant endophyte, *C. gloeosporioides* f. sp. *Camelliae*, was isolated from all cultivars but showed the lowest CF in Yabukita (22, 2, 6, and 48% in bark, xylem, new leaf, and old leaf, respectively). The second most dominant endophyte, *Pleosporales* sp., colonized all cultivars equally. *P. glomerata* was also isolated from all cultivars but showed a CF of 2% in Hokumei bark and new leaf tissues. The fourth most dominant species, *B. dothidea*, was isolated from the cultivars Hokumei and Yabukita but not from Sayamakaori.

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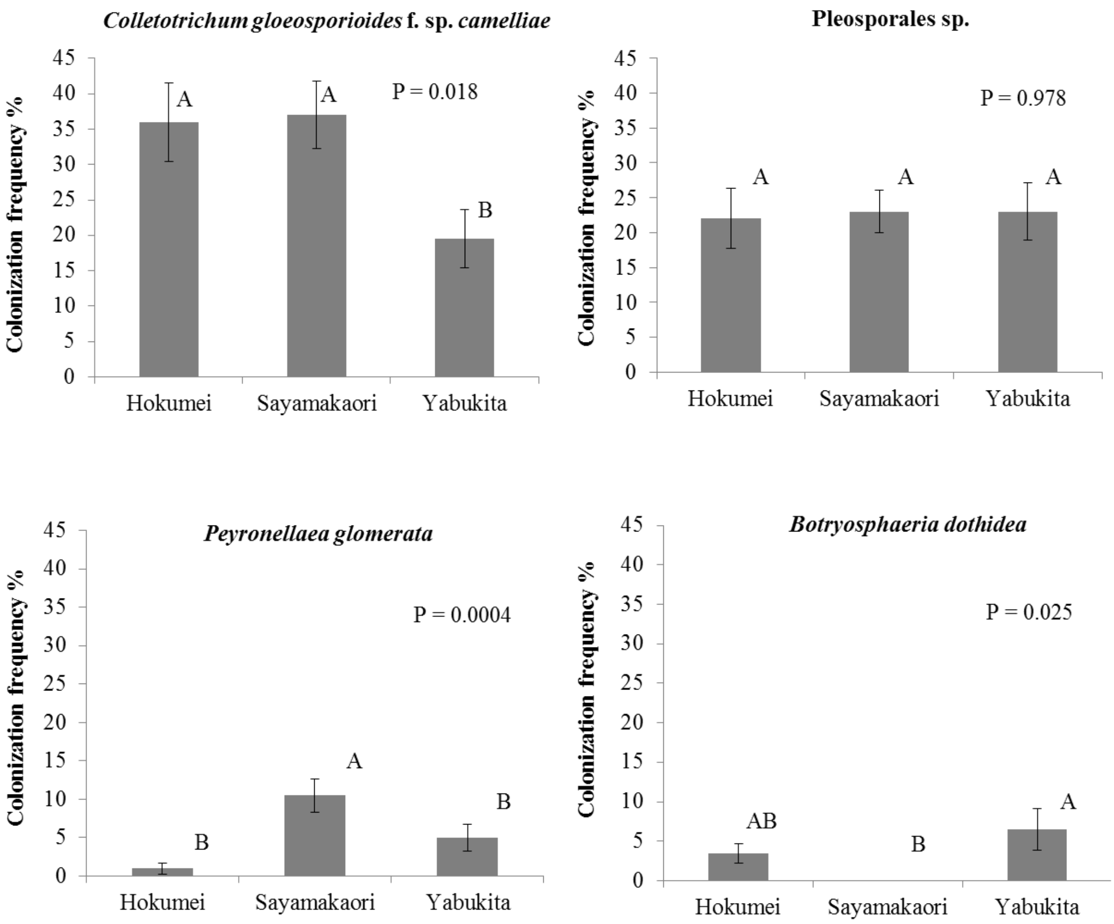

***Figure 3.** Mean CFs of four endophytic fungi with a D > 7% in three cultivars (different letters above columns indicate significant differences among cultivars). Tukey-Kramer HSD test, α = 0.05. Error bars = SE*

Tissue preference

The CF of endophytes differed markedly among the tissue types (*Table 2; Fig. 4*). Most of the endophytes showed a strong tissue preference. The CF of *C. gloeosporioides* f. sp. *camelliae* was significantly higher in bark and old leaf tissues than in new leaf and xylem tissues. Pleosporales sp. showed a significantly higher CF in new and old leaf tissues than stem tissues (bark and xylem); the highest CF was in new leaf tissues. The CF of *Peyronellaea glomerata* did not differ significantly among the tissues. *Botryosphaeria dothidea* showed the highest CF in bark tissues. *G. mangiferae* and *Glomerella* sp. were tissue-specific endophytes, as they were detected in only leaf and stem tissues, respectively.

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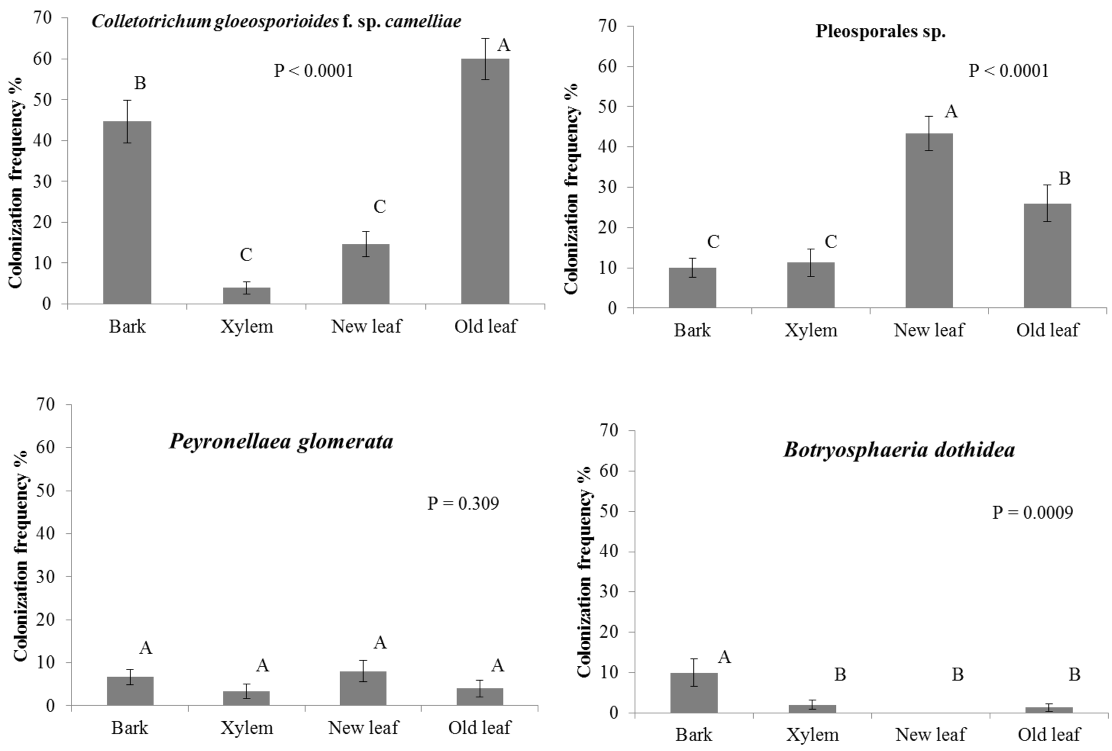

Figure 4. *Mean CFs of four endophytic fungi with a D > 7% among the tissue types (different letters above columns indicate significant differences. Tukey-Kramer HSD test, α = 0.05. Error bars = SE*

Diversity and evenness of endophytic mycoflora

The Shannon–Wiener diversity index (H') of endophytic fungi varied among the cultivars as well as the tissue types (*Table 2*). The values ranged from 2.48 in the bark of Yabukita to 0.79 in the old leaf of Hokumei. Among the tested tissues, old leaf tissues showed the lowest diversity indexes in all cultivars; 0.79, 1.23, and 1.54 in Hokumei, Sayamakaori, and Yabukita, respectively. New leaf tissues had a higher diversity index than old leaf tissues; 1.53, 2.01, and 1.93 in Hokumei, Sayamakaori, and Yabukita, respectively. Bark tissues exhibited the highest diversity index in Yabukita (2.48), followed by Hokumei (1.98) and Sayamakaori (1.51). Xylem tissues also showed high diversity indexes, with the highest in Hokumei (2.27) and the lowest in Yabukita (1.85). Among the tissues, the evenness index was highest in xylem in all cultivars (0.71, 0.8, and 0.7 in Hokumei, Sayamakaori, and Yabukita, respectively).

The rarefaction curve for the endophytes indicated the highest species richness in bark tissues and the lowest in old leaf tissues (*Fig. 5*). Of 44 species, 24 were detected in bark, 19 in xylem, 20 in new leaf tissues, and 14 in old leaf tissues. Among the cultivars, Yabukita showed the highest species richness (29 species), followed by Hokumei (26) and Sayamakaori (24) (*Fig. 5B*). Both rarefaction curves were steep, indicating that more endophytic fungal species remain to be discovered in tea plants.

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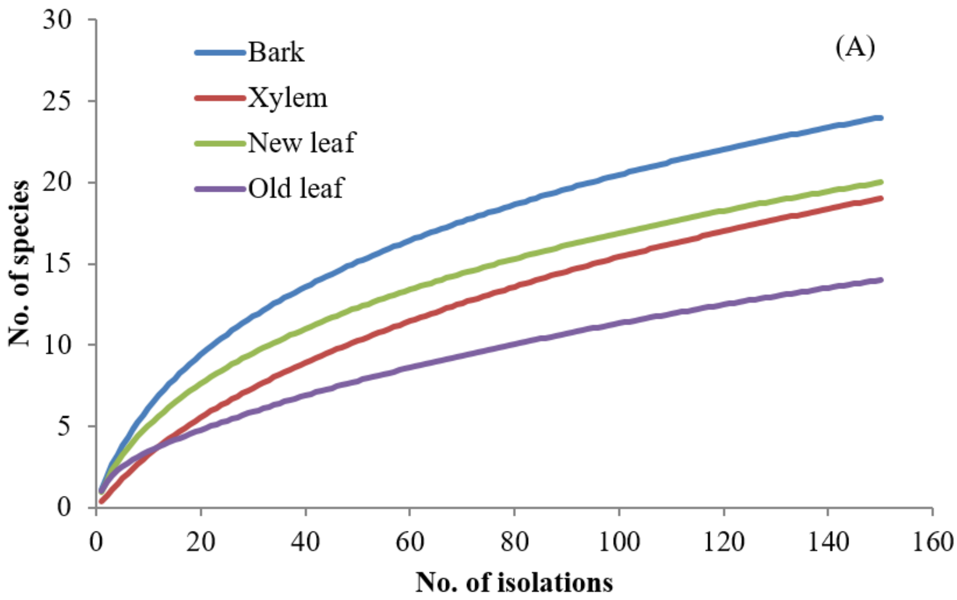

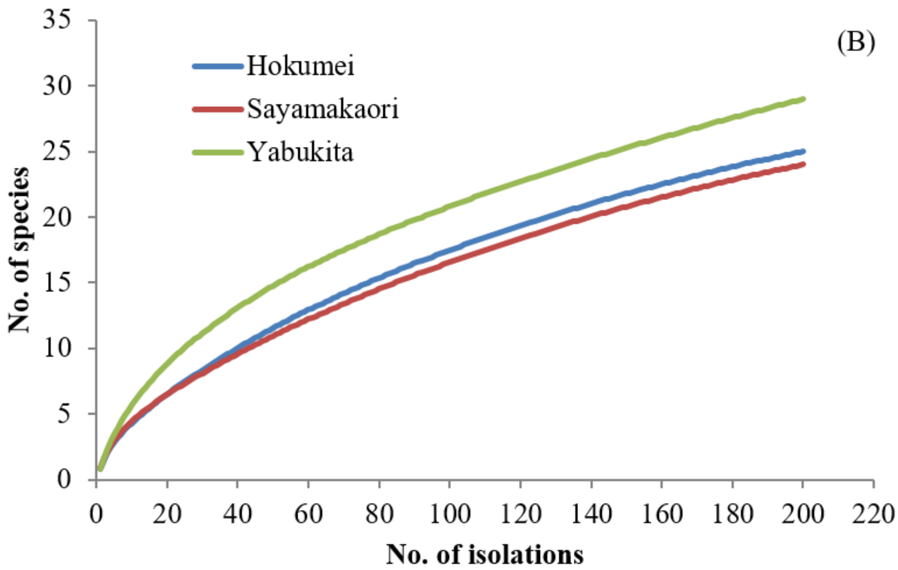

Figure 5. *Rarefaction curves showing endophytic fungal species isolated from (A) different tissues and (B) cultivars*

Effect of cultivar and tissue on endophytic fungal community structure

According to the PERMANOVA results, cultivar did not exert a significant effect on the tea endophytic fungal community structure (P = 0.0955; R^2 = 0.13). In contrast, tissue type had a significant impact on fungal endophyte community structure (P = 0.0001; R^2 = 0.66). The NMDS plot shows that the endophytic fungal community was similar among the cultivars but significantly different among the tissue types (*Fig. 6*).

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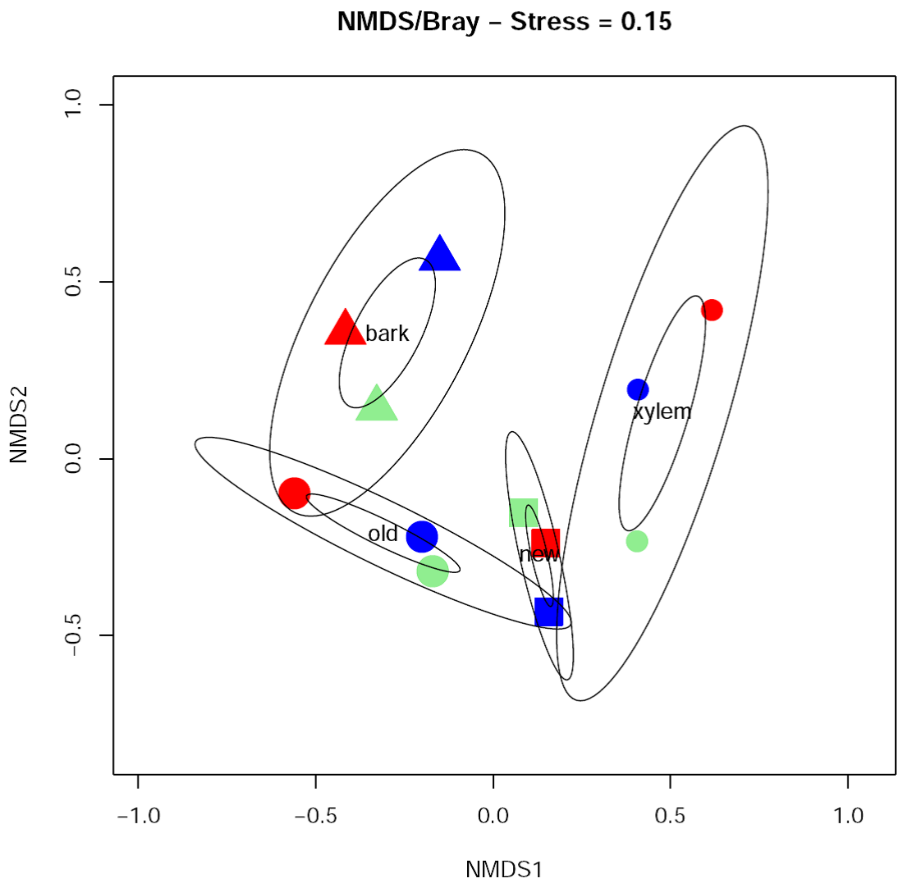

Figure 6. *Non-metric multidimensional scaling (NMDS) plot of endophytic fungal communities (red = Hokumei, green = Sayamakaori, blue = Yabukita; triangle = bark, square = new leaf, small cycles = xylem, large cycles = old leaf). Cultivars: R^2 = 0.13, P = 0.0955; tissues: R^2 = 0.66, P = 0.0001; confidence level = 0.95*

Discussion

Most endophytic fungi in this study belonged to the phylum Ascomycota. Lu et al. (2007) reported a similar result in tea plants in southern Henan province, China. According to Rodriguez et al. (2009), endophytic fungi from woody plants are non-clavicipitaceous endophytes. They are highly diverse, and most belong to the phylum Ascomycota and a minority to Basidiomycota.

Diaporthales was the most diverse order in this study, of which the genera *Phomopsis* and *Diaporthe* predominated. These genera have a wide host range; conversely, the same plant can host several species of the *Phomosis-Diaporthe* complex (Rehner and Uecker, 1994; Van Niekerk et al., 2005).

The fungal endophytic community in this study was somewhat different from those in southern Henan Province (Lu and Wu, 2006; Lu et al., 2007), Fujian Province (Chen 2007), and Zijin hill, Nanjing City, China (Fang et al., 2013). Three dominant endophytic fungi of tea plants, *Neurospora* and *Rosellinia* (Fang et al., 2013), and *Chaetomium* (Lu and Wu, 2006), were not detected in the present study. Therefore, the endophytic fungal community of tea plants varies according to geographical location.

The endophytic genus *Colletotrichum* reportedly predominates in tea plants (Lu et al., 2007; Fang et al., 2013). *Colletotrichum* endophytes of tea plants as well as other

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Camellia plants were identified only to the genus level by Liu et al. (2015), and tended to be regarded as host-specific endophytic fungi of *Camellia* plants. *C. gloeosporioides* f. sp. *camelliaeas* was also the predominant endophytic fungus in this study.

Cultivar preference

The IR did not differ significantly among the cultivars. The cultivars were grown at the same time in the same plot and were subjected to the same agricultural management practices. Although the tested cultivars have specific morphological and genetic characteristics, these differences did not affect the rate of infection by endophytic fungi; the average infection rate was ~ 80%. Such a high infection rate is likely because the study site is surrounded by tea plantations, which results in a dense inoculum.

The endophytes exhibited cultivar-specific colonization. According to Tian et al. (2004) and Naik et al. (2009), the fungal endophytic community can vary markedly among cultivars, and resistance to disease may be the major determinant of endophytic community structure. Leaf morphology and pigment content also affect endophytic community composition (Sanchez-Azofeifa et al., 2012). Endophyte richness is related to leaf traits such as water content, chlorophyll content, fresh:dry weight ratio, and polyphenol/leaf specific weight. The tested cultivars exhibited different morphological characteristics such as leaf color, shape, thickness and size, branching style, and resistance to disease. These factors could also be related to the differences in fungal composition and diversity among the cultivars.

Tissue preference

The IR differed significantly among the tissue types, likely due to differences in the nutrient content and anatomy of the tissues and the duration of aerial spore exposure. The lowest IR was in xylem tissues in all cultivars. The nutrient content of xylem tissues is lower than that of other tissues (Siebrecht et al., 2003; Yadeta and Thomma, 2014), which explains why few endophytes colonize this tissue. Xylem possesses highly structured and rigid secondary xylem walls and pit membranes with extremely small pores. The lignified walls of xylem tissue function as a structural barrier to colonization by endophytic fungi. Bark tissue showed a 100% IR in all cultivars. Bark tissues are rich in sugars (Yadeta and Thomma, 2014), which favors colonization by an endophytic mycoflora. Tea plants are perennial and the outermost tissues are exposed to aerial spores of fungal endophytes for longer than xylem tissues. This may also explain the lower IR of new leaves compared with old leaves. Endophytes tend to be transmitted horizontally between woody plants (Fisher et al., 1993).

Most of the fungal species isolated in this study showed tissue specificity. Fisher and Petrini (1988) reported that the xylem-specific endophytes *Phomopsis* sp., *Coniothyrium*, and *Coniochaeta* were the predominant endophytes in the stem tissues of *Ulex europaeus* and *Colletotrichum* sp. In the present study, the predominant endophyte *Colletotrichum* sp. showed a high colonization frequency in bark and old leaf tissues; this is in agreement with the findings of Fang et al. (2013).

Pleosporales sp. was the second most frequently isolated endophyte and showed a strong preference for new leaf tissues. *Pleosporales* sp. was first reported as a tea endophyte with a preference for leaf tissues by Fang et al. (2013) in the tea plantations of Zijin hill of Nanjing City, China. This endophyte may have coevolved with tea plants

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and is a rare species. However, *Pleosporales* sp. was the second most dominant endophyte in this study.

G. magniferae also exhibited leaf specificity. *G. magniferae* is a sexual stage of *Phyllosticta* that is usually isolated as an endophyte and rarely as a plant pathogen that can infect 70 plant families (Wikee et al., 2013). *Guignardia* was isolated as an endophyte from tea plants (Fang et al., 2013), *Quercus myrsinifolia* (Matsumura and Fukuda, 2015), and *Platycladus orientalis* (Wijeratne et al., 2008).

Old leaf tissues showed the lowest diversity index in all cultivars, and new leaf tissues had a higher diversity index than old leaf tissues. According to Herre et al. (2007), the diversity of endophytic fungi usually declines as the leaves age, but the fungal density (infection rate) increases. The endophytic fungal community of old leaves was dominated by *C. gloeosporioides* f. sp. *camelliae*. *Colletotrichum* species are normally aggressive and fast-growing. Ding et al. (2007) reported that *Colletotrichum* can grow easily under various environmental conditions. The evenness index was highest in xylem tissues in all cultivars, which suggests that endophytes are distributed more evenly in xylem than in other tissues.

Effect of cultivar and tissue type on endophytic community structure

According to the PERMANOVA and NMDS results, tissue type exerted a greater effect on endophytic fungal community structure than did cultivars. Most of the predominant endophytes were isolated from at least two cultivars; no individual cultivar had a unique fungal community. The predominant endophytes belonged to the genera *Colletotrichum*, *Phomopsis*, *Phoma*, and *Guignardia*. These endophytes have a wide host range and can colonize several taxonomically unrelated plant hosts (Murali et al., 2006; Sieber, 2007). Moreover, the cultivars are closely genetically related; Sayamakaori and Hokumei are derived from Yabukita tea plants. The level of similarity of endophyte communities increases with increasing genetic relatedness of the host plants (Sieber 2007).

Conclusion

The composition and diversity of the endophytic fungal community of tea plants were affected by tissue type and cultivar. The predominant endophytes showed preferences for certain tissue types or cultivars. However, tissue type played a more important role in shaping endophytic fungal community structure than did cultivar. Few studies have evaluated the endophytic fungi of Japanese tea cultivars; this is to our knowledge the first report of the diversity of endophytic fungi in Japanese tea cultivars. These results will facilitate further research on the growth and development of tea plants and sustainable tea production.

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Conflicts of Interest. The authors declare no conflicts of interest. All experiments undertaken in this study comply with the current laws of the country in which they were performed.

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ANALYSES OF TURBIDITY AND ACOUSTIC BACKSCATTER SIGNAL WITH ARTIFICIAL NEURAL NETWORK FOR ESTIMATION OF SUSPENDED SEDIMENT CONCENTRATION

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Abstract. The commonly used sampling method is restrictive for the spatial and temporal measurement of suspended sediment and requires intensive labor. These limitations and technological advances have led to methods based on sound or light scattering in water. In this study, the turbidity and acoustic backscattering signal (ABS) values were used with the aim of improving these methods with different artificial neural network (ANN) models; Multilayer Perceptron (MLP), Radial Basis Neural networks (RBNN) and General Regression Neural Network (GRNN). Measurements were taken in a vertical sediment tower for two different sediment sizes (< 50 µm and 50–100 µm) and concentrations (0.0–6.0 g L^{-1}). In the results of the regression analyses, turbidity values had strong relationships with sediment concentration for both sediment size groups (R^2 = 0.937 and 0.967). Although the ABS values had a reasonable R^2 value (0.873) for the 50–100 µm group, the < 50 µm group did not produce a significant R^2 value with regression analyses. The remarkable differences were not observed among MLP, RBNN and GRNN model for this sediment size group, and the reasonable R^2 and RMSE results were not produced with any ANN model that had a single ABS input for the < 50 µm sediment group. On the other hand, for the other sediment group (50–100 µm), ABS values were used as a single input, and the highest R^2 (0.917) value was obtained with MLP model and it was improved with the turbidity input (up to R^2 = 0.999). The results show that the ANN model could be considered as an alternative method because it was applied successfully to estimate suspended sediment concentration using with turbidity and ABS under different particle size conditions.
Keywords: *environmental, water quality, sediment transport, acoustic algorithm, particle size*

Introduction

Sediment transport in river is the crucial technical problem for many environmental and engineering practices. But its monitoring has many difficulties especially during high discharge and rough water-level conditions. The direct water sampling method is generally used as traditionally. But it is restrictive to represent spatial and temporal analysis of suspended sediment concentration, (Thorne and Hanes, 2002; Guerrero et al., 2016). Because sediment concentration high variable parameter depends on flow, climate and basin conditions (Gray et al., 2002). The size and concentration of sediment are mainly affected by intensity and volume of precipitation, texture and erodibility of soil, topography and land cover properties (Melesse et al., 2011). In addition; time and labor consumed for sampling and filtering processes are the other limitations of this method (Wren et al., 2000; Schoellhamer and Wright, 2003; Tfwala and Wang, 2016). Besides these limitations, continuous and precision sediment-monitoring requirements have led to new devices, especially in terms of using light and sound scattering or

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attenuation by particles in water. This new technique has gained importance within the sedimentological community, and its validation has been tested in many laboratory and field studies (Pedocchi and Garcia, 2012).

Turbidity as a physical value is defined as an optical property that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample (APHA, 2012). Scattering and absorption of light occurs on mineral suspended particles, colloids and bacterioplankton units, air bubbles and other suspended materials in the water sample (Lewis and Eads, 2001). The possibility of simultaneous observations of turbidity and its relationship between suspended sediment concentrations (SSC) has led to the use of turbidimetry to monitor sediment transportation (Uncles and Stephens, 2010). The use of turbidity values for SSC is an indirect method and based on determination of the statistical relationship between these values; the relationship could be linear, non-linear or polynomial (Sun et al., 2001). In addition the Regression equation should be considered individually for each stream condition with variations following the change of hydrological seasons (Williamson and Crawford, 2011). Changes in sediment size, mineral composition and water quality properties are the main limitations of this method. These effects should be considered and defined for different conditions to more accurately estimate sediment concentration with turbidity values (Ziegler, 2002). Tananaev and Debolskiy (2014) reported that the effecting factors of turbidity and sediment grain size should be considered in multivariate models, to minimize errors and acquire an understanding of its response. Mitchell et al. (2003) conducted a turbidity study using river conditions, and they reported that water quality and sediment properties were strongly affected, leading to errors in turbidity measurements, especially in spring season conditions. Chanson et al. (2008) conducted a laboratory study and produced a strong relationship ($R^2 = 0.992$) between sediment concentration and turbidity (Nephelometric Turbidity Units, NTU) at low concentrations (0.8 g L^{-1}) for silt and sand sediment materials. Slaets et al. (2014) used the cumulative rainfall values as additional input parameter to estimate sediment concentration with turbidity measurement. Pearson’s correlation coefficient was improved with Multiple linear regression analyses up to 0.87 compared with single (turbidity) parameter.

The acoustic sediment measurements, as other new technology, are based on sound waves spreading through the water column. The strength of the sediment particles’ backscattered signal is used to estimate the particles’ properties. The sediment particle size and concentration in the water can be computed using multi-frequency acoustic backscattering signal (ABS). For this purpose, three or four frequencies in the range 0.5–5 MHz are usually used in transceiver and receiver mode (Thorne and Meral, 2008). Acoustic sediment measurement studies have been applied successfully under different field and laboratory conditions. These studies’ results confirmed that particle size and concentration can be estimated relatively non-intrusively and with high spatial and temporal resolution using ABS (Thorne and Hurther, 2014; Ruessink et al., 2011; O’Hara Murray et al., 2012; Aagaard, 2014; Thosteson and Hanes, 1998; Thorne et al., 1998). However ABS is an indirect method and an inversion algorithm is required to convert the backscattered signal to a sediment concentration (Wilson and Hay, 2015; Clay and Medwin, 1997).

The main principle of ABS can be defined using the backscattering and attenuating characteristics of the particles in suspension, which are used for the acoustic inversion algorithm. The backscattering characteristic of the sediment particles is represented with

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the form function, and the attenuation characteristic is represented with the normalized total scattering cross-section. Both are non-dimensional parameters, and their origins are based on the acoustic properties of sphere particles (Neubauer et al., 1974). The sphere scattering properties, using the form function and normalized total scattering cross-section, were first reported by Sheng and Hay (1988). They used a solid sphere model and formulated a simple expression agreed with the data. Many researchers have adopted a similar expression (Thorne and Hurther, 2014; Hay and Sheng, 1992; Crawford and Hay, 1993; Thorne and Hardcastle, 1993; Schaafsma and Hay, 1997; Kisi, 2005). Thorne and Meral (2008) produced an expression for backscattering and attenuating characteristics that compared well with all the data sets available and that could be used with a reasonable degree of confidence to interpret ABS data collected above sandy sediments.

Many researchers have applied the backscattering signal to estimate suspended sediment, but difficulties and complexity remain in the acoustic algorithm. This requirement has led to alternative approaches to formulating the inversion (Thorne and Hurther, 2014). An artificial neural network (ANN) can be used to estimate a suspended sediment concentration based on the measured backscattered signal strength. The ANN technology has provided reasonable results in many complex nonlinear models used in hydrological studies. Kisi (2005) investigated the performance of the neural network method for modeling suspended sediment transportation. Nourani and Kalantari (2010) used rainfall and runoff parameters to determine spatiotemporal modeling of sediment with Artificial Neural Network. Maanen et al. (2010) used an ANN model to predict the suspended sediment with the input variables flow velocity, water depth and wave height, and they reported that using ANN models can improve sediment transport monitoring. Wang et al. (2009) used flow rate and turbidity values as input parameters for an ANN model to estimate sediment concentration, obtaining reasonable results. Similar ANN models studies have been used for many different types of input data, and strong correlations have been obtained for sediment prediction (Abrahart and White, 2001; Nagy et al., 2002; Yitian and Gu, 2003; Cigizoglu, 2004; Alp and Cigizoglu, 2007).

In this study, an acoustic backscattering signal (ABS) and the turbidity method were used for two different sediment-size groups in laboratory conditions. The study aimed to improve both methods by using regression and different ANN models to reduce the complexity of the acoustic algorithm.

Materials and methods

This study was conducted in a 50-L sediment tower under laboratory conditions. The sediment tower was used to prepare homogenous suspended sediment, which was mixed with a propeller operated by an electric motor. Natural sediment materials were used for both the 0–50 µm and 50–100 µm groups. Nearly 60 different concentrations were prepared, up to 6.0 g L^{-1} for both sediment-size groups.

An AQUAscat-L (Aquatec Group) device was used with 2 MHz frequency for the acoustic backscattering measurements (*Fig. 1*). The transducer was fixed vertically at the top of the tower, and backscattered signals were measured at 0.01 m intervals for each second during a 2-min period for both sediment groups. Turbidity measurements were made simultaneously with ABS using a Seapoint Turbidity Meter (*Fig. 1*). This device detects light scattered by suspended particles in water and produces an output

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voltage. The output voltage is calibrated to turbidity in formazine turbidity units (FTU). The unique optical design confines the sensing volume to within 5 cm of the sensor, allowing near-bottom measurements and minimizing errant reflections in restricted spaces (Smerdon, 2006). The measured data were saved by an external data logger. The turbidity sensor read 60 values per minute and a total of 120 turbidity values were taken during a 2-min period for each concentration. In addition, tree water samples (250 mL) were taken to determine the real sediment concentration with the gravimetric method.

Figure 1. *The AQUAscat-L (Aquatec Group) device and Seapoint Turbidity Meter sensor*

The regression analyses were applied to obtain the relationship between the sediment concentration with the ABS signal and the turbidity values. These relationships were evaluated using determination coefficient (R^2), root mean squared error (RMSE) and mean absolute error (MAE). As an alternative method, the ANN method was carried out to estimate sediment concentration using the ABS and turbidity values. In addition, a single input parameter was performed to get a simple alternative for sediment measurement. Different network topologies with single or double hidden layers and varying numbers of neurons were created using the Neural Network Toolbox for MATLAB software. Structures of ANN models are given in *Figure 2*. The measured data set was used 70% for training and 30% for testing, producing an ANN model. Finally, the ANN models were evaluated using R^2, RMSE and MAE.

Although there are many alternative models of ANN, in this study the Multilayer Perceptron (MLP), Radial Basis Neural networks (RBNN) and General Regression Neural Network (GRNN) were applied. A feed forward MLP network is formed by simple neurons called perceptron. The perceptron computes a single output from multiple inputs by making a linear combination according to its input weights and then determining the output through a nonlinear transfer function (Singha et al., 2012). The RBNN network is feed-forward network trained using a supervised training algorithm. The RBNN has connection weights between the hidden layer and the output layer only and an activation function is used as radial basis. It does not perform parameter learning as in MLP, performs linear adjustment of the weights fort the radial bases. The RBNN usually train much faster than back propagation networks. They are less susceptible to problems with non-stationary inputs because of the behavior of the radial basis function hidden units. The GRNN is a variation of the RBNN and is based on kernel regression networks. It consists of four layers: input layer, pattern layer, summation layer and

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output layer. The GRNN is based on the non-linear regression model. It estimates most probable output values for given input training set with the minimum mean-squared error (Cigizoglu, 2004; Alp and Cigizoglu, 2007).

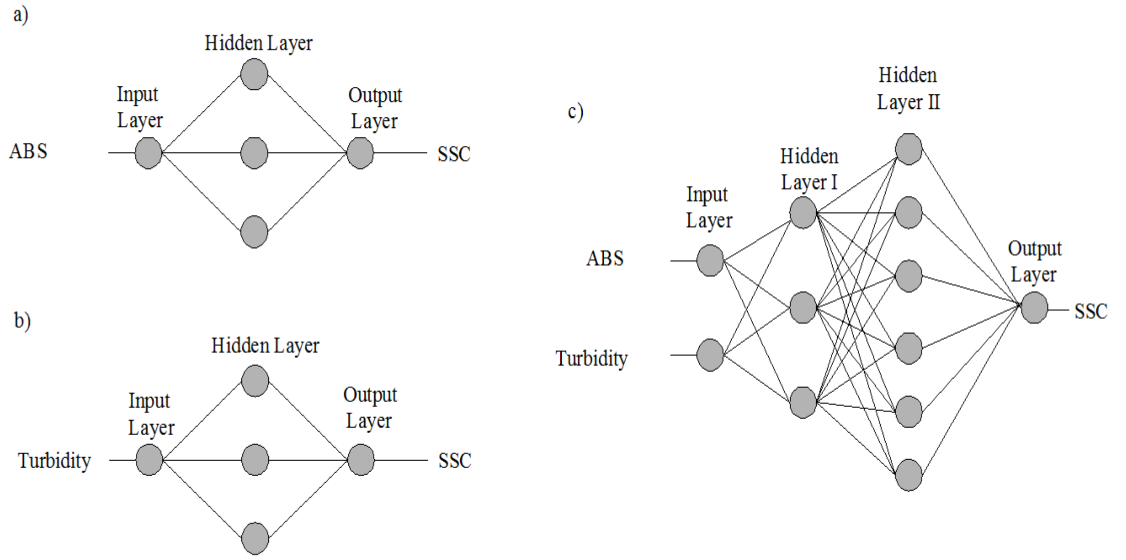

***Figure 2.** Structures of ANN models for sediment concentration estimation. a) Single input layer (ABS). b) Single input layer (Turbidity). c) Double input layer (ABS and Turbidity)*

Results and discussion

The results show that small particles had the greatest turbidity and decreasing in turbidity values depended on increases in sediment size (*Fig. 3*).

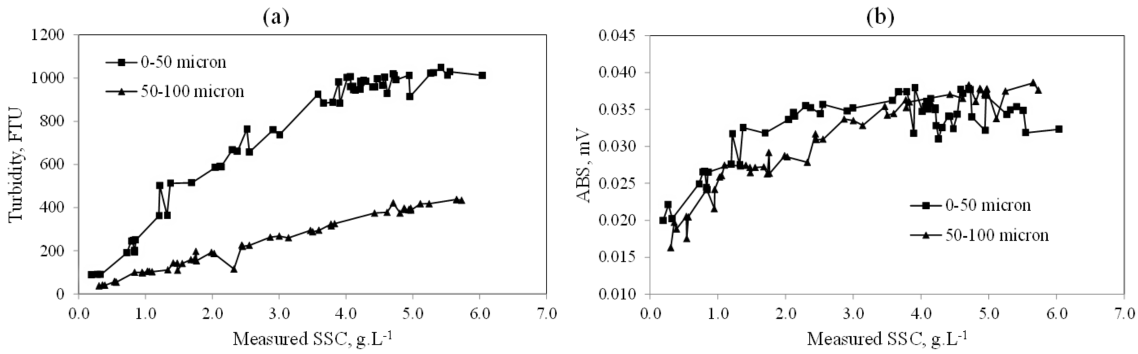

***Figure 3.** Observed turbidity (a) and ABS values (b) for different sediment concentrations*

Similarly, Gao et al. (2008) reported that turbidity is more sensitive to fine particles than to coarse particles. This property has led to investigate the relationship between particle size distribution and turbidity. Yao et al. (2014) used different particle size and investigate its relationship with turbidity. They obtained strong correlation for bigger than 5 micron sediment size but reasonable relation was obtained only for low turbidity (0–40 NTU) under smaller sediment condition. Pavanelli and Bigi (2005) reported that a large deviation in sediment size can lead to serious errors in estimating. They prepared sediment sample groups with narrow intervals (19–31, 58–81 and 124–149 µm), and

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they obtained good relationships. They concluded that sediment size problems can be eliminated with sensitive calibration but it should be consider the flow regime and water color for each river condition.

ABS measurements were observed in a large and unstable range for fine-sediment materials. Previous studies stated that clay's existence in the sediment suspension negatively effects of ABS values. This problem is essentially explained with the shape of a fine sediment (Moate and Thorne, 2009), for particle coagulation depends on the clay material, which causes unstable backscattering (Thorne and Hanes, 2002). In addition, this study's results showed that the negative effect of clay depends on the sediment concentration, especially above the level of 4.0 g L^{-1}. This situation limits the uses of the acoustic method in certain clay-content conditions.

Regression analyses

The average FTU and ABS values for each known sediment concentration were used for regression analyses. The statistical parameters (RMSE, MAE and R^2) values of the linear regression analysis results between sediment concentration and FTU and between sediment concentration and ABS are presented *Table 1* for training and testing data. In addition estimated and measured SSC values were plotted in *Figure 4* to show comparison of models for testing data.

Table 1. *The statistical parameters (RMSE, MAE and R^2) values of the linear regression analysis results*

Inputs	Training			Testing		
	RMSE	MAE	R^2	RMSE	MAE	R^2
	For smaller than 50 µm sediment group					
ABS	1.338	1.107	0.311	1.480	1.253	0.274
FTU	0.467	0.365	0.934	0.428	0.370	0.937
FTU and ABS	0.430	0.326	0.947	0.491	0.417	0.918
	For the 50–100 µm sediment group					
ABS	1.094	0.938	0.904	1.210	1.050	0.873
FTU	0.185	0.139	0.988	0.340	0.210	0.967
FTU and ABS	0.175	0.130	0.990	0.360	0.200	0.961

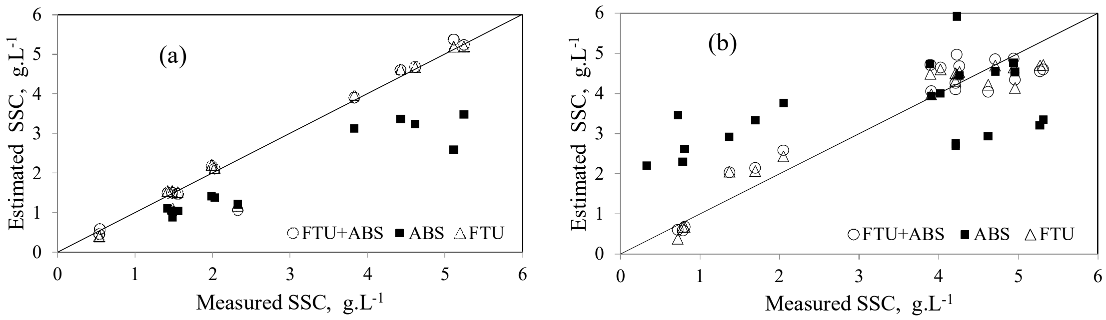

Figure 4. *Regression analyses between sediment concentration (SSC) with turbidity (FTU) and acoustic backscattering signal (ABS), (a) < 50 µm, (b) 50–100 µm sediment group*

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The turbidity values had good relationships with the SSC values ($R^2 = 0.937$ and 0.967) for testing data of each sediment groups respectively. However, the linear relationship was destroyed for values higher than 700 FTU, and this critical level was reached at the 3 g L^{-1} sediment concentration for the small particle size. The Seapoint Turbidity manual reported that the sensor response becomes nonlinear above 750 FTU; however, the useful range can be extended by calibrating the sensor and fitting the response to a second-order polynomial equation. Similarly, Wang et al. (2014) reported that values up to 600 FTU had good relations with SSC. It can be concluded that turbidity measurements should be conducted for lower sediment concentrations and that subsamples with dilution should be used for high concentrations (Foster et al., 1992). Due to negatively effect of small particle size, relatively lower R^2 values were obtained for ABS measurements compared to turbidity values. Although ABS had a reasonable R^2 value (0.873) for the 50–100 µm sediment group, but a significant R^2 (0.274) was not produced for < 50 µm sediment group. In addition, all regression equations can be used for the low-sediment concentration condition. Increasing sediment size can provide these equations for up to a 4 g L^{-1} concentration. Medalie et al. (2014) obtained a moderately strong relation ($R^2 = 0.650–0.810$) was found for concentrations of fine-grained suspended sediments and backscatter signals up to 500 mg L^{-1} sediment concentration. However, a quite weak relationship ($R^2 = 0.220–0.370$) was observed for fine sediment for some river conditions in the same study. This problem is not related to the regression method; rather, it is a result of the problems observed in the acoustic measurement.

ANN models

The statistical parameters (RMSE, MAE and R^2) of each ANN models were used to evaluate the alternative approaches (*Tables 2 and 3*). The reasonable R^2 and RMSE results were not produced with the any ANN model that had a single ABS input for the < 50 µm sediment group, and it was not considered for discussion in *Table 3*. This problem is caused by the negative clay particles on the ABS sediment, as mentioned above, and it was not improved with ANN models. These results show that ABS is strongly affected by sediment grain size, thus supporting the findings of previous studies. Similarly, De Falco et al. (2010) reported that backscatter signal strongly affected by sediment grain size; being directly correlated to the weight percent of the coarse fraction (1000–1600 µm) and inversely correlated to weight percent of the finer sediments (16–500 µm). Goff et al. (2004) did not find a significant relationship between backscatter intensity and the mean grain size of sands (> 4000 µm), although they found an inverse relationship with finer sediments (0.063 µm). This can be explained deviation of size and distribution. Therefore, in this study; strong relationships were obtained using a single turbidity input, with the highest R^2 (0.970) for the MLP, and R^2 (0.999) values in RBNN and GRNN model for testing. The use of ABS values as additional inputs (along with turbidity) did not improve the statistical parameters of this relationship. The remarkable differences were not observed among MLP, RBNN and GRNN model for this sediment size group. On the other hand, for the other sediment group (50–100 µm), ABS values were used as a single input, and the highest R^2 (0.917) and lowest RMSE (0.521) values were obtained for the MLP among all ANN models (*Table 2*). De and Chakraborty (2012) concluded that estimating the mean grain size using an acoustic inversion algorithm is computing-intensive, but this value could be estimated using an ANN-based approach in a much shorter computing

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time and with high determination coefficients (R^2 up to 0.998). Similarly this study shows that single ABS input values can be used with any ANN model to estimate SSC instead of the complex acoustic algorithm. The using single turbidity for input for the 50–100 µm sediment group produces good relationships with the highest R2 (0.986) for the MLP, and R^2 (0.996) values in RBNN and GRNN model for testing. In addition, for the relatively coarse sediment group, the using ABS values as an additional input with turbidity showed an improved relationship with R^2: 0.973 0.999 and 0.999 for MLP, RBNN and GRNN models respectively.

Table 2. *The statistical evaluation of different ANN models for the < 50 µm sediment group*

Inputs	Model	Training			Testing		
		RMSE	MAE	R^2	RMSE	MAE	R^2
FTU	MLP	0.209	0.151	0.983	0.397	0.296	0.970
FTU and ABS	MLP	0.272	0.167	0.971	0.417	0.329	0.940
FTU	RBNN	0.183	0.106	0.988	0.280	0.120	0.990
FTU and ABS	RBNN	0.187	0.093	0.987	0.290	0.160	0.990
FTU	GRNN	0.116	0.042	0.995	0.250	0.100	0.990
FTU and ABS	GRNN	0.160	0.080	0.990	0.260	0.130	0.990

Table 3. *The statistical evaluation of different ANN models for the 50–100 µm sediment group*

Inputs	Model	Training			Testing		
		RMSE	MAE	R^2	RMSE	MAE	R^2
ABS	MLP	0.286	0.222	0.972	0.400	0.187	0.936
FTU	MLP	0.131	0.102	0.994	0.278	0.202	0.986
FTU and ABS	MLP	0.142	0.094	0.993	0.297	0.170	0.973
FTU	RBNN	0.068	0.032	0.998	0.110	0.050	0.996
FTU and ABS	RBNN	0.079	0.035	0.998	0.050	0.030	0.999
FTU	GRNN	0.051	0.035	0.999	0.060	0.040	0.999
FTU and ABS	GRNN	0.032	0.023	0.999	0.370	0.280	0.953

Conclusions

Many researchers have used the acoustic method for estimating sediment concentration in both laboratory and river conditions. Sound-scattering properties are known to become more complex with different particle sizes and sediment concentrations (Thorne and Hanes, 2002; Mouraenko, 2004). Although the acoustic algorithm is the basic method for evaluating ABS, many parameters – such as sediment and water properties, flow regime and acoustic device settings – are required to estimate sediment concentration, This method is quite complex, requires user expertise, and has some disadvantages (Meral et al., 2008). Comparatively, the ANN model is not complicated, and it can be a reasonable alternative for evaluating ABS values, In addition, turbidity values have a strong relationship with a wide range of sediment concentrations at different particles sizes, and these turbidity values can be used as a single input parameter to estimate sediment concentration as an alternative to the

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acoustic method. This study's results show that the acoustic method has potential for instantaneous and continuous sediment concentration analysis with reasonable precision, and it can be improved upon using regression and ANN models, The ANN model is a powerful tool for input/output mapping, and it can facilitate the acoustic method by removing complex algorithms. A single input parameter (turbidity or ABS) can be used with the ANN model for all waters not containing clay, to get more accurate results, further regression and ANN model studies should be conducted with alternative particle sizes, shapes and densities and for varying water properties.

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INTRA- AND INTER-REGIONAL TECHNOLOGY TRANSFER AND CO_2 EMISSIONS IN CHINA: COMPARING THE EFFECTS OF ENERGY AND ENVIRONMENTAL TECHNOLOGIES

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Abstract. The purpose of this paper is to reveal the role of regional technology transfer in reducing carbon dioxide emissions in China. By collecting a panel data of 150 observations from 30 regions of mainland China during 2006–2010, this paper extends the STIRPAT model to explore the relationship between intra- and inter-regional technology transfer and CO_2 emission intensity, and meanwhile compares the effects of energy and environmental technologies in different regions of China. The results show that, in the national level, only intra-regional transfer of energy technologies can significantly reduce the CO_2 emission intensity. When it comes to the regional level, intra-regional transfer of energy technologies has a negative influence on CO_2 emissions in the eastern and western regions. In contrast, intra-regional transfer of environmental technologies negatively affects CO_2 emissions in the central regions, and even leads to a significant increase of CO_2 emissions in the eastern regions. However, neither inter-regional transfer of energy technologies nor inter-regional transfer of environmental technologies has significant effect on CO_2 emissions reduction in China. Our findings reveal the critical role of intra-regional rather than inter-regional technology transfer, and also energy technologies rather than environmental technologies in reducing CO_2 emissions, which has important implications for future environmental policy-making in China.
Keywords: *energy and environmental technologies; regional technology transfer; CO_2 emissions; China; STIRPAT model*

Introduction

As the largest developing economy in the world, China is now facing enormous pressure in reducing carbon emissions (Li et al., 2011). In the past decade, the CO_2 emissions in China has increased continuously, accounting for more than 25% of the world's total CO_2 emissions since 2009 (*Fig. 1*). During the Copenhagen Climate Change Conference in 2009, China has promised to significantly cut down its CO_2 emissions by 40% - 45% in 2020 compared with 2005 (Wang et al., 2015). Consequently, the emission reduction targets have been included as the binding targets in the national and regional economic and social development of China (Sun et al., 2015). All regions in China have to bear huge duty of reducing carbon emissions to accomplish the targets of the national commitment.

In the past few years, technological innovation has been considered an important driver of reducing carbon emissions and promoting environmental sustainability (Wang et al., 2012a). Numerous scholars have explored the roles of different technological channels in improving the environment, which include internal research and development (R&D) activity, international knowledge spillovers and domestic technology spillovers (Yang et al., 2014). Given those studies generally investigated on

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the regional level, however, an important channel of technology transfer–domestic regional technology transaction–is still underexplored. Particularly, due to the uneven distribution of regional innovation resources in China, some regions have to rely on green technologies across regional borders. Thus, the extent to which intra- and inter-regional technology transfer can influence carbon emissions in China is still an important question that needs further investigation.

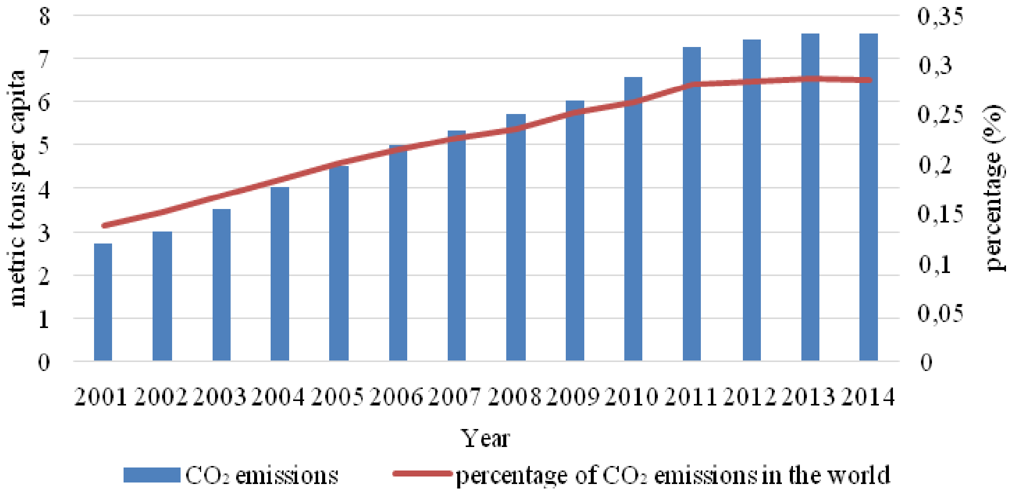

Figure 1. *CO_2 emissions in China from 2001 to 2014*
(Source: World Development Indicators from the World Bank)

Meanwhile, previous studies on the relationship between technological innovation and CO_2 emissions seldom distinguish the effects of different types of green technologies (Yang et al., 2014), such as energy technologies and environmental technologies. In fact, however, energy technologies and environmental technological solutions play roles in different processes of carbon emissions reduction. From a whole process treatment perspective (Zhang, 2013), energy technologies mainly serve as the solution of source prevention and process control, which promote the use of renewable energy and improve the efficiency of traditional fossil energy in the production process. In contrast, environmental technologies mostly act as the end-of-pipe technological solution, which prevent, control and decrease the carbon emissions with technical equipment such as pollution control equipment and environmental monitoring instruments. However, the different effects of energy technologies and environmental technologies on CO_2 emissions in China have been rarely taken into account in previous studies.

To address the above gaps, this paper uses a unique data of regional technology transaction of province-level in China, and explores the relationship between intra- and inter-regional technology transfer and CO_2 emissions, and meanwhile compares the different effects of energy and environmental technologies in different regions of China.

Literature review

As an important environmental issue, the influencing factors of CO_2 emissions have been studied by many scholars. In recent years, scholars have paid more attention to the role of green technological innovation in reducing carbon emissions (Jiang et al., 1998;

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Goulder and Schneider, 1999), especially for those developing countries facing greater pressure to lessen carbon emissions. Specifically, three different technological channels in reducing carbon emissions have been investigated, including indigenous innovation, international knowledge spillover and inter-regional knowledge spillover.

The impact of indigenous innovation on CO_2 emissions

Numerous studies have investigated the impacts of indigenous R&D and technological innovation on CO_2 emissions. At first, some scholars mainly related the R&D investment to CO_2 emissions, aiming to recognize the positive role of internal R&D activity. Cole et al. (2005) found that in the UK, the industry's R&D expenditure was negatively correlated with air pollution intensity. Lee and Min (2015) also revealed the negative impact of green R&D on carbon emissions in Japan. However, Garrone and Grilli (2010) examined 13 advanced economies and indicated that public energy R&D could significantly improve energy efficiency, but had no significant effect on carbon intensity. When it came to China's case, Feng and Yuan (2016) showed that increasing R&D intensity could decrease the carbon intensity.

Apart from the literatures on R&D input, the impact of green patent output on CO_2 emission has also attracted much attention. Yan et al. (2017) used patent data of 15 major economies during 1992-2012, and found no evidence of the influence of low-carbon innovation on CO_2 emission. Wang et al. (2012b) revealed that the carbon-free energy patents were significantly negatively correlated with CO_2 emissions in the eastern regions of China, but had no significant effects in the central and western regions. Ding et al. (2015) showed that green patents had positive influences on the CO_2 emission reduction in the whole country as well as the eastern and western regions of China, except the central region. Wang et al. (2012c) also confirmed the negative relationship between energy technology patents and CO_2 emissions in Beijing city.

The impact of international knowledge spillovers on CO_2 emissions

In addition to indigenous R&D and innovation for improving the environment, international knowledge spillovers through trade and foreign direct investment (FDI) have also been investigated. Researchers suggested that trade and FDI can lead to knowledge spillovers by means of competition effects, demonstration effects, employee turnover and vertical linkages (Yang et al., 2014), which may result in local technological improvement. However, trade and FDI can also lead to scale and structure effects, which may be harmful to local environment sustainability because of more energy use and more carbon emissions (Zhang, 2012).

Several studies examined the impact of trade or FDI on CO_2 emissions separately. For instance, Yan and Yang (2010) revealed that the rapid growth of China's CO_2 emissions was mostly caused by the manufacture of exports, confirming the scale and structure effects of international trade in China. Zhang and Zhou (2016) found that the FDI's impact on CO_2 emissions decreased from the western region to the eastern and central regions. Moreover, many scholars have explored the effects of trade and FDI simultaneously. Ren et al. (2014a) revealed that trade surplus and FDI inflows in China contributed to the increasing CO_2 emissions. Ren et al. (2014b) suggested that China should reduce the scale of FDI inflows, and import and export more green products with other countries. However, Yang et al. (2014) did not find the positive spillover effects of FDI and trade in reducing carbon emissions.

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The impact of inter-regional knowledge spillovers on CO_2 emissions

Another important technological channel–inter-regional knowledge spillovers within a country–has also been explored in recent years. First of all, just like international trade, domestic trade within a country could also improve regional technology capabilities through positive embodied knowledge spillovers, and thus reduce its CO_2 emissions. But the side effect of domestic inter-regional trade may also exist, which is known as “the pollution haven hypothesis” (Ren et al., 2014a, b). A developed region may shift its intensively polluting industry to lagged regions through inter-regional trade, which instead increases the CO_2 emissions of lagged regions. Zhang et al. (2014) showed that inter-regional imports and exports can lead to more carbon emissions in the coastal and inland provinces. Guo et al. (2012) confirmed that the eastern area has transferred embodied CO_2 emissions to the central area, and inter-regional trade in China has an effect on regional CO_2 emissions. Zhang (2017) also discovered that inter-regional trade has significant spillover effects on provincial CO_2 emissions in China.

Furthermore, many scholars argued that inter-regional trade is not the only way of regional knowledge spillovers, the R&D activities of neighboring regions can also result in inter-regional knowledge spillovers, thus benefiting local innovation. Yang et al. (2014) revealed that R&D intensity of neighboring regions have statistically significant spillover effects in China, confirming the role of inter-regional R&D spillovers in decreasing CO_2 emissions. Yang et al. (2014) was the first to consider domestic inter-regional R&D spillover effect in environmental issues. However, their focus is only the indirect spillovers of neighboring regions. In fact, however, the channel of technology transaction is a more direct way to transfer technologies outside. Recently, some studies began to examine regional technology transfer through the channel of technology transaction. For example, Sun and Liu (2016) investigated the evolution of inter-regional technology transactions in China. However, there is still few research on the effect of direct technology transaction on regional CO_2 emissions.

To sum up, studies on the first channel of indigenous innovation mainly focused on the impact of R&D input and patent output, while paid little attention to intra-regional technology transfer, thus neglecting the diffusion and commercialization of regional green technologies. Meanwhile, literature on the second and the third channels took external knowledge spillovers across the regional borders as important ways to reduce carbon emissions. However, compared with the direct technology transaction between regions, the effects of international and inter-regional knowledge spillovers were relatively indirect. Therefore, it can be summarized that prior research on the relationship of “technological innovation-CO_2 emissions” ignored the direct channel of intra- and inter-regional technology transaction through technology market, which has been considered as an important channel for local innovation in technological innovation and regional studies. Meanwhile, previous literature seldom compared the effects of energy and environmental technologies on CO_2 emissions, which actually act as different types of technological solutions in the whole process of carbon emissions. Thus, this paper aims to fill the above gaps. In the following parts, we will investigate the impacts of intra- and inter-regional technology transfer on CO_2 emission intensity, and meanwhile compare different effects of the energy technologies and environmental technologies in different regions of China.

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Methodology and data

STIRPAT model

Ehrlich and Holdren (1971) firstly proposed the IPAT model (Human impact on the environment, Population, Affluence, and Technology) to address the factors influencing environmental pressure. The general form of IPAT model is I=PAT. Here, I denotes human impact on the environment, P denotes population, A denotes affluence, T denotes technological factor. However, the determinants in IPAT model are limited, and the equal ratio relationship also limits its generality (Ding, 2015). So some scholars like Dietz and Rosa (1994) extended the IPAT model to the STIRPAT model (Stochastic Impacts by Regression on Population, Affluence and Technology), and used it to investigate multiple influencing factors of environmental issues. The STIRPAT model is an equation with the random form as follows.

$$I = aP^b A^c T^d e \tag{Eq.1}$$

In this equation, a represents the constant term, b, c, d represent the exponential terms of the P, A and T, and e means the error term. *Eq. (1)* is often transformed into a logarithmic form in empirical studies:

$$\ln I = a + b\ln P + c\ln A + d\ln T + e \tag{Eq.2}$$

By reviewing the literatures on “technological innovation-CO_2 emissions”, we find that most scholars used the STIRPAT model to empirically study the environmantal issues, such as Li et al. (2011), Ding et al. (2015) and Wang et al. (2012c). Although these studies did not reach the consistent result due to their focus on different technological channels, they generally proved the fitness of the STIRPAT model in “technological innovation-CO_2 emissions” research. In this study, we also use the STIRPAT model to study the effect of regional technology transfer on CO_2 emissions. Specifically, I means CO_2 emission intensity, P means population size, A is measured by per capita GDP (PGDP). As for the technological factor T, some scholars like York et al. (2003) suggest that it can be broken down into several measurable indicators, such as the industrial structure, energy consumption structure and energy intensity. Later, more and more researchers begin to add more direct indicators like R&D investment and patent output into the model to measure the impact of technological level (Wang et al., 2012; Ding, 2015). In this paper, apart from the decomposed factors like industrial structure (IS), energy intensity (EI) and energy consumption structure (ES), we take regional technology transfer as a central technology channel of improving technological level. According to the regional and sectoral boundary of technology transfer activity, we set up four variables to extend the model, including intra-regional transfer of energy technologies (IntraEY), inter-regional transfer of energy technologies (InterEY), intra-regional transfer of environmental technologies (IntraET) and inter-regional transfer of environmental technologies (InterET). Then, the extended STIRPAT model is as follows.

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$$\begin{aligned}\ln I_{it} &= a + b\ln P_{it} + c\ln \mathrm{PGDP}_{it} + d_1 \ln IS_{it} + d_2 \ln EI_{it} + d_3 \ln ES_{it} \\ &+ d_4 \ln IntraEY_{it-1} + d_5 \ln InterEY_{it-1} + d_6 \ln IntraET_{it-1} + d_7 \ln InterET_{it-1} + e\end{aligned} \quad \text{(Eq.3)}$$

In the equation, i represents regions, t represents year; a represents the constant term, b, c, d_1, d_2, d_3, d_4, d_5, d_6, and d_7 represent the exponential terms of the P, PGDP, IS, EI, ES, IntraEY, InterEY, IntraET and InterET, and e means the error term. Considering the possible lag effect of regional technology transfer, all technology transfer variables are lagged for one year in the model.

Variables and data

The dependent variable in the model is the CO_2 emission intensity (I). We measure it with the ratio of CO_2 emissions to GDP. So far, China has no direct statistics about CO_2 emissions on regional level, thus most scholars have to estimate it on the basis of energy consumption in each region (Ren et al., 2014a, b). According to the China Statistics Bureau, there are 8 major types of energy sources in China, which are coal, coke, crude oil, gasoline, kerosene, diesel, fuel oil and natural gas. Therefore, we use the method proposed by IPCC (Intergovernmental Panel on Climate Change) Guidelines for National Greenhouse Gas Inventories to estimate CO_2 emissions of Chinese regions (IPCC, 2006).

$$CO_{2jt} = \sum_{j=1}^{8} E_{jt} \times NCV_j \times CEF_j \times COF_j \times \frac{44}{12} \quad \text{(Eq.4)}$$

In *Eq.4*, E_j represents the consumption of energy type *j*. NCV_j is net calorific value, CEF_j is carbon emission factor, and COF_j is carbon oxidation factor. 44 and 12 represent the molecular weight of CO_2 and carbon. Data of NCV_j is collected from China Energy Statistical Yearbook, and CEF_j and COF_j are collected from IPCC (IPCC, 2006).

The industry structure (IS) is measured by the percentage of industry added value to GDP, the energy intensity (EI) is measured by the ratio of energy consumption to GDP, and the energy consumption structure (ES) is measured by the percentage of coal consumption to total energy consumption. All types of energy consumption data derive from China Energy Statistical Yearbook, and the data of P, GDP, and industry added value are from the China Statistical Yearbook.

Moreover, this study uses technology transaction data to measure regional technology transfer. In China, there are four types of technology transaction in technology markets, including technology development, service, transfer and consulting (Sun and Liu, 2016). A Chinese official institution, the Technology Market Management & Promotion Centre, is responsible for organizing and managing technology transaction activities, thus owning all contract data on regional technology transaction. We obtain the unique inter-regional technology contract data in 2006-2010 from this official source. This dataset contains intra- and inter-regional technology transaction data in the province level of China. And it is also divided into 11 technology sectors, in which the energy technologies and environmental technologies are the focus of this paper. Specifically, intra-regional transfer of energy technologies (IntraEY) is measured by the contract value of energy technology transaction within the same region, while inter-regional transfer of energy technologies (InterEY) is measured by the contract value of energy technology transaction across different regions. Similarly, intra-regional transfer of environmental technologies (IntraET) is measured by the

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contract value of environmental technology transaction within the same region, and inter-regional transfer of environmental technologies (InterET) is measured by the contract value of environmental technology transaction across different regions. The detail description and interpretation of all variables is presented in *Table 1*.

Table 1. *Description and explanation of variables*

Variables	Definition and measurement	Unit
I	The ratio of CO_2 emissions to GDP	ton per Yuan
P	The number of total population	Million
PGDP	per capita GDP	Yuan
IS	The percentage of industry added value to GDP	%
EI	The ratio of energy consumption to GDP	ton per Yuan
ES	The percentage of coal consumption amount to energy consumption	%
IntraEY	Contract value of energy technology transaction within the same region	Million Yuan
InterEY	Contract value of energy technology transaction across different regions	Million Yuan
IntraET	Contract value of environmental technology transaction within the same region	Million Yuan
InterET	Contract value of environmental technology transaction across different regions	Million Yuan

Our data of regional technology transfer is between 2006 and 2010, so we collect the data between 2007 and 2011 of other variables with the consideration of one-year lag effect of technology transfer activity. Meanwhile, the mainland China has 31 administrative regions, including provinces and municipalities. Due to the lack of statistics of the Tibet in "China Energy Statistical Yearbook", we only collect data from 30 regions of the mainland China. So the sample size includes a panel data of 150 observations.

In order to study the influence of regional technology transfer in different groups of regions, we divide 30 provinces and municipalities into three sub-sample economic regions like other studies (Wang et al., 2012b, c; Ding et al., 2015), including the eastern, central and western regions. Specifically, in China, the eastern regions include 11 provincial administrative regions (Beijing, Fujian, Guangdong, Hainan, Hebei, Jiangsu, Liaoning, Shandong, Shanghai, Tianjin, Zhejiang), the central regions include 8 provincial administrative regions (Anhui, Henan, Heilongjiang, Hubei, Hunan, Jilin, Jiangxi, Shanxi), and the western regions include 11 provincial administrative regions (Gansu, Guangxi, Guizhou, Inner Mongolia, Ningxia, Qinghai, Shaanxi, Sichuan, Xinjiang, Yunnan, Chongqing). The specific location of the eastern, central and western regions in mainland China is shown in *Fig. 2*.

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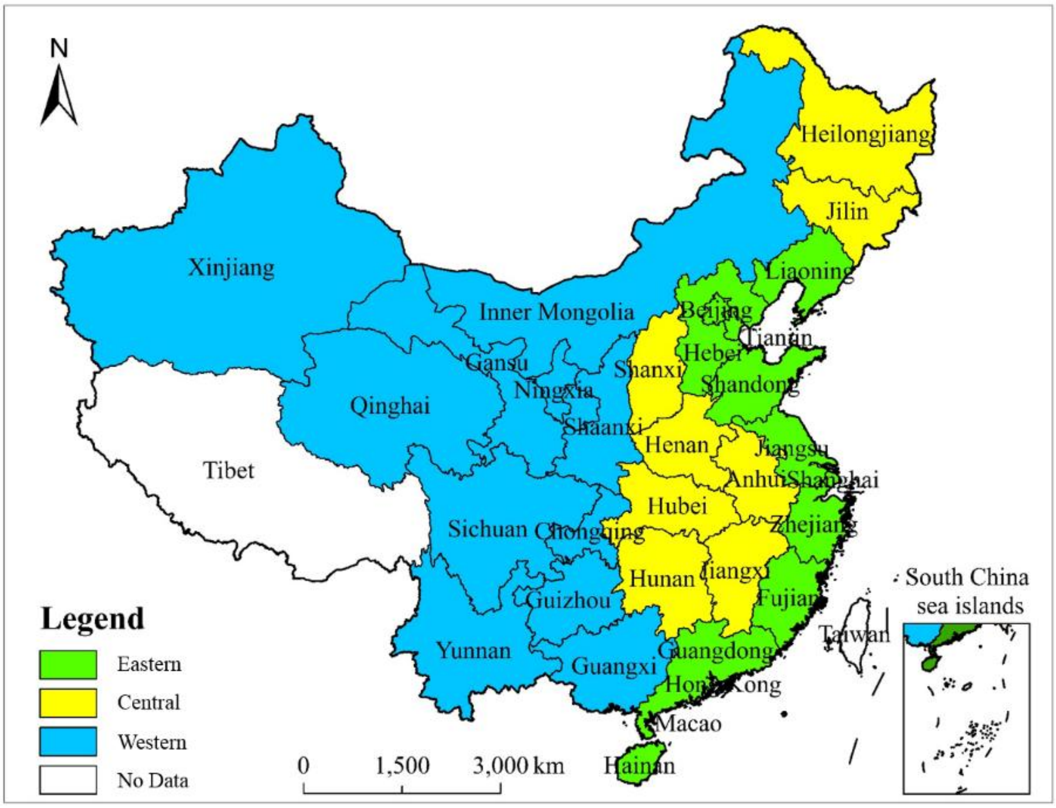

Figure 2. *The location of the eastern, central and western regions in mainland China*

Estimation procedure

Considering the panel data of 5 years and 30 sections, this research uses traditional panel regression model to empirically study the role of regional technology transfer in reducing CO_2 emissions for 30 Chinese regions from 2006 to 2010. We will firstly estimate the relationship between regional technology transfer and CO_2 emissions within all regions of China. Then, we split the full dataset into three sub-samples, and estimate the corresponding relationships in the eastern, central and western regions.

In each panel data estimation, we use the Hausman's test to decide on the fixed-effect or random-effect model. After the tests of all regression models, the results all support the fixed-effect models. Meanwhile, in order to overcome the heteroscedasticity problem, the cross-section weighted generalized least squares (GLS) technique is employed in all panel estimations.

Results

Descriptive statistics

Firstly, we present the descriptive statistics of all variables, and we specifically describe the difference of variables in three sub-sample regions of China, just as shown in *Table 2*.

From *Table 2* we can see that, the intensity of CO_2 emissions in China during 2007-2011 is 4.25 on average, and the western regions have the highest CO_2 emissions (5.58

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on average), followed by the central regions (4.56 on average), and then the eastern regions (2.69 on average). Obviously, in China, the eastern regions have the least environmental problems of CO_2 emissions during economic development. The average population of China in 2007-2011 is 44 million, and is mainly concentrated in the central and eastern regions, 52.67 and 49.05 million, respectively. The average per capita GDP in 2007-2011 is 30343 Yuan. As the most developed regions in China, the eastern regions' per capita GDP reaches up to 45577 Yuan, far higher than that in the central and western regions (22436 and 20860 Yuan, respectively). The ratio of industry sector added value is 0.49 on average, and there is only small difference of industry structure among the eastern, central and western regions. The regional distribution of energy intensity is very similar to CO_2 emission indicator. The highest energy intensity is in the western regions (1.72), followed by the central regions (1.34), and then the eastern regions (0.96). As for the energy consumption structure, there is 62% coal consumption on average, and the central regions rely more on coal consumption (72%), followed by the western and eastern regions.

Table 2. *Descriptive statistics of all variables in different regions of China*

Variables	All regions		Eastern regions		Central regions		Western regions	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
I	4.25	2.75	2.69	1.37	4.56	3.03	5.58	2.83
P	44	26.53	49.05	31.97	52.67	20.20	32.64	20.30
PGDP	30343	17525	45577	18786	22436	6245	20860	9595
IS	0.49	0.08	0.47	0.11	0.51	0.05	0.49	0.05
EI	1.34	0.75	0.96	0.44	1.34	0.79	1.72	0.78
ES	0.62	0.16	0.49	0.15	0.72	0.08	0.67	0.13
IntraEY	316.90	484.79	557.15	708.10	212.52	185.93	152.55	166.58
InterEY	456.64	483.11	629.30	576.92	301.68	249.92	396.70	462.88
IntraET	188.11	322.25	365.71	464.33	104.84	139.57	71.08	75.15
InterET	296.95	282.33	292.11	238.37	381.37	392.06	240.40	208.66

Finally, in general, the average value of intra- and inter-regional transfer of energy technologies is higher than that of environmental technologies. Specifically, the eastern regions are most active in both intra- and inter-regional transfer of energy technologies, and the western regions are more dependent on inter-regional technology transfer of energy technologies than the central regions. By contrast, in environmental technologies, the eastern regions are more active in intra-regional technology transfer, while the central and western regions are more dependent on inter-regional technology transfer.

Table 3 shows the correlation coefficients of all variables. We can see that the population (P) and per capita GDP (PGDP) show significant negative correlations with CO_2 emission intensity, while the industrial structure (IS), energy intensity (EI) and energy consumption structure (ES) have significant positive correlations with CO_2 emission intensity. For all technology transfer variables, only the intra-regional transfer of energy technologies (IntraEY) and intra-regional transfer of environmental

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technologies (IntraET) are significantly negatively related to CO_2 emission intensity. Furthermore, most independent variables have correlations with each other, but all the coefficients are lower than 0.8, meaning the multicollinearity does not have any effect on the following regression analysis.

Table 3. *Correlation coefficients of all variables (after logged)*

	1	2	3	4	5	6	7	8	9	10
1.I	1.00									
2.P	-0.22**	1.00								
3.PGDP	-0.55**	-0.04	1.00							
4.IS	0.35**	0.28**	-0.01	1.00						
5.EI	0.97**	-0.36**	-0.51**	0.22**	1.00					
6.ES	0.50**	0.37**	-0.34**	0.62**	0.28**	1.00				
7.IntraEY	-0.14*	0.34**	0.38**	0.30**	-0.22**	0.29**	1.00			
8.InterEY	0.02	0.27**	0.41**	0.08	0.001	0.08	0.48**	1.00		
9.IntraET	-0.16*	0.27**	0.42**	0.25**	-0.20*	0.14*	0.77*	0.51*	1.00	
10.InterET	0.02	0.28**	0.26**	0.12	-0.03	0.15*	0.39**	0.46**	0.32**	1.00

Notes: * and ** mean significance at 5%, and 1% level (two tailed).

Panel regression results

Then, we implement the panel regressions with Eviews 7.0 software to reveal the effects of intra- and inter-regional technology transfer on CO_2 emissions. *Table 4* presents all panel regression results.

In the first model of all regions (Model 1), the population (ln P) is significantly and negatively correlated with CO_2 emission intensity, indicating that as the population size increases, the growth rate of GDP exceeded the growth rate of CO_2 emissions in China. The Energy intensity (ln EI) and the energy consumption structure (ln ES) have significant positive impacts on CO_2 emissions, which means that the greater the energy consumption and the higher ratio of coal consumption is, the greater the CO_2 emission intensity will be. As for the regional technology transfer variables, only intra-regional technology transfer of energy technologies (ln IntraEY) has significant negative effect on CO_2 emission intensity (d_4= -0.00083, $p<0.05$), implying that intra-regional technology transfer in energy technologies can significantly decrease a region's CO_2 emissions in economic development.

Then, we observe the regression models of three sub-samples of the eastern, central and western regions (Model 2-4). The results show that, the Population (ln P) is significantly negatively correlated with CO_2 emission intensity in the eastern regions, but positively affects CO_2 emission intensity in the western regions. The per capita GDP (ln PGDP) only has significant negative effect on CO_2 emission intensity in the eastern regions. And the industry structure (ln IS) is significantly positively correlated with CO_2 emission intensity in the eastern regions, but negatively affects CO_2 emission intensity in the western regions. The above significant differences among the three regions might be related to their diverse level and stage of economic development. As the most developed regions in China, the eastern regions' population increase, economic growth and industrial upgrading are more related with low-carbon green development. By contrast, the central and western regions are still in an investment-dependent and factor-

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driven development stage; the population expansion and economic growth are usually accompanied by an increase in carbon emissions. Furthermore, the energy intensity (ln EI) and the energy consumption structure (ln ES) are significantly positively correlated with CO_2 emissions in the eastern, central and western regions, which is consistent with theoretical and practical expectations.

The impacts of regional technology transfer on CO_2 emission intensity also show some differences among the three regions. In the eastern regions, intra-regional transfer of energy technologies (ln IntraEY) has a significant negative effect on CO_2 emission intensity (d_4= -0.004969, p<0.01), while intra-regional transfer of environmental technologies (ln IntraET) is significant positively correlated with CO_2 emission intensity (d_6=0.005445, p<0.01). This suggests that in the eastern regions, energy technologies do play important roles in reducing carbon emissions by focusing on source prevention and process control, while environmental technologies as end-of-pipe treatments, may instead encourage more polluting activities, thus exacerbating carbon emissions.

Table 4. *Panel regression results of different regions in China*

Variables	Model 1 All regions	Model 2 Eastern regions	Model 3 Central regions	Model 4 Western regions
Constant	1.925734***	2.450626***	1.651618***	0.643102*
	(0.115844)	(0.297816)	(0.421081)	(0.334736)
ln(P)	-0.071447***	-0.107773***	-0.028888	0.086713**
	(0.012129)	(0.033599)	(0.046515)	(0.042275)
ln(PGDP)	0.000138	-0.021526**	0.000226	0.002912
	(0.003576)	(0.009525)	(0.006489)	(0.004787)
ln(IS)	-0.008151	0.038317*	-0.022962	-0.032934***
	(0.006199)	(0.019727)	(0.013454)	(0.011737)
ln(EI)	1.008786***	0.957938***	0.994867***	1.015423**
	(0.006196)	(0.015285)	(0.011739)	(0.009291)
ln(ES)	0.426151***	0.403168***	0.498978***	0.432017**
	(0.007728)	(0.015962)	(0.025271)	(0.015872)
ln(IntraEY)	-0.000830**	-0.004969***	0.000323	-0.001534**
	(0.000414)	(0.000515)	(0.000658)	(0.000513)
ln(InterEY)	-0.000235	-0.000530	-0.000471	-0.000502
	(0.000576)	(0.000981)	(0.000685)	(0.000648)
ln(IntraET)	-0.000079	0.005445***	-0.002888***	0.000104
	(0.000428)	(0.000598)	(0.000890)	(0.000263)
ln(InterET)	0.000479	0.000593	0.000019	-0.000447
	(0.000374)	(0.000997)	(0.000518)	(0.000675)
R-squared	0.999987	0.999973	0.999985	0.999981
Adjusted R-squared	0.999983	0.999958	0.999975	0.999970
F-statistic	233576	68231	96895	94708
Prob(F-statistic)	0.000000	0.000000	0.000000	0.000000
Observations	150	55	40	55

Notes: *, **, and *** mean significance at 10%, 5%, and 1% level. Standard errors are in parentheses.

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With regard to the central and western regions, only intra-regional transfer of environmental technologies (ln IntraET) is significant negatively correlated with CO_2 emission intensity in the central regions (d_6= -0.002888, $p<0.01$). In contrast, only intra-regional transfer of energy technologies (ln IntraEY) has a significant negative effect on CO_2 emission intensity in the western regions (d_4= -0.001534, $p<0.05$). The results reveal that the central regions are inclined to commercialize their own environmental technologies to reduce CO_2 emissions by focusing on the end-of-pipe treatment, while the western regions are just similar to the eastern regions, relying more on energy technologies within regional boundary in terms of source prevention and process control.

Discussion

According to the above results, in addition to indigenous R&D, international and inter-regional knowledge spillovers (Yang et al., 2014), intra- and inter-regional technology transfer through direct technology transaction is confirmed as another important channel to reduce carbon emissions on the regional level of China. We get some new findings and enrich our understanding about the technological channels in reducing carbon emissions.

First, in addition to the confirmed positive effects of energy R&D and patent output (Wang et al., 2014a, b; Ding et al., 2015), our findings suggest that the diffusion and commercialization of intra-regional green technologies are also critical for reducing a region's CO_2 emissions. Previous studies have paid much attention to the input and output of green technological innovation, while our study highlights the crucial role of the transfer and commercialization of green technologies, which obviously calls for more research on the roles of international and domestic technology market in the low-carbon economic development.

Second, Yang et al. (2014) has compared the effects of indigenous R&D, spillovers through increasing openness, and interregional R&D spillovers on CO_2 emissions. However, they have not compared the different roles of intra- and inter-regional technology transfer. Our findings reveal that although the contract value of inter-regional technology transfer is generally higher than that of intra-regional technology transfer, only intra-regional transfer of green technologies has significant effects on CO_2 emission intensity, and there are certain barriers to impede the effective use of technology across different regions.

Finally, we followed the whole process treatment perspective (Zhang, 2013), and compared different roles of energy and environmental technologies in reducing CO_2 emissions. The results show that intra-regional transfer of energy technologies can significantly reduce CO_2 emissions in the national, eastern and western regions, while intra-regional transfer of environmental technologies can only decrease CO_2 emissions in central regions. It indicates that in reducing CO_2 emissions, source prevention and process control with energy technologies are much more effective than the end-of-pipe treatment solutions using environmental technologies.

Conclusions

In this paper, using regional technology transaction data in energy and environmental technologies of mainland China during 2006–2010, we extended the STIRPAT model to investigate the relationship between intra- and inter-regional technology transfer and

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CO_2 emission intensity, and meanwhile compared the effects of energy and environmental technologies in different regions of China. We found that on the national level, only intra-regional transfer of energy technologies can significantly reduce the CO_2 emission intensity. When it comes to the regional level, intra-regional transfer of energy technologies has a negative influence on CO_2 emissions in the eastern and western regions. By contrast, intra-regional transfer of environmental technologies negatively affects CO_2 emissions in the central regions, but leads to a significant increase of CO_2 emissions in the eastern regions. However, neither inter-regional transfer of energy technologies nor inter-regional transfer of environmental technologies plays a significant role in reducing CO_2 emissions.

The contribution of this paper can be summarized in three aspects. First, in addition to the indigenous technological innovation and indirect knowledge spillovers, this paper confirms another important technological channel of reducing CO_2 emissions–domestic regional technology transfer through technology transaction, which not only offers a new insight into the research on “technological innovation-CO_2 emissions” relationship research from the perspective of technology transfer, but also has important implications for the development of domestic technology market to improve environmental performance in China. Second, this paper identifies the significant role of intra-regional technology transfer rather than inter-regional technology transfer in reducing CO_2 emissions, which verifies the significance of regional boundary in absorbing and utilizing technology transfer for green development, and therefore calls for more efforts to put into improving absorptive capability of regions in China to make full use of external technological resources. Third, this paper reveals the much more essential role of energy technologies than environmental technologies in reducing CO_2 emissions, which not only advances our understanding of the heterogeneous effects of different types of green technology from the perspective of whole process treatment, but also has practical significance for the technology choice in developing green low-carbon economy of China. Obviously, more attention should be paid to the source- and process-oriented technological solutions in energy technologies.

The findings of this study may also provide some implications for the policy-makers in China. First, in the context of green and low-carbon development, Chinese government should further optimize the environment of technology market development, and establish the market-oriented service system of technology market, thus promoting technology transfer in energy and environmental sector. Second, on the basis of utilizing intra-regional technology transfer, local governments should place more emphasis on the establishment of cross-regional information exchange and communication platforms, and improve regional absorptive capability to fully use advanced technology across regional boundaries. Third, it’s difficult and insufficient to fundamentally ease the pressure of carbon emissions in China by relying on the end-of-pipe environmental protection solutions, so local governments should pay more attention to new energy and energy-saving technology development and application, and reduce CO_2 emissions in the source and the process of economic production activities.

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IMPACT OF SOIL SALINITY ON DIVERSITY AND COMMUNITY OF SUGARCANE ENDOPHYTIC PLANT GROWTH PROMOTING BACTERIA (*SACCHARUM OFFICINARUM* L. VAR. CP48)

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Abstract. The aim of this study was to isolate and identify culturable bacterial endophytes from sugarcane grown in saline and non-saline soil and assay some plant growth-related traits and evaluate selective isolate potential to improve growth, yield and grain phosphorus uptake of wheat. Endophytic bacteria associated with sugarcane grown in saline and non-saline soil were isolated from roots, stems and leave and tested for their ability to fix nitrogen, indole acetic acid (IAA) production and dissolution of insoluble phosphorus. A total of 55 endophytic bacteria were isolated from leaves, stems, and roots of sugarcane from saline and non-saline soil. The results revealed a prevalence of Bacillaceae, with *Bacillus* sp. being the most frequently isolated bacterium. Salinity had the effect on bacterial community structure with the higher diversity of root entophytic bacteria in saline soils. From the isolates, four strains were shown to produce IAA and 19 strains showed the ability to solubilize phosphate, but twelve isolates having the best characteristics tests were identified using 16S rRNA gene sequencing. From the isolates *Pseudomonas* sp. SugS_49 with more phosphorus dissolution ability selected for evaluation on wheat growth. Application of *Pseudomonas* sp. SugS_49 caused increased growth, grain yield and phosphorus uptake of wheat.
Keywords: *biofertilizer, phosphorus dissolution, pseudomonas, Shannon index, wheat*

Introduction

Endophytic bacteria are ubiquitous colonizers of the inner plant tissues, viz., roots, stems, leaves or seeds where they do not normally cause any substantial morphological changes and disease symptoms (Hallmann et al., 1997). It was traditionally thought that endophytic bacteria cause mild diseases in plants, but recent research has proved that these bacteria can improve plant growth and increase their resistance against plant pathogens (Berg, 2009). The most predominant and studied endophytes belong to three major phyla (Actinobacteria, Proteobacteria, and Firmicutes) and include members of *Azoarcus* (Krause et al., 2006), *Acetobacter* (Bertalan et al., 2009), *Bacillus* (Deng et al., 2011), *Enterobacter* (Taghavi et al., 2010), *Pseudomonas* (Taghavi et al., 2009), and *Stenotrophomonas* (Ryan et al., 2008). Endophytic and rhizospheric bacteria improve plant growth through several mechanisms. They can increase plant growth by dissolving insoluble phosphorus, producing hormones such as indole acetic acid (IAA) and amino cyclopropane carboxylate deaminase (ACC), antimicrobial agents such as antibiotics

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and siderophore, nitrogen fixation, and reducing environmental stresses. They have beneficial effects on their host by preventing the spread of disease through synthesizing new compounds and antifungal metabolites (Ryan et al., 2008). Application of PGPR that was auxin producer (*Bacillus thuringiensis*, *Enterobacter asburiae*, and *Serratia marcescens*) caused to enhance nutrient uptake, seedling and root growth in wheat (Selvakumar et al., 2008). Improvement of grain yield, phosphorus uptake, and tillers number in wheat was observed after using a mixture of phosphate solubilizing bacteria and organic fertilizer (Afzal and Bano, 2008). The recent study showed an increase of wheat root length, root fresh and dry weight due to inoculation of bacterial in hydroponic culture (Singh et al., 2017). Although agricultural practices that use high amounts of external inputs, such as inorganic fertilizers, pesticides, and other amendments, can overcome specific soil constraints to crop production, this has resulted in continuous environmental degradation, particularly of soil, vegetation and water resources, especially in the most intensively managed systems. Therefore, we should now use alternative fertilizers without affecting the environment to reach sustainable agriculture. A potential option to increase the growth and yield of plants and reduce the destructive impact of chemical fertilizers on the environment can be the application of plant growth promoting bacteria (Krause et al., 2006). Beneficial properties of endophytes in increasing yields of agricultural crops, controlling plant diseases or pests, and adapting plants against stress conditions can encourage their increased application in sustainable agriculture practice (Mei and Flinn, 2010).

Sugarcane (*Saccharum officinarum*) is one of the five strategic products in the world and is considered as one of the best converters of solar energy to biomass and sugar (Yadav and Solomon, 2006). Sugarcane has an important role in the agriculture tradition of Khuzestan. This industry developed in the region and produces more than half of the country's sugar. Numerous reports are available on isolation of PGPR and their effects on growth and yield of crops. However, no information exists on the isolation and identification of endophytic bacteria from sugarcane cultivated in Iran. On the other hand salinity of soil under sugarcane cultivation due to irrigation practices and application of chemical fertilizer is now being seriously issue. There have been relatively few studies that have analyzed the effects of different environmental variables on endophyte diversity. Therefore it is necessary to pay attention to the effect of soil salinity on the diversity of sugarcane endophytic bacteria. The present study was carried out to isolate endophytic bacteria from the healthy roots, stems and leave of *Saccharum officinarum* L. var. CP48 grown in saline and non-saline soils with this hypothesis that endophytic bacterial communities within *Saccharum officinarum* vary among different tissues of the plant and are affected by the salinity of the soil in which the plant is growing.

After evaluation of some traits related to plant growth promotion of isolated bacteria, the impact of a selected halo tolerant isolate with phosphate dissolution ability was investigated on wheat plant growth parameters. This study may be further used for subsequent researches of isolates effect on plant growth as biological fertilizer.

Materials and methods

Sample preparation

Healthy roots, stems and leaves of 20 plants of sugarcane (*Saccharum officinarum* L.) cultivar CP48 near one-year-old plant cultivated in clay loam soils of two fields (with salinity levels of around 1.5-5dS/m) in Debal-Khazaei agro-industrial unit located

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in Khuzestan province of Iran (latitude 31°05′; longitude 48°30′) were sampled in October 2015. Fields have been under sugarcane cultivation for more than four years and managed using typical conventional farming practices appropriate for the region. The plants were collected in plastic bags and stored at 4°C within 72 h of collection. The roots, stems, and leaves of each plant were washed with tap water (to remove their mud and adhering soil). Then tissues of each plant were separated into three parts and cut into 2-4 cm pieces. About 10 g of each organ were weighed and placed in separate plates. Subsequently, the plant tissues were immersed in 70% ethanol for 1 min. They were then transferred into 2% sodium hypochlorite solution for 3 min, and 70% ethanol for 30 s. Finally, they were rinsed three to five times with sterile distilled water until they were completely free of disinfectants (Mendes et al., 2007). The disinfection steps were carried out under the laminar flow hoods.

Isolation of culturable endophytic bacteria

Disinfected plant organs were macerated separately in sterile physiologic saline (0.7% NaCl) and serially diluted to 10^{-8}. 50 µl of 10^{-3} to 10^{-8} dilutions were spread on nutrient agar medium in triplicates and incubated at 28°C for 72 h. The appeared colonies were differentiated on the basis of colony morphology such as shape, margin, color, elevation, texture, and size of colony and pigmentation, thereafter the number of colonies that were similar in appearance was counted. Thereafter the isolates were classified to genus level according to Bergey's Manual of Determinative Bacteriology according to their physiological and biochemical properties (Holt et al., 1994). Microbial diversity was assayed by Shannon's diversity index (H'). When diversity indices are used in ecology, the types of interest are usually species, but they can also be other categories, such as genera, families, functional types or haplotypes. The Shannon diversity index (H) of isolated bacteria from each tissue was calculated by *Equation 1*. The proportion of isolate i relative to the total number of isolates (p_i) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln p_i$). Shannon's equitability (Evenness) was calculated by dividing H by H_{max} (here $H_{max} = \ln N$).

$$H = -\sum_{i=1}^{S} \mathrm{Pi} * \ln \mathrm{Pi}\ ,\ \mathrm{Pi} = \frac{\mathrm{ni\ (Sample)}}{\mathrm{N\ (SUM)}} \qquad \text{(Eq. 1)}$$

where:
H = the Shannon diversity index
n_i = number of the ith isolate
N = number of total isolates in the sample
S = numbers of isolates encountered
∑ = sum from isolate 1 to isolate S

Screening for traits related to plant growth promotion

Nitrogen fixation ability of isolates was tested on nitrogen-free Doberenier's medium (Döbereiner et al., 1972). About 7 µl of an overnight culture of isolates (16-18 h) was inoculated (in triplicate) on nitrogen-free agar medium and incubated for two days at 30°C. The developed bacterial colonies changed medium color to yellow and were considered as nitrogen-fixing species (Bashan et al., 1993; Olivares et al., 1996).

To test the ability of all the bacterial isolates in dissolving phosphate, the researchers used National Botanical Research Institutes Phosphate - Bromophenol blue (NBRIP-

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BPB) medium (Nautiyal, 1999) containing (g/l): glucose (10), Ca_3 $(PO_4)_2$ (5), $MgCl_2{\cdot}6H2O$ (5), $MgSO_4{\cdot}7HO$ (0.25), KCl (0.2) and $(NH4)_2SO_4$ (0.1), BPB (0.025), agar (15). About 10µl of the overnight culture of isolates, containing10^7 cells mL^{-1} was inoculated on NBRIP-BPB medium and was then incubated for 3 days at 30°C. The bacteria's ability to solubilize insoluble phosphates calculated via the formula of halo zone diameter/colony diameter (Sarkar et al., 2012). To carry out the quantitative estimation of phosphate solubilization by isolates, Erlenmeyer flasks (50 ml) were used. They contained 10 ml of NBRIP broth medium inoculated with 1% (v/v) of an overnight culture of each isolates containing around 10^8CFU/ml. The researchers utilized the autoclaved uninoculated NBRIP medium as a control. The flasks were incubated for 5 days at 30°C on a shaker at 150 rpm. After 5 days, the pH of the medium was measured and the supernatants were collected by centrifugation for 15 min at 8000 rpm. In order to estimate the available phosphorus content of harvested supernatant, the vanado-molybdate colorimetric method was used in which the absorbance was measured at a wavelength of 420 nm. Finally, a standard curve prepared from KH_2PO_4 was used to determine the phosphorus content of samples (Inui-Kishi et al., 2012). The ability of isolates in producing auxin was tested on LBT medium (Luria-Bertani tryptophan containing: 0.5% glucose, 0.5% yeast extract, 0.5% NaCl, 1% tryptone, 0.05% tryptophan and 2% agar). The bacterial isolates were streaked on LBT solid media. They were then covered with filter papers and were incubated at 30°C for 18-24 h. Subsequently, filter papers were removed and a few drops of (Salkowski reagent) were added to them. The isolates were found to produce auxin when the color of papers turned into pink (Bric et al., 1991). The best isolates (with regard to their ability in nitrogen fixation, phosphate solubilization, and IAA synthesis) were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences. Bacterial DNA was extracted and purified following the instructions of the DNA extraction Kit (SinaClone). The quality of extracted DNA was determined by electrophoresis on 1% agarose gel. Polymerase chain reaction was carried out in a volume of 50 µl consisting of 1x PCR buffer, 2.5 U/µlTaq Polymerase (Fermentas), 0.2 mM of each dNTP, 2 mM magnesium chloride, 10 pM of each primer (Fermentas) and 10 ng DNA.

Primers FD1 (5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3') and reverse primer RP1 (5'CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT3') were used for PCR (Weisburg et al., 1991). The amplification was initiated at 94ºC for 5 min, followed by 30 cycles each containing denaturation (94°C, 1 min), annealing (58°C,40s), elongation (72°C, 150 s), and a final extension (72°C, 10 min). Target amplification was confirmed by electrophoresis in 1% (w/v) agarose containing DNA. Partial 16S rRNA gene of selective isolates was sequenced (Macrogen, South Korea). 16S rRNA sequence of the isolate was compared with that of other microorganisms via BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Based on the results of the database searches, sequences were aligned with representative bacterial sequences from the GenBank database by using ClustalX (Thompson et al., 1997). Distance matrices and phylogenetic trees were calculated by the Hesegawa-Kishino-Yano model and neighbor-joining (NJ) algorithms using the program MEGA (version 6) by bootstrap analysis of 1000 replications (Tamura et al., 2013). The partial 16S rDNA sequences of the representatives were deposited in GenBank.

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Plant growth and phosphorus uptake by wheat

Plant growth promotion was evaluated in wheat variety Chamran in a greenhouse, using soil collected from the research area of Agriculture Faculty of Shahid Chamran University of Ahvaz. The collected soil was air-dried and passed through 4mm sieve. The soil chemical analysis showed the following results: Electrical conductivity = 3.2 dS/m, pH 7.8, organic matter = 0.52%, available P = 8 (mg/kg), available K = 270 mg/kg and total nitrogen = 0.04% with clay loam soil texture. A greenhouse experiment consisted of a 2 × 3 factorial in complete randomized design with four replications was arranged. The factors included two levels of inoculation (B0 without inoculant and B1 with inoculant) and three levels of phosphorus fertilizer. The nitrogen fertilizer used 400 kg/ha urea. Phosphorus from the source of triple superphosphate applied at three levels of 100 kg/ha (P100), 50 kg/ha (P50) and without phosphorus application (P0). The soil was completely mixed and irrigated by distilled water to field capacity (70%). Seeds of wheat (*Triticum aestivum*) Chamran cultivar were surface sterilized in 10% sodium hypochlorite solution for 10 min, then rinsed with sterilized distilled water and planted at about 2cm soil depth. The seeds were planted in pots containing 4kg of steam sterilized clay loam soil. An overnight culture of the isolate was diluted to10^6CFU/mL and 1mL of inoculum (isolate S-49) was applied under each seed. During the experiment chlorophyll index by spad was measured. At the end of experiment plant height, dry weight of root and grain yield was measured. The digested grain samples analyzed using spectrophotometer at 470 nm for the quantification of phosphorus (Jaiswal, 2004) and then phosphorus uptake by grain was calculated. The data was analyzed statistically via SAS version9.1. Mean comparisons were done using Duncan test at 5%.

Results

A total of 55 endophytic bacteria were isolated from the sugarcane tissues (26 isolates from saline soil and 29 isolates from non-saline soil), from which 18.18% belonged to roots (8 isolates from saline soil and 2 isolates from non-saline soil), 38.18% to stems (7 isolates from saline soil and 14 isolates from non-saline soil) and 43.63% to leaves (11 isolates from saline soil and 13 isolates from non-saline soil). In comparing the Shannon diversity index value of bacterial endophytic communities at genus level based on the sampling soil salinity situation, diversity (Shannon's Index) and evenness of isolated bacteria from saline soil was H = 1.95 and E = 0.6, respectively, and those was recorded H = 1.58 and E = 0.47 for non-saline soil. The most commonly isolated bacteria were in the phylum Firmicutes, which included 15 isolates and20 isolates belong to saline and non-saline soil, respectively and constituted 57% of the total isolates in the tissues of plant grown in saline soil and 68% of the total isolates in the tissues of plant grown in non-saline soil. Genera in phylum Firmicutes included *Staphylococcus*, *Paenibacillus and Bacillus*. Other phyla identified as Actinobacteria included *Corynebacterium*, *Arthrobacter*, *Rhodococcus*, *Streptomyces*, *Rhodococcus*, *Arthrobacter* and another phyla Proteobacteria included *Enterobacter*, *Pseudomonas* and *Aureimonas*. We found that higher diversity of isolated bacteria from sugarcane roots grown in saline soil than non-saline soil that indicated high severity of salt stress increases diversity in the bacterial communities (*Table 1*).

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Table 1. *Number and Shannon diversity components of isolated bacteria from sugarcane in saline and non-saline soil*

Genus	Number		Pi		ln Pi	
	Saline soil	Non-saline soil	Saline soil	Non-saline soil	Saline soil	Non-saline soil
Isolated bacteria from sugarcane root						
Enterobacter	3	-	0.37	-	-0.99	-
Staphylococcus	3	-	0.37	-	-0.99	-
Corynebacterium	1	-	0.12	-	-2.48	-
Paenibacillus	1	-	0.12	-	-2.48	-
Arthrobacter	-	1	-	0.5	-	-0.69
Bacillus	-	1	-	0.5	-	-0.69
Isolated bacteria from sugarcane shoot						
Bacillus	3	7	0.43	0.5	-0.84	-0.69
Corynebacterium	3	-	0.43	-	-0.84	-
Pseudomonas	1	-	0.14	-	-1.96	-
Rhodococcus	-	2	-	0.14	-	-1.96
Enterobacter	-	1	-	0.071	-	-2.64
Staphylococcus	-	1	-	0.071	-	-2.64
Aureimonas	-	1	-	0.071	-	-2.64
Paenibacillus	-	1	-	0.071	-	-2.64
Streptomyces	-	1	-	0.071	-	-2.64
Isolated bacteria from sugarcane leaf						
Bacillus	6	9	0.54	0.69	-0.61	-0.37
Staphylococcus	2	1	0.18	0.07	-1.71	-2.65
Rhodococcus	1	2	0.09	0.15	-2.4	-1.89
Arthrobacter	1	1	0.09	0.07	-2.4	-2.65
Enterobacter	1	-	0.09	-	-2.4	-

Diversity of isolated bacteria from sugarcane shoots in saline soil were lower than non-saline soil, but those data from sugarcane leave were recorded higher in saline soil. From 55 isolated bacteria almost 21.81% of them were grown on N-free medium and changed the color of medium to yellow, which suggests that they were able to fix atmospheric nitrogen (*Tables 2, 3, 4*), while 41, 33 and 25% of the isolated bacteria from roots, stems, and leaves indicated nitrogen fixation ability, respectively. The isolates from the roots with the codes R-1, R-9, and R-10, which were identified as *Enterobacter*, *Paenibacillus* and *Enterobacter* respectively, had the ability to fix nitrogen. All the endophytic isolates were initially tested by an agar assay using NBRIP medium to observe their phosphate solubilizing activity. The screened bacteria could solubilize TCP on solid medium by creating clear halozones around the colony. In fact, they represented different degrees of solubilization, depending on the type of organism involved (*Tables 2, 3, 4*). A clear halo zone was formed around the colony by almost 7, 7 and 5 entophytic bacteria isolated from roots, stems, and leaves, respectively. Isolate S-49, which was taken from stems and was identified as *Pseudomonas*, exhibited the highest phosphate solubilization index on NBRIP plates which was followed by S-14 and B-13 isolates identified as *Bacillus*.

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Table 2. *The results of some growth-promoting assays of isolated bacteria from the root*

Strain code	Genus	Source	N-fixation	P-solubility index (SI)	Auxin production
R-1	*Enterobacter*	Saline soil	++[1]	0.125	+[4]
R-2	*Arthrobacter*	Non-saline soil	-[2]	-	-[5]
R-3	*Bacillus*	Non-saline soil	-	-	-
R-4	*Staphylococcus*	Saline soil	+[3]	0.285	-
R-5	*Corynebacterium*	Saline soil	-	0.2	-
R-6	*Staphylococcus*	Saline soil	-	-	-
R-7	*Enterobacter*	Saline soil	+	0.27	+
R-8	*Staphylococcus*	Saline soil	-	0.22	-
R-9	*Paenibacillus*	Saline soil	++	0.14	-
R-10	*Enterobacter*	Saline soil	++	0.2	+

[1]Growth of bacterium on N-free medium and change the colour of medium to yellow
[2]Lack of bacterium growth on N-free medium
[3]Poor growth of bacterium on N-free medium
[4]Auxin production ability by changing the paper colour to pink
[5]No colour change of paper

Table 3. *The results of some growth-promoting assays of isolated bacteria from the stem*

Strain code	Genus	Source	N-fixation	P-solubility index (SI)	Auxin production
S-14	*Bacillus*	Non-saline soil	-	0.43	-
S-18	*Bacillus*	Saline soil	-	-	-
S-25	*Bacillus*	Saline soil	-	-	-
S-27	*Bacillus*	Non-saline soil	-	-	-
S-28	*Bacillus*	Non-saline soil	-	-	-
S-29	*Rhodococcus*	Non-saline soil	-	-	-
S-30	*Bacillus*	Saline soil	-	-	-
S-33	*Enterobacter*	Non-saline soil	+	0.12	-
S-36	*Corynebacterium*	Saline soil	-	-	-
S-38	*Bacillus*	Non-saline soil	-	-	-
S-40	*Staphylococcus*	Non-saline soil	-	-	-
S-41	*Aureimonas*	Non-saline soil	-	0.25	+
S-42	*Corynebacterium*	Saline soil	-	0.18	-
S-43	*Paenibacillus*	Non-saline soil	-	-	-
S-45	*Bacillus*	Non-saline soil	-	-	-
S-46	*Bacillus*	Non-saline soil	-	-	-
S-48	*Bacillus*	Non-saline soil	-	-	-
S-49	*Pseudomonas*	Saline soil	+	0.75	-
S-50	*Streptomyces*	Non-saline soil	+	0.21	-
S-54	*Rhodococcus*	Non-saline soil	-	0.33	-
S-59	*Corynebacterium*	Saline soil	+	-	-

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Table 4. *The results of some growth-promoting assays of isolated bacteria from leave*

Strain code	Genus	Source	N-fixation	P-solubility index (SI)	Auxin production
B-11	*Bacillus*	Non-saline soil	-	-	-
B-12	*Bacillus*	Saline soil	+	0.36	-
B-13	*Bacillus*	Non-saline soil	-	0.42	-
B-15	*Staphylococcus*	Saline soil	+	0.25	-
B-16	*Rhodococcus*	Non-saline soil	-	-	-
B-17	*Bacillus*	Saline soil	-	-	-
B-19	*Bacillus*	Saline soil	-	-	-
B-20	*Bacillus*	Non-saline soil	-	-	-
B-21	*Bacillus*	Non-saline soil	-	-	-
B-22	*Bacillus*	Non-saline soil	+	0.15	-
B-23	*Staphylococcus*	Saline soil	-	0.22	-
B-24	*Bacillus*	Saline soil	-	-	-
B-26	*Bacillus*	Non-saline soil	-	-	-
B-34	*Bacillus*	Non-saline soil	-	-	-
B-35	*Bacillus*	Saline soil	-	-	-
B-37	*Rhodococcus*	Saline soil	-	-	-
B-39	*Rhodococcus*	Non-saline soil	-	-	-
B-47	*Bacillus*	Saline soil	-	-	-
B-51	*Arthrobacter*	Saline soil	-	-	-
B-52	*Pseudomonas*	Saline soil	-	-	-
B-53	*Arthrobacter*	Non-saline soil	-	-	-
B-55	*Staphylococcus*	Non-saline soil	-	-	-
B-56	*Bacillus*	Non-saline soil	-	-	-
B-57	*Bacillus*	Non-saline soil	-	-	-

The ability of all isolates in solubilizing phosphate was further evaluated in NBRIP liquid broth medium. In both liquid broth and agar assays (*Table 5*) consistent results were obtained in regard with solubilizing phosphate from calcium phosphate. Various amounts of phosphate solubilization, from 4.35 to15.36 mg L^{-1}, were reported. Isolate S-49 showed maximum phosphate solubilizing activity in NBRIP medium with consistency result in agar assay which was followed by isolates S-14 and B-13. In this study, a decrease of pH was observed in the supernatant of the bacterial incubations.

According to the results of auxin production test in *Tables 2, 3* and *4* only three isolates from the root and one isolate from the stem showed auxin production ability by changing the paper color to pink. Isolate R-1 (*Enterobacter*) had a higher ability to produce auxin than the other strains. Phylogenetic tree of bacteria based on 16S rRNA genes are shown in *Figure 1*. Comparative analysis of 16S rRNA sequence could help to identify isolates 1(R-1), 2(R-4), 3(R-7), 4(R-9), 5(R-10), 6(S-41), 7(S-49), 8(S-50), 9(B-12), 10(B-13), 11(B-15) and 12(B-22) as *Enterobacter cloacae*, *Staphylococcus hominis*, *Enterobacter cloacae, Paenibacillus lactis, Enterobacter cloacae, Aureimonas altamirensis, Pseudomonas* sp., *Streptomyces roseofulvus, Bacillus endophyticus, Bacillus pumilus*, *Staphylococcus gallinarum* and *Bacillus pumilus,* respectively. The obtained 16S rRNA sequences were deposited in Gene Bank under accession No. KX262849, KX898441, KX262850, KX898442, KX262851, KX898446, KX262853,

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KX898447, KX898443, KX898444, KX898445 and KX262852 for each of the mentioned isolates.

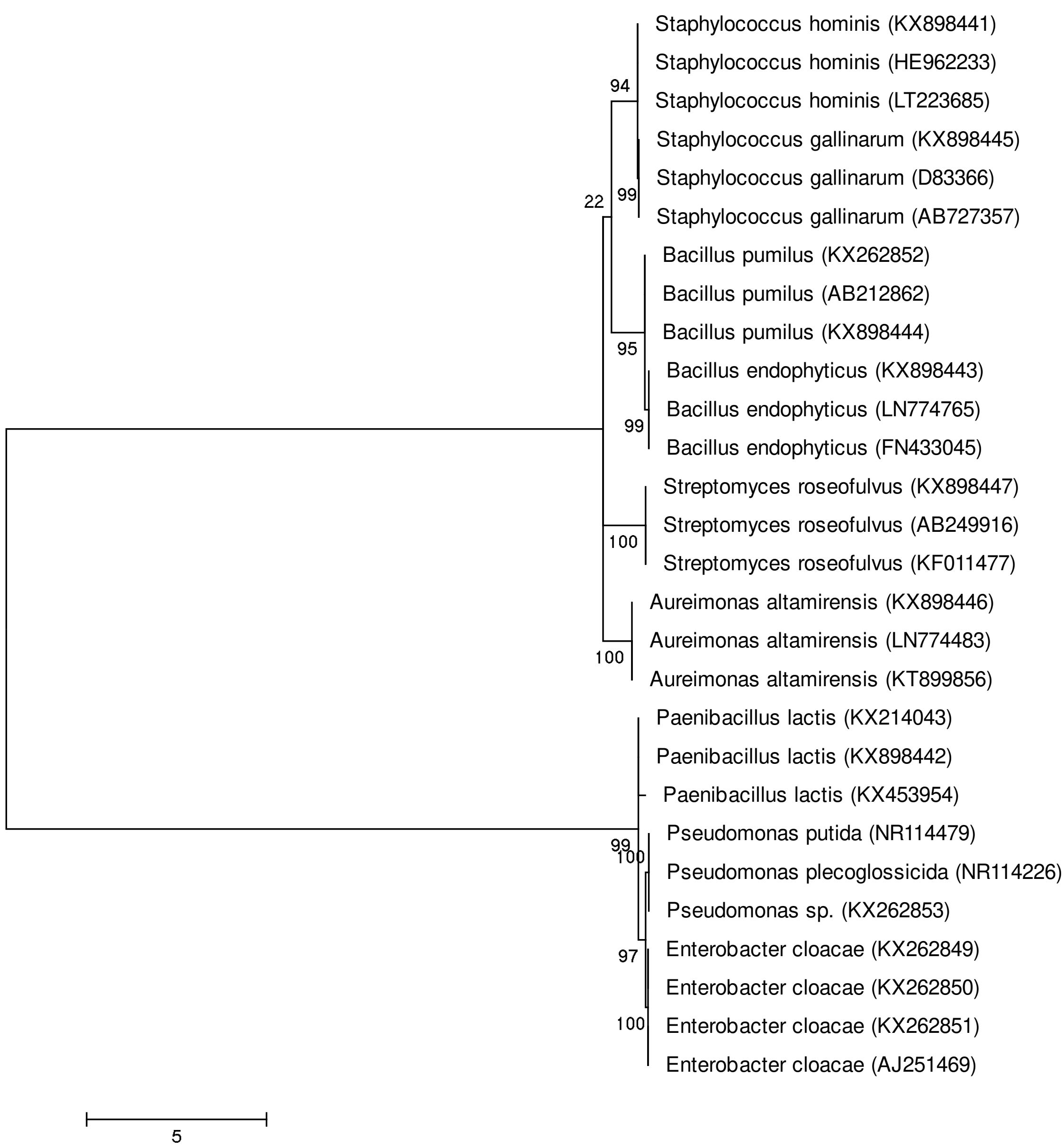

Figure 1. *Phylogenetic tree of selected isolates based on 16S rRNA sequences*

An overview of the effect of the selective isolate (*Pseudomonas* sp. sugS_49) according to insoluble phosphorus solubilization on some growth characteristic, grain yield and phosphorus uptake of wheat are presented in *Table 6*. The interaction effect of *Pseudomonas* sp. sugS_49 inoculation and phosphorus level was significant ($P \leq 0.05$) on chlorophyll index. The maximum chlorophyll index was obtained by application of *Pseudomonas* sp. and the second level of phosphorus fertilizer (P_{50}). Significant synergetic effect on the dry weight of root and spike and also grain phosphorus uptake of wheat ($P < 0.05$) was noticed due to combined application of phosphorus fertilizer and bacterial inoculation than phosphorus fertilizer application individually.

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Significantly greater amount of noticed properties was obtained at *Pseudomonas sp.* treated soils with 100 kg/ha of phosphorus application.

Table 5. *pH and phosphate solubilization in liquid media (Initial broth pH = 7.34)*

	Strain code	Genus	pH	P (mg l^{-1})
Control	-	-	6.82	0.95
Root	R-1	*Enterobacter*	5.85	10.03
	R-4	*Staphylococcus*	6.15	13.01
	R-5	*Corynebacterium*	4.44	10.12
	R-7	*Enterobacter*	5.8	10.32
	R-8	*Staphylococcus*	5.94	5.31
	R-9	*Paenibacillus*	6.12	5.21
	R-10	*Enterobacter*	5.98	11.96
Stem	S-14	*Bacillus*	5.08	11.66
	S-33	*Enterobacter*	5.2	9.33
	S-41	*Aureimonas*	5.02	10.57
	S-42	*Corynebacterium*	4.98	10.10
	S-49	*Pseudomonas*	4.02	15.36
	S-50	*Streptomyces*	5.5	9.31
	S-54	*Rhodococcus*	5.1	9.64
Leave	B-12	*Bacillus*	4.06	6.05
	B-13	*Bacillus*	4.75	9.64
	B-15	*Staphylococcus*	4.07	13.53
	B-22	*Bacillus*	4.55	4.35
	B-23	*Staphylococcus*	4.07	11.66

Table 6. *Mean comparison of treatments effect on some characteristics of wheat*

Treatment	Chlorophyll index (Spad)	Root dry weight (g/pot)	Spike dry weigh (g/pot)	Grain yield (g/pot)	Phosphorus uptake (mg/pot)
P_0 B_0	41.47^{e}	0.51^{e}	7.11^{d}	5.37^{c}	16.07^{d}
P_{50} B_0	42.62^{d}	0.57^{d}	7.57^{c}	5.7^{c}	19.56^{c}
P_{100} B_0	42.52^{d}	0.62^{c}	7.19^{d}	6.25^{b}	19.36^{c}
P_0 B_1	44.8^{b}	0.61^{c}	8.43^{b}	5.84^{c}	20.165^{c}
P_{50} B_1	45.7^{a}	0.69^{a}	9.08^{a}	6.67^{a}	28.653^{a}
P_{100} B_1	43.9^{c}	0.65^{b}	9.1^{a}	6.58^{a}	23.423^{b}

Means followed by the same letters are not significantly different based on Duncan at $\alpha = 5\%$

Discussion

Diversity and evenness of isolated endophytic bacteria from sugarcane in saline soil were higher than non-saline soil indicated the high severity of salt stress increases diversity in the bacterial communities. According to earlier research (Lamizadeh et al., 2016), Shannon index of isolated bacteria from rhizosphere of sugarcane in saline soil was also higher than that in the non-saline soil. Bacterial density in the stems and leaves of plant grown in non-saline soil was more than in the roots. This distribution pattern

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was against the results of Mendes et al. (2007) which reported more isolates in roots than in stems and leaves. *Bacillus* (almost half of the isolated bacteria) was the most frequently observed genus in the roots, stems, and leaves. The result of the effectiveness of the surface sterilization protocol was also determined. The rinsed water of each sample showed no microbial growth on nutrient agar medium after incubation, indicating that the epiphytic microbes were completely removed by this surface sterilization procedures. In the present research, nitrogen fixing bacteria were mostly isolated from sugarcane roots and leaves. Similarly, Hongrittipun et al (2104) isolated high numbers of nitrogen-fixing bacteria from roots and leaves of rice. Most researches on sugarcane endophytic bacteria have focused on diazotrophicus bacteria among which there are important representatives such as *Acetobacter diazotrophycus* and species like *Herbaspirillum* (Muthukumarasamy et al., 1999). In India, research on sugarcane endophytic bacteria showed that diazotrophicus bacteria had less population than the other bacteria (Suman et al., 2001). In the same way, in Brazil, research on isolation and identification of endophytic bacteria from sugarcane showed that from 32 isolates, 14 isolates were from Enterobacteriaceae (Magnani et al., 2010). The ability of *Enterobacter* to fix nitrogen was also reported by Tam and Deep (Tam and Diep, 2014).

Isolate S-49, which was taken from stems and was identified as *Pseudomonas*, exhibited the highest phosphate solubilization index on NBRIP plates. This index was followed by S-14 and B-13 isolates, respectively and both were identified as *Bacillus*. According to previous studies *Pseudomonas, Bacillus, Rhizobium, Micrococcus* and *Erwinia* are reported as phosphate solubilizer bacteria (Rodriguez and Fraga, 1999). *Pseudomonas* and *Bacillus* have been screened inorganic phosphate solubilizing isolates from *Pisumsativum* L. plants (Oteino et al., 2015). Among the reported phosphate solubilizing bacteria, *Bacillus* and *Pseudomonas* are the most important bacteria (Chauhan et al., 2013; Sharma et al., 2013). An inverse relationship between pH and phosphorus solubility has been observed in the present research that confirmed by other researchers (Chen et al., 2006). The decreased pH of the cultures indicates that phosphorus has been dissolved and released in the medium (Asis et al., 2000). The consumption of glucose from the growth media and the production of organic acids can explain the resulting decrease in pH. Results of auxin production by isolates are indicating the ability of Isolate R-1 (*Enterobacter*) to produce auxin than the other strains. Previous researches have shown that endophytic bacteria isolated from sugarcane also produce IAA (Chauhan et al., 2013; Tam and Diep, 2014). Most studies of sugarcane endophytes have focused on the diazotrophic bacteria (Olivares et al., 1996; Asis et al., 2000; Magnani et al., 2010). The *Enterobacter* genus was the most frequently found genus in the root. *Enterobacter* has been identified as endophytes of several plants such as *Zea mays* (Asis et al., 2000), soybean (Dalal and Kulkarni, 2013), and sugarcane (Tam and Diep, 2014). *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas putida* have been isolated from the stalk of sugarcane (Viswanathan et al., 2003). The *Bacilli* genera identified in sugarcane as endophytes: *Bacillus pumillus* was found to be associated with leaves. Chauhan et al. (2013) isolated *Pseudomonas* spp., *Bacillus* spp., *Azospirillum* spp. and *Gluconacetobacter* spp. from different varieties of sugarcane. The results reported here suggest that the population of sugarcane endophytes can vary depending on the plant organ analyzed. Further studies will be necessary to thoroughly analyze the endophytic population of sugarcane, including a collection of plants from different geographic origins and using the acetylene reduction assay to detect diazotrophicus bacteria.

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Application of *Pseudomonas* sp. SugS_49 alone and in combination with P fertilizer significantly increased growth, grain yield and phosphorus uptake of wheat. Similar results due to PGPR applications in wheat reported by Ponmurugan and Gopi (2006) and Afzal and Bano (2008). From this obtained results, it is clear that bio-fertilizer application was more effective to increase grain yield. The important finding was the measured properties in wheat under *Pseudomonas* sp. SugS_49 treatment without P fertilizer were almost more than those recorded in the second level of P, i.e. P_{50}. Nutrient uptake enhances by inoculated plants may attribute to the production of plant growth regulators by the bacteria at the root interface, which stimulated root development and resulted in better absorption of water and nutrients from the soil (Abbasi et al., 2011). Increasing the bioavailability of P and N in soils with inoculation of PGPR which may lead to increased their uptake and plant growth, was reported by many researchers (Zaidi et al., 2003; Wani et al., 2007).

Conclusion

The microbial communication in salt-influenced soils may have a biotechnological potential, which illustrated a reserve for future use in biotechnological applications expecting they could be utilized as a part or the like of restoration or protection methods of saline conditions, but they can serve as model frameworks for investigating the relationships between assorted variety and activity at the soil level in selective/restricting circumstances. On completing this investigation, we are inspired by the wide diversity of microorganisms introduce in saline soils. Although the presence of endophytic microorganisms in roots and stems of various plants has been accounted previously, there are no providing details regarding the occurrence of endophytic microbes inside sugarcane in Iran. The endophytic bacterial community related with sugarcane harbors numerous genera with potential for plant advancement. On the basis of 16SrRNA gene, the greater part of the chose endophytic isolates acquired from sugarcane plants to belong to the genus Bacillus. Results of this investigation exhibit the presence of diazotrophic endophytic and phosphate solubilizing microorganisms inside the roots and leaves of *Saccharum officinarum* CP48. The utilization of phosphorus solubilizing *Pseudomonas* sp. SugS_49. in wheat cultivation has given an important choice to upgrading its yield and preserving inherent characteristic of some strains to which require impressive regard for creating biofertilizer for sugarcane cultivation in Iran for the future.

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Conflict of Interest. The authors have declared that no competing interest exists.

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LIPOPHILIC AND HYDROPHILIC EXTRACTIVES FROM STRAWBERRY TREE (*Arbutus andrachne* L.) AND ORIENTAL PLANE (*Platanus orientalis* L.) WOOD

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Abstract. Extractives compose 5-10 % of wood by weight and they have important roles in trees. Extractive composition of different plant species has to be investigated in order to get the broadest sense. Thus, hexane and acetone:water (95:5, v:v) extracts of *Arbutus andrachne* and *Platanus orientalis* wood were analysed by GC-MS and GC-FID to determine lipophilic and hydrophilic extractive composition of these species. Total amount of lipophilic extractives was found 3,39 mg/g in *A. andrachne* wood, whereas it was determined as 16,91 mg/g in *P. orientalis* wood. However, total amount of hydrophilic extractives in *A. andrachne* had higher values. Fatty acids were detected as the dominant group in lipophilic extractives in both wood species. Acid 18:3 in *A. andrachne* wood (0,17 mg/g) and acid 16:0 in *P. orientalis* wood (0,19 mg/g) were determined as the highest fatty acids.
Keywords: *Arbutus andrachne, Platanus orientalis, fatty acids, phenolics, sugars*

Introduction

Plants consist of compounds with high molecular weight such as cellulose, hemicelluloses and lignin. They are essential for survival on organism and called primary metabolites. Primary metabolites compose 90-95 % of wood by weight. Absence or deficiency of primary metabolites can cause the death of organism. However, extractives are secondary metabolites of plants that affect such functions as texture, odor, taste and color of the tree. Extractives compose 5-10 % of wood chemical substances by weight and they can be classified according to their morphological site in the tree and the function. In addition, extractives can also be determined according to solubility in some solvents and polarity of those solvents (Fengel and Wegener, 1989; Sjöström, 1993; Holmbom, 1999).

In general, extractives can be divided into two sub-groups: lipophilics and hydrophilics. The lipophilic extractives, water-insoluble extractives, in another term, include the substances soluble in neutral solvents such as dichloromethane, diethyl ether and some hydrocarbon solvents. Lipophilic extractives are mainly composed of fats, fatty acids, sterols, and some chemical substances mostly found in softwoods like resin and resin acids (Holmbom, 1999). Hydrophilic extractives are substances that are soluble in such polar solvents as water, alcohol and acetone. Carbohydrates, phenols, lignans and chalkones are some of the hydrophilic extractives (Holmbom, 1999). There are many papers about plant extractives. In last years, studies have been focused on the wood and bark extractives of trees though (Cunha-Queda et al., 2007; Dönmez et al., 2012a; Ekman and Eckerman, 1985; Hafızoğlu, 1982; Hafızoğlu, 1989; Hafızoğlu and Holmbom, 1987; Balaban and Uçar, 2003; Pietarinen et al., 2006; Ferreira et al., 2015), recently, fruit, berry and cone extractives of the trees have been gaining interest (Kılıç et al., 2010a, 2010b, 2011; Dönmez et al., 2012b; Gönültaş and Uçar, 2013). As it is a

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renewable material and the chemicals found in it, tree has important utilization potential in every industrial area.

Analyses of a tree, especially extractives that have minor amount and some sugar compounds like monosaccharides, determines its possible use for other industries (Baptista et al., 2013; Feng et al., 2013). To date, it is known that, extractives can protect wood from decay, add color and odor to wood and enhance the strength properties of wood as well as acting for production of some medicinal products for human health. Thus different kinds of plant species and also different tissues of same species have to be taken into account for more research of chemical composition of trees.

Arbutus andrachne is one of the plant species in Turkey that it is not seen as stand but occurred as soliter along riversides. Moreover it is not utilized in any industrial area except burning its wood in bread producing factories. It is called "sandal" tree in Turkish, but it is officially named as strawberry tree due to its fruits in general. *A. andrachne* is an evergreen tree and has edible fruits that are consumed as jam, jelly and beverage. Fruits of *A. andrachne* have been investigated for their chemical composition because they have rich sugar content. Moreover, protein, ash content and antioxidant properties of *A. andrachne* fruits, extractive composition and suberin monomers of A. andrachne bark were previously presented (Alarcao-E-Silva et al., 2001; Şeker and Toplu, 2010; Serçe et al., 2010; Dönmez et al., 2016). However, any papers about chemical composition of wood of *A. andrachne* is out of our knowledge.

Platanus orientalis is a large deciduous tree and named as oriental plane. It is a natural tree species that is grown around the rivers and used in landscapes and parks (Khan et al., 2013). It is named as "çınar" in Turkish and known as a symbol of long and healthy life. Since it has been growing up in parks in city centers, phenolic composition of its leaves and buds and the effects of environmental stress have been studied but there is limited knowledge of the chemical composition of *P. orientalis*. Most studies on its chemical composition have focused on anticancer or antiseptic properties and activity against urinary infections (Dimas et al., 2000; El-Alfy et al., 2008; Mitrocotsa et al., 1993; Mitrocotsa et al., 1999).

It was aimed to determine the amount and the composition of lipophilic and hydrophilic extractives of *A. andrachne* and *P. orientalis* wood. Thus new data about extractive composition of *A. andrachne* and *P. orientalis* was presented to the literature.

Material and Method

A. andrachne and *P. orientalis* samples were taken from the altitude of 375 m. from Aşağıgökdere-Eğirdir, Isparta, Turkey. Diameters of the trees were 27 cm. and 29 cm. for *A. andrachne* and *P. orientalis* respectively. Samples were taken according to TAPPI-T-257 cm-02 standard. Debarked samples were stored at -24 °C until analyses. All of the samples were freeze-dried and grounded by Wiley mill to 1 mm (Ekman, 1983). To prevent the effect of volatile compounds, a second drying procedure was treated to the grounded samples.

Extraction

Approximately 5 g of grounded wood from each sample was sequentially extracted with an accelerated solvent extractor (ASE) apparatus (ASE 200, Dionex Inc., Sunnyvale, CA, USA). Samples were successively extracted with n-hexane,

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acetone:water (95:5, v:v), and finally distilled water. After extraction, aliquots of extractives were completed to 100 ml with the same solvent used in extraction so gravimetric analyses were carried out. For gravimetric analyses, 10 ml of the aliquot was evaporated to dryness, i.e, constant weight, leaving a film of extractives in the solvent container. The containers were weighed before and after extraction to determine the extractive yield in mg/g. An internal standard (heneicosanoic acid and betulinol) was added to the hexane and acetone:water (95:5, v:v) extracts. The mixture was evaporated under nitrogen prior to silylation. Hexane and acetone:water (95:5, v:v) extracts were injected to GC-MS and GC-FID for identifying lipophilics and hydrophilics.

Identification

Quantitative analyses of both extractives from the wood of A. andrachne and P. orientalis were performed with a Pelkin Elmer Autosystem XL gas chromatograph (GC-FID; PerkinElmer Inc., Waltham, MA, USA) equipped with an HP-1 25 m × 0.2 mm (0.11 µm film thickness) column and flame ionization detector. The carrier gas was H_2 added at 0.8 mL/min. The temperature program began at 120 °C, which was raised by steps of 6 °C/min to 320 °C. The injector temperature was 260 °C, and the FID temperature was 320 °C. One µL of each sample was injected (split ratio: 1:24). Individual compounds were identified with an HP 6890-5973 gas chromatography/mass spectrometry (GC-MS) instrument (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an HP-1 column. The temperature program was the same as above.

Results and Discussions

Lipophilic and hydrophilic components of *A. andrachne* and *P. orientalis* wood were analysed by GC-MS and GC-FID after extraction with different solvents. The amount of soluble substances in the solvents and water was determined gravimetrically and shown in *Figure 1*.

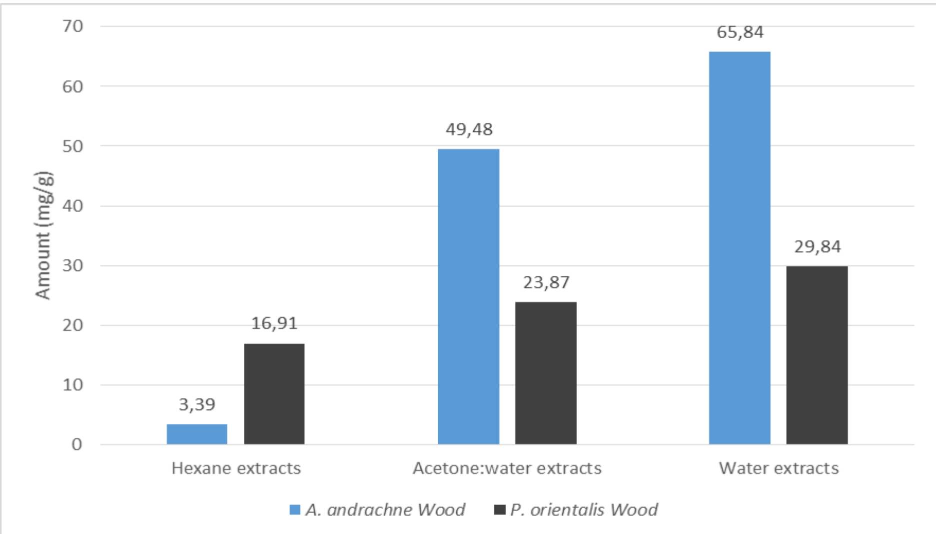

Figure 1. *Gravimetric amount of extractives from A. andrachne and P. orientalis wood (mg/g of dry weight)*

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Gravimetric analyses results of *A. andrachne* wood were found higher than *P. orientalis* wood results. It was also observed in our previous study that bark samples of both species had the same results for gravimetric analyses (Dönmez et al., 2016). Acetone:water (95:5, v:v) and water solubility in *A. andrachne* wood was seen two-times more comparing to *P. orientalis* wood. While acetone:water (95:5, v:v) was determined as 49,48 mg/g in *A. andrachne* wood, it was found as 23,87 mg/g in P. orientalis wood. Hot water solubility in *A. andrachne* wood was determined as 65,84 mg/g though, it was identified as 29,84 mg/g in *P. orientalis* wood. It is known that fruits and bark of *A. andrachne* have rich sugar content (Serçe et al., 2010; Şeker and Toplu, 2010; Dönmez et al., 2016). Moreover, this work shows that wood of A. andrachne is rich in its sugar content. Contrary to this results, only *P. orientalis* wood hexane solubility (16,91 mg/g) was found higher than A. andrachne wood hexane solubility (3,39 mg/g).

Hexane and acetone:water (95:5, v:v) extracts were firstly injected to GC-MS for qualitative identification, secondly to GC-FID for quantative determination just after suitable amounts of mixture of solvent and extracts was determined for chromatographic analyses. Extractive composition and the amount can be seen in *Table 1.*

Table 1. *Extractive composition of A. andrachne and P. orientalis wood (mg/g of dry weight)*

	A. andrachne	*P. orientalis*
Fatty Acids		
Acid 16:0	0.05	0.19
Acid 18:3	0.17	0.08
Acid 18:1	0.02	0.08
Acid 18:0	0.09	0.10
Acid 18:2	0.05	0.09
Sugars		
D-xylose	0.03	0.03
D-fructose	0.29	0.22
Sorbopyranose	1.15	0.46
α-d-glucopyranose	0.07	0.03
Pinitol + D-glucose	1.39	4.61
L-rhamnose	0.03	0.10
Mannose	0.02	0.04
Sucrose	0.48	0.04
Myo-inositol	0.14	0.08
Unidentified sugar compounds	4.36	1.91
Maltitol	0.02	0.04
Phenolics		
3,4-dihydroxybenzoic acid	0.01	0.03
Catechin	3.74	1.46
Phytosterol		
Sitosterol	0.17	0.15
Other		
Hydroquinone-beta d glucopyranoside	0.01	0.08
Betulinic acid	0.01	0.98
Sinapyl alcohol	0.08	0.08
Lupeol	0.02	0.01

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5 different fatty acids were detected in both samples. While acid 16:0 was determined as the highest amount in *P. orientalis* wood (0.19 mg/g), acid 18:3 was seen as the dominant fatty acid in *A. andrachne* wood (0.17 mg/g).

β-sitosterol was found as the only phytosterol in the sterols group that was the second lipophilic substances group after fatty acids detected in samples. It was found both in *A. andrachne* (0.17 mg/g) and in *P. orientalis* (0.15 mg/g) wood.

The compounds in sugar group, a hydrophilic group, was determined by injecting of acetone:water (95:5, v:v) extracts. Total amount of sugar group was found as 7,98 mg/g and 7,56 mg/g in *A. andrachne* and *P. orientalis* wood respectively. There are not any research on the literature about extractive structure of *A. andrachne* and *P. orientalis* wood. However, chemical composition of bark of same trees was reported by Dönmez et al. (2016). It was seen that total amount of sugar group in wood was lower than their bark.

Phenolics were the other hydrophilic group and only two components (3,4-dihydroxybenzoic acid and catechin) were detected in both samples. Catechin was seen as the dominant component and had the highest proportion in both *A. andrachne* (3,74 mg/g) and *P. orientalis* (1,46 mg/g) wood.

Besides, hydroquinone-β-d glucopyranoside, a glycoside, betulinic acid, a triterpenic acid, and sinapyl alcohol, an organic compound, were also determined in all samples.

Conclusions

Hexane, acetone:water (95:5, v:v) extracts of *A. andrachne* and *P. orientalis* after successive extraction were analysed by GC-MS and GC-FID to obtain lipophilic and hydrophilic extractives. Total amount of lipophilic extractives was lower (3,39 mg/g) in *A. andrachne* wood than *P. orientalis* wood (16,91 mg/g). However, hydrophilic extractives was found two-times more in *A. andrachne* wood (49,48 mg/g) than *P. orientalis* wood (23,87 mg/g). Fatty acids were the dominant lipophilic extractive group in both wood species. While acid 18:3 in *A. andrachne* wood was detected as the most abundant compound, acid 16:0 had the highest amount in *P. orientalis* wood. Sugars were seen as the main hydrophilic extractive group both in *A. andrachne* and in *P. orientalis* wood. It is seen that wood of the both species have same extractive composition with their bark but the amount of wood extractives is lower comparing to their bark (Dönmez et al., 2016).

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FOLIAR SPRAY OF NUTRIENTS AFFECTS FRUIT QUALITY, POLYGALACTURONIC ACID (PECTIN) CONTENT AND STORAGE LIFE OF PEACH FRUITS IN TURKEY

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Abstract. Fruit flesh softening is generally accompanied by changes in pectin structure in most fruits. In cell wall, pectin polysaccharides cross-linked with Ca^{2+}. Especially Calcium chloride application is environmentally friendly and is also very effective on fruit quality. An experiment was performed by foliar spraying of calcium, magnesium and manganese alone and in combination to assess their effects on fruit quality, storage life and the polygalacturonic acid (pectin) content in peach (*Prunus persica* cv. JH. Hale). Experiment was conducted in commercial peach orchard located in Çanakkale, Turkey. Treatments included 1.5% $CaCl_2$, 2% $MgSO_4$, 1% $MnSO_4$, combination (0.6% $CaCl_2$, 1% $MgSO_4$ + 0.1% $MnSO_4$), and control (water) applications. Spray applications were started 30 days after the full bloom, and continued at four-week intervals up to four weeks before the harvest. The amount of pectin in the peach fruits was determined by FT-IR spectroscopy. Fruit weight loss, firmness, color, titratable acidity, pH and soluble solids contents were determined. Foliar nutrient-treated trees showed improved resistance to compression and penetration, as well as a decrease in weight-loss during postharvest storage. A similar response was obtained from Mg treatments. The smallest weight loss occurred in calcium and manganese applications, which were 0.71 and 0.75%, respectively. Calcium and magnesium treatments had a positive effect on fruit flesh firmness. The highest polygalacturonic acid content was obtained with $CaCl_2$ treatment, which was 414% greater than that of control treatment. Therefore, $CaCl_2$ application can be safely used to increase pectin content as well as fruit quality of peach fruit.
Keywords: *pectin, polygalacturonic acid, FT-IR spectra, peach, calcium chloride, storage*

Introduction

Short shelf life in peach fruit after harvest is due to rapid ripening which results in a limitation for efficiency in handling and transportation. Climacteric fruit species practically undergoes softening after textural changes during postharvest storage (Harker et al., 1997). Physicochemical and biochemical changes affect fruit's final flesh firmness (Brummell et al., 2004). Consumer acceptability is mostly influenced by fruit texture, which is largely established by cell wall and middle lamella polysaccharides (Roeck et al., 2008).

Treatments such as wrapping, cold storage and high amount of CO_2, calcium compounds, aminoethoxyvinylglicyne and 1-MCP have been used to improve fruit quality (Sisler and Serek, 1997; Byers, 1997; Fan et al., 2002; Manganaris et al., 2007). Pre and postharvest treatments with calcium chloride has been proven to be safe and environmentally friendly (Karabulut et al., 2003). Additionally, application of foliar sprays is environmentally friendly fertilization method since the nutrients directly contact to the plant without little contamination to soil (Farrag et al., 2015). Integrated fruit production systems frequently utilize preharvest calcium sprays to improve fruit characteristics and to minimize fungicide applications towards the end of harvest by improving resistance to brown rot (Conway et al., 1994). Calcium deficiency is associated with many physiological disorders in fruits. Although calcium level can be

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increased with foliar sprays, in many cases, achieving it proves to be difficult due to its limited uptake and absorption by the fruit and transport into the fruit (Mengel, 2002).

In plant cells, pectin, a structural polysaccharide, is mostly present as protopectin. The backbone of pectin consists of, in part, 1-4 linked galacturonic acid residues. Quantitative and structural analyses of complex polysaccharides are required for accurate evaluation of the content of galacturonic acid (Gary, 2004). Cell wall strength is affected by calcium in the cell wall because it involves in producing cross-bridges and is considered to be the last barrier before cell degradation (Fry, 2004). Plant cell wall is stabilized by exogenous Ca applications and protected from cell wall degrading enzymes (White and Broadley, 2003). Marscher (1995) stated that stability of cell wall is increased with Ca ions by linking non-esterified pectins and that fruit senescence might be due to its applications. Beavides (2011) reported delayed softening in apple and pear fruits during storage period, following Ca applications before harvest.

Mg^{2+}, a bivalent cation like Ca^{2+}, might sustain cell wall and plasma membrane integrity through acting as a bonding agent between pectin substances in the cell wall (Farag and Nagy, 2012). Lester and Grusak (1999) reported that Ca and Mg treatments influenced weight loss and fruit firmness during storage. Weight loss was linked with fruit deterioration during postharvest handling (Gonzales-Aguilar, 2009). Crisosto et al. (1999) stipulated that Ca sprays may have result in skin discoloration in peach and nectarine.

Literature about the effects of manganese on fruit species are limited to their applications as foliar and/or soil supplement to determine effects on fruit quality in species such as peach and nectarine (Serrano et al., 2004) and orange (Labanauskas et al., 1963). However, these studies did not include its possible effects on fruit quality during storage.

Mansoor et al. (2001) reported that FTIR can be used as an alternative method for determining pectin content in commercial pectin samples and in pectin extract. Since FTIR requires no reagent, rapid, cost and time effective application of FTIR method for fruits could be useful.

The objective of this study was to rapid determination of different foliar nutrient applications ($CaCl_2$, $MgSO_4$, and $MnSO_4$) on pectin content and fruit quality of peach (*Prunus persica* L.) by using FT-IR spectroscopy.

Materials and Methods

Plant material and location

Field experiments were conducted on a commercial orchard located in Lapseki-Çanakkale, Turkey (40° 19.8′ N, 26° 43.8′ E, around 47 m above sea level). The orchard soil is sandy loam and was classified as Typic Xerofluvents according to Soil taxonomy.

The average annual rainfall is about 616 mm and the mean temperature is about 15°C (Anonymous, 2017). Ten-year-old peach trees (*Prunus persica* cv. J.H. Hale) growing under a drip irrigation system, were selected for study. Trees were managed according to commercial practices for fertilization, cultivation, irrigation, pest and weed control, and hand thinning of fruits was also performed.

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Treatments

Eight suitably distant sub-plots (5 trees each) were selected for applications. Trees received each of the following different leaf treatments: 1.5% $CaCl_2$ (75 g/5 L), 2% $MgSO_4$ (100 g/5 L), 1% $MnSO_4$ (5 g/5 L), combination (0.6% $CaCl_2$, 1% $MgSO_4$ + 0.1% $MnSO_4$), and control (water (5 L) was sprayed) (Alcaraz-Lopez et al., 2003 ; Farag and Nagy, 2012).Four leaf applications at 4 week intervals were performed as follows: 1st application on April 26th, 30 day after the full blooming; 2nd application on May 22nd; 3rd application on June 20th, and 4th application on July 18th, 2012. Applications were ended 4 weeks before the harvest. Fruits were harvested at firm-ripe stage depending on the skin ground color as maturity index. After the elimination of defective fruits, all treatments were placed in 5 kg commercial plastic containers and then placed in a storage room at 0°C and 90% relative humidity for 30 days. The fruits were analyzed at 10 day intervals during cold storage. Three replicates were utilized for each trial, with 30 fruits per replicate.

Weight loss (%) was determined using a 0.001 g precision balance (Precisa XB 220A) at 10 days intervals. Weight loss was calculated as: (Wi-Wf) / Wi x 100, where Wi was the initial sample weight and Wf was the final sample weight. The results were expressed as percentage weight loss. Peach tissue firmness was measured with a penetrometer (Chatillon, model DFS-500, USA), after removing skin, using a flat-head stainless-steel cylindrical probe with a 8 mm diameter. Penetration depth of the probe into the flesh of peach fruit was approximately 3 mm (the flesh thickness of the samples ranged between 3.5 and 5.5 mm for cv. J. H. Hale). Maximum force (N) and MT slope (N mm^{-1}, ratio of force to the deformation until the maximum force) extracted from the force/deformation were considered to be measures of fruit firmness.

The color (hue angle) was measured using a Chroma meter (Minolta CR-400, Japan). The color of each fruit was measured in terms of the L*, a* and b* coordinates, and from these values, the hue angle was calculated as $h^{\circ} = \arctan(b/a)$ (Abbott, 1999).

For the purpose of determination of titratable acidity (TA), pH and soluble solids content (SSC), samples were taken from each treatment and pulped by using a blender. SSC (°Brix) was determined by a digital refractometer (Kyoto Electronics Manufacturing Co. Ltd., Japan, and Model RA-250HE) at 22°C. TA was determined by means of titration with 0.1 N NaOH until pH reached 8.1, expressing the results in of g malic acid/100 ml. The pH was determined by potentiometric measurement at 22ºC with a pH meter (WTW, Germany). All measurements were done at the beginning of the trial and every other 10 days for a 30 day storage period.

Determination of galacturonic acid content

The infrared absorption spectra were obtained from a Perkin Elmer BX II spectrometer in KBr discs and were reported in cm^{-1} units. Spectra were collected by co-adding 32 scans at a resolution of 0.5 cm^{-1} in 4000-400 cm^{-1} range. Standards for polygalacturonic acid were purchased as KBr from Sigma-Aldrich (Steinheim, Germany). Polygalacturonic acid (PGA) calibration standards were prepared by mixing polygalacturonic acid with potassium bromide to cover a range of acid concentrations (10-98%). High-purity water from a Millipore Simplicity 185 water purification system (Millipore Iberian S.A., Madrid, Spain) was used for all chemical analyses and glassware washing. A set of 10 calibration polygalacturonic acid standards was prepared by blending polygalacturonic acid with KBr to obtain acid standards with polygalacturonic acid content of 10, 20, 30,

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40, 50, 60, 70, 80, and 98%, respectively. Pectin (PGA) was extracted by the method of Kratchanova et al. (2004) from peach samples.

The experiment was factorial and completely randomized design with 3 replicates for each treatment. The data was evaluated by using SAS® (1990) statistical package program and groupings were done using Duncan's multiple range test, at 5% importance level.

Results

Table 1 shows the weight loss during a 30 day-cold storage period at 0°C. The smallest weight loss occurred in calcium and manganese applications (0.71 and 0.75%, respectively), whereas highest weight losses were obtained from the magnesium, combination, and control fruits (1.00, 0.91and 0.89%) respectively.

Table 1. *Effects of preharvest foliar applications on weight loss (WL%), fruit flesh firmness (N) of peach (cv. J. H. Hale) fruits during the cold storage conditions (0^0C, 30 days).*

Parameters	Treatment/days	0	10	20	30
WL (%)	Control	0^{NS}	1.25a*	0.98ab	1.34ab
	Calcium	0	0.76bc	0.85b	1.22b
	Magnesium	0	1.28a	1.15a	1.55a
	Manganese	0	0.69c	0.86b	1.42ab
	Combination	0	0.96b	1.17a	1.51a
	Mean	0	0.99	1.00	1.41
Flesh Firmness (N)	Control	55.00c	34.43b	20.00b	6.64^{NS}
	Calcium	65.00a	40.38a	32.00a	7.53
	Magnesium	64.67a	31.75b	25.04b	7.23
	Manganese	61.67ab	29.76b	22.03b	6.39
	Combination	58.33bc	29.33b	21.03b	6.24
	Mean	60.93A	33.13B	24.02C	6.81D

*Means within columns with the same small letter are not significantly different at the $p < 0.05$ level. NS: Non-significant.

Significant reduction in fruit flesh firmness was detected during the cold storage period for all treatments (*Table 1*). However calcium and magnesium treated fruits had less reduction in fruit flesh firmness compared to other treatments. At harvest, applications of calcium (65 N) and magnesium (64.16 N) greatly improved fruit flesh firmness. On the other hand, the rest of the treatments, including the control, provided the lowest firmness in the fruits. The reduction was significantly greater in the combination, manganese and control fruits compared to those in the calcium and magnesium treatments.

An interaction effect was found on L* color parameter (*Table 2*). This effect was observed only in the Ca treated fruits at the 30th day of the storage period. L value reached 68 at the end of the storage period (*Table 2*). Ca positively affected the brightness of the fruits and enabled them to be brightest at the end of the storage. Hue*, on the other hand, was influenced by the treatments but prolonged time of storage caused a significant decrease at the end of the storage. Hue* values were between 112.50 and 115.30 but the differences were not statistically significant (*Table 2*).

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***Table 2.** Effects of preharvest foliar applications on L* and Hue* angle of peach (cv. J. H. Hale) fruits during the cold storage conditions (0^oC, 30 days).*

Parameters	Treatment/days	0	10	20	30
L*	Control	63.17a*	62.02a	61.58a	63.60b
	Calcium	62.06b	66.50a	79.92a	68.47a
	Magnesium	64.16a	62.90a	63.06a	63.23b
	Manganese	65.72a	63.26a	60.85a	66.27b
	Combination	63.33a	62.10a	60.81a	62.60b
	Mean	63.69	63.36	65.24	63.83
Hue*	Control	116.36^{NS}	116.56^{NS}	115.81^{NS}	114.77^{NS}
	Calcium	114.90	115.01	115.04	113.90
	Magnesium	116.09	115.87	115.46	115.31
	Manganese	116.15	114.99	118.92	115.15
	Combination	115.21	115.29	115.43	112.50
	Mean	115.74A	115.54A	116.1A	113.58B

*Means within columns with the same small letter are not significantly different at the $p < 0.05$ level. NS: Non-significant.

SSC and pH of the fruits were dependent on both applications and storage period (*Table 3*). SSC showed an increase and decrease trend during storage period. It seems that nutrient treatments caused a delay in accumulation of sugar components until 20th day of the storage, after which all treatments had similar ratios of SSC. Differences among the treatments showed that no specific treatment sustained its SSC throughout the storing period. Interestingly, the gradual increase was more prominent in the $CaCl_2$ and combination treated fruits compared to the control ones. Calcium treated fruits contained the highest pH (4.11) throughout the storing time, followed by the control fruits (3.90) up to the 20th day but later it drastically stayed the lowest (3.92) compared to the rest of the applications. Titratable acidity in the fruits was affected by the treatments. The highest titratable acidity was obtained from the magnesium (0.825%) followed by calcium (0.758%), control (0.728%), combination (0.698%) and manganese (0.655%) treatments (*Table 3*).

***Table 3.** Effects of preharvest foliar applications on SSC(%), pH and TA of peach (cv. J. H. Hale) fruits during the cold storage conditions (0^oC, 30 days).*

Parameters	Treatment	0	10	20	30
SSC (%)	Control	13.13bc*	13.75a	13.00b	13.00cd
	Calcium	13.25bc	13.50b	13.60a	13.75a
	Magnesium	13.50a	13.58b	13.50a	13.50b
	Manganese	13.50a	12.75c	12.75c	12.90d
	Combination	13.00d	12.25d	13.00b	13.10c
	Mean	13.28	13.17	13.17	13.25
pH	Control	3.78b	3.89a	3.90b	3.92d
	Calcium	3.87a	3.87a	4.00a	4.11a
	Magnesium	3.56d	3.78c	3.80d	3.98c
	Manganese	3.74c	3.82b	3.85c	3.95cd
	Combination	3.74c	3.79bc	3.84c	4.05b
	Mean	3.74	3.83	3.88	4.00

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TA (g L^{-1} malic acid)	Control	0.805^{NS}	0.750^{NS}	0.751^{NS}	0.607^{NS}
	Calcium	0.645	0.850	0.790	0.748
	Magnesium	0.860	0.830	0.813	0.798
	Manganese	0.720	0.650	0.630	0.620
	Combination	0.720	0.701	0.690	0.682
	Mean	0.750A	0.756A	0.735A	0.691B

*Means within columns with the same small letter are not significantly different at the $p < 0.05$ level. NS: Non significant.

FT-IR spectra and total carbonyl absorption peak area at 1740-1635 cm^{-1}, from free COO- and esterified COO-R groups of polygalacturonic acid samples, were obtained using the method described for polygalacturonic acid standards (*Table 4*). A linear relationship between polygalacturonic acid content and carbonyl absorption band area was found ($R^2=0.982$).

Table 4. *The carbonyl absorption area of the polygalacturonic acid (PGE) standards*

Polygalacturonic acid (%)	**FTIR carbonyl peak area**
10	22,87
20	29,97
30	40,43
40	47,52
50	55,57
60	63,17
70	69,59
80	78,41
90	81,66
98	83,23

Polygalacturonic acid contents of peach fruits were calculated from the linear fit equation (*Table 5*, *Fig. 1*). The calculated polygalacturonic acid (PGA) content of 17 peach samples were; 87.67, 83.79, 78.23, 74.42, 70.45, 65.94, 58.78, 53.58, 52.56, 46.67, 45.23, 43.36, 21.13, 20.18, 19.14, 15.78, 15.75 %, respectively . Polygalacturonic acid content was affected by $CaCl_2$, $MgSO_4$, $MnSO_4$ and $CaCl_2$-$MgSO_4$-$MnSO_4$ mixture applications and subsequently increased an average of 414% with $CaCl_2$, 340% with $MgSO_4$, 302% with $MnSO_4$ and 262% with $CaCl_2$-$MgSO_4$-$MnSO_4$ compared with the control (*Fig. 2*, *Table 5*).

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Table 5. *The calculated polygalacturonic acid (PGE) content of the peach samples.*

Peach samples[a]	Polygalacturonic acid (%)
1	87,67
2	83,79
3	78,23
4	74,42
5	70,45
6	65,94
7	58,78
8	53,58
9	52,56
10	46,67
11	45,23
12	43,36
13	21,13
14	20,18
15	19,14
16	15,78
17	15,75

[a] Peach trees were sprayed with containing 1.5% $CaCl_2$, 2% $MgSO_4$, 1% $MnSO_4$ and 0.6% $CaCl_2$-1% $MgSO_4$-0.1% $MnSO_4$. Day 0: 1 (Ca^{2+}), 2 (Mg^{2+}), 3 (Mn^{2+}), 4 (Combination), Day 10: 5 (Ca^{2+}), 6 (Mg^{2+}), 7 (Mn^{2+}), 8 (Combination), Day 20: 9 (Ca^{2+}), 10 (Mg^{2+}), 11 (Mn^{2+}), 12 (Combination), Day 30: 13 (Ca^{2+}), 14 (Mg^{2+}), 15 (Mn^{2+}), 16 (Combination), 17 (Control, Chemical-free).

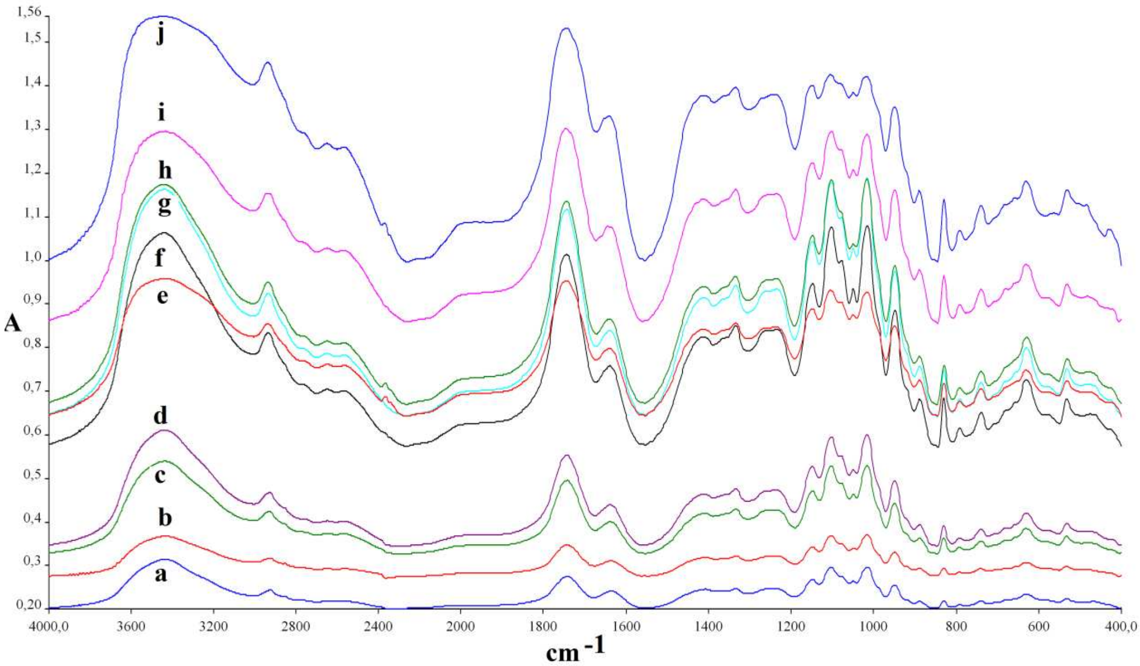

Figure 1. *FTIR spectra of the 4000-400 cm^{-1} region of polygalacturonic acid standards diluted with KBr : (a) 10, (b) 20, (c) 30, (d) 40, (e) 50, (f) 60, (g) 70, (h) 80, (i) 90, (j) 98%.*

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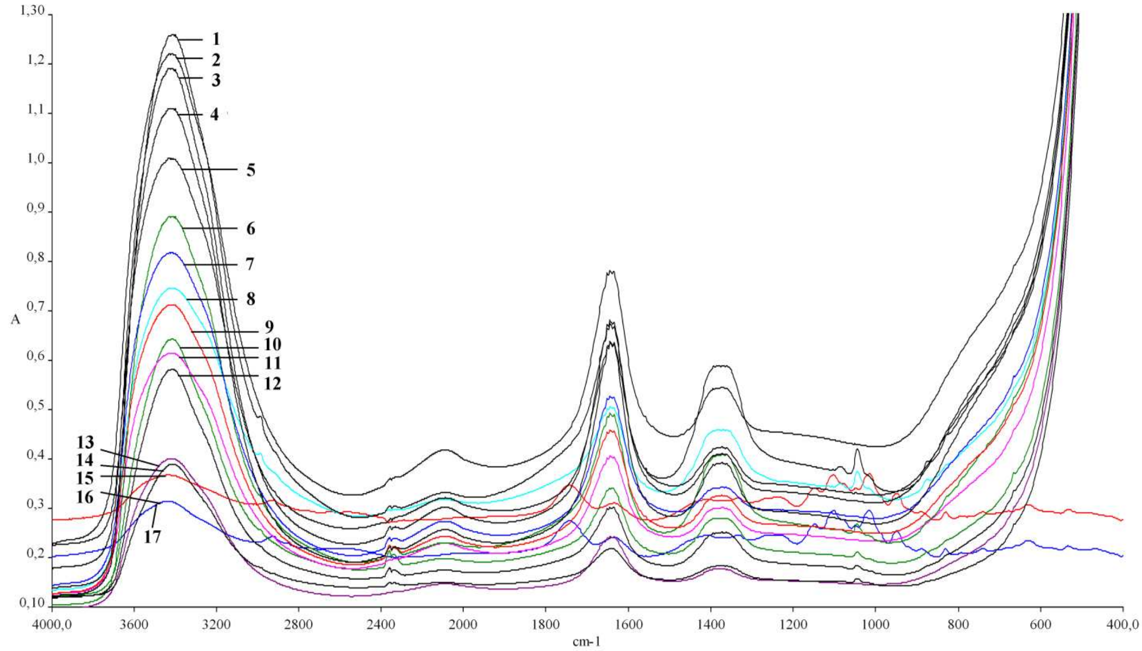

***Figure 2.** FTIR spectra of the peach samples sprayed with 1.5% $CaCl_2$, 2% $MgSO_4$, 1% $MnSO_4$ and 0.6% $CaCl_2$-1% $MgSO_4$-0.1% $MnSO_4$. Day 0: 1 (Ca^{2+}), 2 (Mg^{2+}), 3 (Mn^{2+}), 4 (Combination), Day 10: 5 (Ca^{2+}), 6 (Mg^{2+}), 7 (Mn^{2+}), 8 (Combination), Day 20: 9 (Ca^{2+}), 10 (Mg^{2+}), 11 (Mn^{2+}), 12 (Combination), Day 30: 13 (Ca^{2+}), 14 (Mg^{2+}), 15 (Mn^{2+}), 16 (Combination), 17 (Control, Chemical-free).*

Discussion

Effects of the treatments on the commercial quality of cv. J. H. Hale fruits are discussed with specific focus on improving their mechanical properties. Parameters related to fruit quality were evaluated starting from harvest at ten day intervals up to 30^{th} day under storage. Weight loss is a consequence of fruit dehydration and leads to loss of quality and associated fruit deterioration during postharvest handling (Gonzales-Aguilar et al., 2009). In this research, calcium and manganese treatments reduced weight loss compared to other treatments. Treatments might have delayed fruit senescence, therefore limiting the water loss. Calcium was reported to retard ripening (Liu et al., 2009) and protect cell membrane integrity (Guimarães et al., 2011).

The increased water loss in Mg, combination and control fruits might have affected cell turgor and be related to the lower firmness detected. According to Marschner (1995), calcium ions increase the stability of cell walls by binding non-sterified pectins even though plant cell walls are permeable to water. In melons, fruit firmness was affected by Ca and Ca-Mg applications (Lester and Grusak, 1999). Preharvest calcium treatment of trees was found to be useful in delaying pear and apple softening during the storage (Benavides et al., 2001).

Ca applications increased the L value of peaches . Crisosto et al. (1999) stated that Ca spray formulations may contribute to peach and nectarine skin discoloration depending on application rates. Ground color of the skin in these genotypes is closely associated with the ripeness of the fruit in general and the flesh firmness in particular (Kader, 1999). Kao et al. (2012) reported a decrease in H* value with progressing of

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flesh softening throughout the ripening. In our research, the lowest Hue* value was obtained from the combination treatments, however, they were not statistically different.

$CaCl_2$ and combination treatments increased SSC contents during the storage in this study. Similarly, Manganaris et al. (2007) reported that effects of both 62.5 mM and 187.5 mM $CaCl_2$ applications increased SSC at the end of the storage period. Values for the pH also reacted the same, being lower at the harvest and gradually increasing towards the end of the storage.

Foliar applications of mineral nutrient elements had significant effects on the biochemical and physical properties of the fruits. FTIR spectroscopy analysis showed that the structure of pectin extracted from fruit is similar to the commercial pectin spectrum. For the analysis of pectin, the absorbance peaks at 1000- 1600 cm^{-1} was focused. Polygalacturonic acid content was affected by $CaCl_2$, $MgSO_4$, $MnSO_4$ and combination applications and subsequently increased an average of 414-262% compared with the control. It also decreased during the cold storage. Ekinci and Yildiz (2015) determined PGA contents of sweet cherry fruits using FTIR and showed that pre-harvest foliar Ca applications after bloom resulted in highest PGA content at harvest. Monsoor et al. (2001) reported the usefulness of FTIR method to determine pectin content of commercial samples.

The reduced weight losses in the treated fruits could be due to maintaining the cell membrane integrity. Lester and Grusak (1999) observed positive effects of Ca and Ca-Mg treatments for weight loss in melons during storage.

TA losses during the peach storage show that fruit quality is decreasing. TA losses in Ca and Mg applications are less than in other applications. However, these values were not statistically significant. Manganaris et al. (2007) reported that all storage time reduced TA of peach fruits in all treatments.

In this research it was shown that polygalacturonic acid content changed during the storage. At the harvest, the fruits contained the highest content during which the storage time it gradually diminished. In consistent with our results, Mignani (1995) showed that polygalacturonase in tomato pericarp was reduced by calcium.

Conclusion

Preharvest foliar nutrient applications were important for peach fruit quality. Especially 1.5% $CaCl_2$ sprays provided an increase in pectin (PGA) content of fruits at harvest. Calcium applications increased tissue firmness and pH at harvest. It maintained tissue firmness, pH, and TA during the cold storage. Additionally, Ca application decreased weight loss of fruit. Fruit color was preserved during the storage. The smallest weight loss occurred in 1.5% $CaCl_2$ and 1% $MnSO_4$ applications.

$MgSO_4$ (2%) application helped to keep fruit flesh firmness and TA value but it was not as effective as Ca application. Combination treatments had greater concentration than other treatments and this caused a deformation of fruit epidermis, leading to an increase in both weight loss and fruit color. Lower concentrations of $CaCl_2$ needs to be studied for future studies. A linear relationship between polygalacturonic acid content and carbonyl absorption band area was found. Using FTIR method to determine PGA contents of fruits is suggested since FTIR requires no reagent, and is rapid, cost and time effective compared to standard chemical methods.

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CO_2 EMISSION AND GWP OF ENERGY CONSUMPTION IN THE COTTON PRODUCTION IN GOLESTAN PROVINCE OF IRAN

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Abstract. Careful choosing of appropriate methods in farming operations reduces fuel consumption, energy, and Greenhouse Gas (GHG) emissions in agricultural production. The present study was conducted to investigate the input and output energies and GHG emissions in cotton production in Golestan Province of Iran and the cities of Ali Abad Katool and Aqqala. For this purpose, agricultural operations were randomly selected from 100 farms for the years 2014 and 2015 and the related data on all farms were recorded. Various inputs and comprehensive information at every phase from planting to harvesting were collected, recorded, and processed and then extracted from multiple sources for each equivalence operation during different crop operations for cotton production using a conversion ratio of energy and GHG emissions. Afterward, the energy and GHG emissions for each input and operation were calculated. According to the obtained results, the mean power output was 154 GJ per ha, which is approximately six times of the average input energy (26 GJ/ha). The output and input energies for cotton production were 49 to 243 and 15 to 43 GJ/ha, respectively. Moreover, Global Warming Potential (GWP) of various activities in the cotton fields varied between 741 up to 7790 kg CO_2 equivalent per ha. The maximum GHG emissions were related to fertilizers with manure and fuel ranked in the next orders. The comparison between input energy and GWP revealed their direct relation in cotton production farms. Irrigation, feeding, and preparation operations had the highest fuel consumption and led to an increase in GHG release. Based on these results, it can be concluded that energy consumption and GHG emissions can be reduced by lowering fuel consumption and using chemical fertilizers.
Keywords*: cotton, GHG emissions, GWP, input energy, output energy*

Introduction

Many factors, whether natural or human-made affect the climatic changes. The sunlight hitting the earth and the reflection of infrared rays from the earth create a balance in the earth's atmospheric system that varies from place to place due to the environmental factors. One of the factors influencing climate change is the excessive absorption of infrared rays by greenhouse gases. Agriculture has led to the production of greenhouse harmful gases (Johnson et al., 2007). Greenhouse gas (GHG) emission reduction is possible by minimizing fossil fuels burning and applying effective strategies to reduce global warming (Tzilivakis et al., 2005a). The reduction in fuel consumption is also important for the production of sustainable agricultural products, in order to return economic optimization and the preservation of fossil fuel reserves (Pervanchon et al., 2002; Rathke and Diepenbrock, 2006).

Cotton (*Gossypium hirsutum* L.) is one of the most important and valuable agricultural products that are planted in more than 100 countries and plays a crucial role in economies of some countries in Asia and Africa. This product has been found to be

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of great economic importance and a particular agricultural and commercial status in the world, to an extent that it is called as the “white gold” (Marashi and Vaghif, 1981).

Energy as an input is of particular significance in the agricultural sector. According to the latest statistics, nearly 49% of the registered industrial projects are related to energy (renewable and renewable resources) and 24% to waste disposal. Estimates also suggest that agricultural activities contribute to the emission of 15% of global pollutants worldwide (Monthly Clean Development Mechanism, 2009). Today, due to population growth, reduction in arable land and improvement of the living standards, energy consumption has increased in agricultural sectors. Presently, the intensive uses of chemical fertilizers, pesticides, agriculture machinery, electricity, and natural resources are required to supply the food of the growing population (Barut et al., 2011). After transportation, the agricultural sector is the largest consumer of gasoline in Iran (Hydrocarbon balance sheet, 2011).

Rajabi et al. (2013) investigated GHG emissions and Global Warming Potential (GWP) in six wheat fields in Gorgan and reported that the average GWP production was 662 kg equivalent to CO_2 per ha in total farms. Furthermore, the highest and lowest amounts of GWP production were reported 923 and 268 kg, respectively, equivalent to 0.9 and 0.3 tons CO_2 per ha. These values showed that there is a direct relationship between GWP values and consumption of the crops inputs (input energy). In this regard, chemical fertilizers (especially nitrogen) and fossil fuels with 45.8% and 22.5%, respectively, have the highest share in energy consumption. Moreover, the largest share of the GHG emissions and GWP were 56.8% and 36.8% for the chemical fertilizers and fossil fuels, respectively. In addition, it was found that the maximum and minimum GWPs in terms of weight were 44.6 and 34.8 kg equivalent to CO_2 in GJ and in the output energy unit, respectively, as 11.7 and 4.5 kg equivalent to CO_2 in GJ. Soltani et al. (2013) showed that the highest value of energy is used to prepare a planting bed with 53%, irrigation with 15%, and harvest with 19% of total energy. Also, in the entire scenarios, more than 99% of GHG emissions was related to CO_2 and less than 1% was related to CH4 and N2O (Soltani et al., 2013). Ahmadi and Aghaalikhani (2013) investigated energy consumption in cotton cultivation in Golestan province of Iran and concluded that the share of energy consumed in cotton cultivation in Golestan province, the share of energy consumed in the fuel inputs of the tractor and engine fuel were 24% and 30%. In general, 54% of the energy consumed was related to diesel fuel, followed by fertilizers with 24% and chemicals with 13%. Furthermore, the total input energy for cotton production in the Alborz province was 31 GJ/ha. Dastan et al. (2014) examined the energy consumption of rice planting systems and CO_2 emissions and stated that the largest share of input energy in production systems was related to electric power for irrigation water pumps that had highest CO_2 emissions and global heating potential. After electric power, the nitrogen fertilizer and fuel had the second and third rank in CO_2 emission, respectively. Nikkhah et al. (2014) examined the GHG emissions in tea production in Golestan province and reported that chemical fertilizer inputs provide the largest share of GHG emissions. CO_2 as the most important GHG plays a significant role in absorbing infrared radiation produced in the atmosphere over the past decades. Feyzbakhsh and Soltani (2014) studied the energy flow and GWP in corn fields in Gorgan and reported that the lowest GWP was obtained from spring cultivation of 2349 kg equivalent to CO_2 per ha. Pathak and Wassmann (2007) considered GHG emissions and the GWP resulting from the conventional rice cultivation system in India and came to the conclusion that agronomic and non-agronomic operations (fertilizer and pesticide

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production) contribute to the GHG emissions as 80 to 98 and 16 to 91 kg equivalent to CO_2 per ha. The totals GWP of rice production was reported to be between 2766 and 4054 kg equivalent to CO_2 per ha. Meisterling et al. (2008) conducted a study on energy assessment and reduction of greenhouse gases in wheat production with two conventional and organic systems and estimated different effects in different stages of the growing season of the seed till transportation to the factory in two methods. They concluded that the GWP produced for the production of 1 kg of organic wheat bread is about 30 grams equivalent to CO_2 per ha.

Due to the increasing trend of energy consumption in Iranian agricultural sector, it is necessary to consider the current state of energy consumption in this sector. Although Golestan province (Iran) has been previously known as the cotton capital of Iran in the world, its planting rate has decreased notably due to the increased production costs. This study aims to evaluate GHG emissions and estimate GWP and to investigate the correlation between energy consumption and GHG emissions.

Materials and methods

Study area

This study was carried out in Golestan province, which is located in northern Iran between 36°30′ to 38° 80′ northern latitude and 53°51′ to 56°220′ eastern longitude (*Fig. 1*).

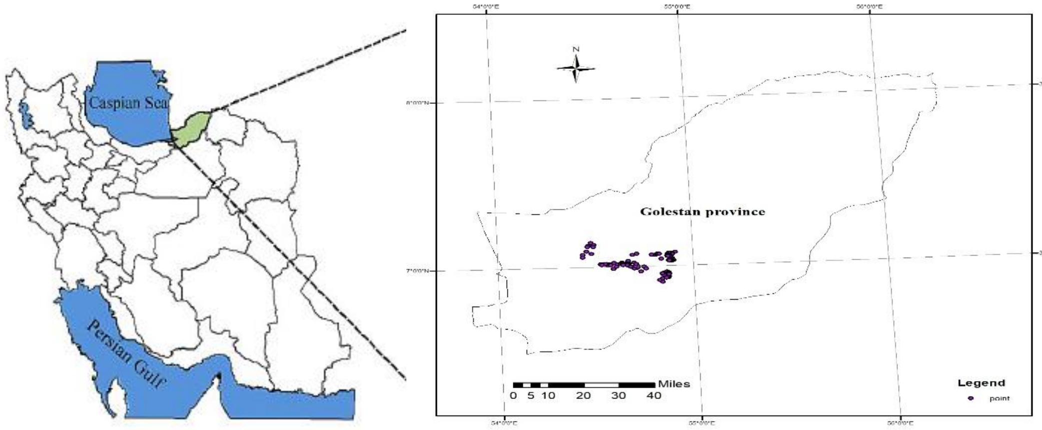

***Figure 1.** Location of the study region Golestan province within Iran (left) and the geographical distribution of the 100 Agricultural fields within Golestan province (right).*

The area of Golestan province is 20,438 km2, which accounts for 1.3% of Iran's total area (Bureau of Statistics and Information Technology, 2015). The province is bordered by Turkmenistan in the north, Mazandaran province and the Caspian Sea in the west, Semnan province in the south, and north Khorasan province in the east. Golestan is geographically characterized by the Alburz mountain range in the south and southeast and flat plain regions in the north and northwest. Accordingly, the climatic conditions range from humid temperate to semi-arid temperate climate. In average the study region's mean annual temperature is 18.1°C, the mean solar radiation is 14.2 MJ m^{-2} d^{-1}, and the total annual precipitation is 565 mm.

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The statistical population of this study consisted of the cotton farmers who provided the seeds from the service centers. Since data were collected during the crop year, farmers who were more likely to cooperate were introduced by the service centers (130 farmers). Accordingly, the number of fields was calculated using Cochran's formula as 96. In order to increase the accuracy, 100 farmers were randomly selected (*Eq. 1*).

$$n = \frac{\frac{Z^2 pq}{d^2}}{1+\frac{1}{N}(\frac{Z^2 pq}{d^2}-1)} \quad \text{(Eq. 1)}$$

where p and $q = 0.5$; $z = 1.96$; $d = 0.05$; N is the volume of statistical population, and n is the sample size.

A total of 100 samples were selected from Aliabad and Aq Qala cities in Golestan province of Iran during two crop years, 2014 and 2015. These farms were selected to include a range of farmers. All operations and events during the growing season were observed in these fields. Also, full details of the typical methods of production and agronomic operations in recent years (e.g., the use of machinery, fuel, fertilizer, and pesticides) were collected. For this purpose, all agricultural practices were first separated into 8 types including land preparation, planting, fertilization, plant protection, weed control, irrigation, harvesting, and transportation to the factory for delivery of the product. Next, different inputs values and more comprehensive information were collected and recorded at each stage from planting to harvest and their initial processing was conducted using Excel software. After that, data analysis was performed in three sectors including (consumed) input energy, (produced) output energy, and the GWP resulting from GHG emissions. The results of energy analysis are presented in *Table 1*. The main focus of the present study is on the GHG emissions and resulting climate change.

Table 1. *Input and output of energy used in the production of cotton*

Type of input and output	Unit (in ha)	Energy equivalent	Reference
Input			
Human labor	h	2.96	Ozkan et al, 2005
Cotton grains	kg	34	Ozkan et al, 2005
Machinery	kg	143.7	Kaltsas et al, 2008
Chemical fertilizer	kg	61.6	
(a) Nitrogen	kg	61.6	Akcaoz et al, 2010
(b) Phosphate	kg	7.7	Akcaoz et al, 2010
(c) Potassium	kg	12.1	Akcaoz et al, 2010
Animal manure	kg	1.3	Ozkan et al, 2005
Gasoline	L	39	Balance sheet hydrocarbon Iran, 2009
Electriciy	kwh	4.6	Pimental & Pimental, 2009
Herbicides	Kgai	279	Tzilivakis et al, 2006
Pesticides	Kgai	238	Tzilivakis et al, 2006
Fungicides	Kgai	100	Strapatsa et al, 2007
Output			
lint	kg	55.5	Ozkan et al, 2005

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(Consumed) input energy: At this stage, all direct (fuel, electricity, and human force) and indirect (seeds, chemical materials, chemical fertilizers, and machinery) inputs during various cropping operations for cotton production were extracted from multiple sources for each operation using energy conversion coefficients, and then, the energy input was calculated for each input and operation.

(Produced) output energy: At this stage, the amount of energy output from cottonseed was equated using the energy conversion coefficients extracted from cottonseed, followed by calculating the total amount of output energy. The energy conversion coefficient for cottonseed was estimated to be 54.5 (Ozkan et al., 2004).

Global Warming Potential (GWP): GWP is the sum of GHG emissions that are expressed as equivalent to CO_2 (IPCC, 1996; IPCC, 2007). To calculate GWP, the production of CO_2, N2O, and CH4 from energy consumption was considered in the production of various inputs and operations. These inputs and operations included the production of nitrogen, phosphorus, and potassium fertilizers, the production of chemical toxins of herbicide, fungicide, and insecticides, consumption of fossil fuels for agronomic operations, irrigation, transportation, production, and maintenance of agricultural machinery and equipment.

The GWP was calculated as follows

The equivalent of coefficients of production and GHG emissions was calculated in *Table 2* for each of the stages including the amount of energy of agricultural machinery, fuel (L), chemical fertilizers (kg), chemicals (kg), and manure (kg). Followed by calculating the total GWP, GWP values were calculate in terms of area (kg equivalent to CO_2 per ha), weight (kg equivalent to CO_2 per tons of wheat), input energy (kg equivalent to CO_2 per GJ), and output energy (Kg equivalent to CO_2 in GJ) (Rajabi et al., 2013).

Initial calculations and plotting some graphs were performed in Excel while plotting cumulative graphs and regression correlation was conducted by SPSS software.

Table 2. *GWP emission factors (kg co2eq unit-1)*

Input	Unit	GWP emission factors	Reference
Machinery	KJ	1.017	Dayer & Desjardins, 2007
Diesel fuel	L	3.76	Dayer & Desjardins, 2007
Nitrogen fertilizer	kg	2.3	Lal, 2005
Phosphate fertilizer	kg	1.2	Lal, 2005
Potassium fertilizer (K2O)	kg	1.2	Lal, 2005
Fungicides	kg	4.9	Lal, 2005
Pesticides	kg	6.1	Lal, 2005
Herbicides	kg	7.3	Lal, 2005
Animal manure	kg	1.126	Pishgar-Komleh et al, 2013; Xiamei & Koltelko, 2004

Results and discussion

Incoming and outgoing energy

According to the results, the average output energy was equal to 154 GJ/ha, which is about 6 times the average input energy with 26 GJ/ha (*Table 3*). The output energy

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range for producing cotton is between 49 GJ/ha to 243 GJ/ha and for input energy varied from 15 to 43 GJ/ha (*Fig. 2*). This amount was reported in another study conducted in this area as 31 GJ/ha (Ahmadi and Aghaalikhani, 2013). Total input energy for cotton production in the Alborz province was reported as 31 GJ/ha (Pishgar-Komleh et al., 2012, 2013) and in the provinces of Antalya and Hataa, Turkey, it was reported as 49 and 19 GJ, respectively (Dagistan et al., 2009; Yilmaz et al., 2005). Mousavi-Avval et al. (2011) reported the amount of input energy for soybean production in Golestan province 35 GJ/ha on average. Rajabi et al. (2013) studied the energy use for wheat production in Gorgan and reported that the average input energy in the understudy fields as 15577 MJ/ha.

Table 3. *The amounts of incoming and outgoing energy in MJ ha for cotton production in Golestan province*

Kind of energy	Average	Minimum	Maximum
Input Energy	26326.60499	15614.81698	43321.65855
Output Energy	154371.25	49050	234350

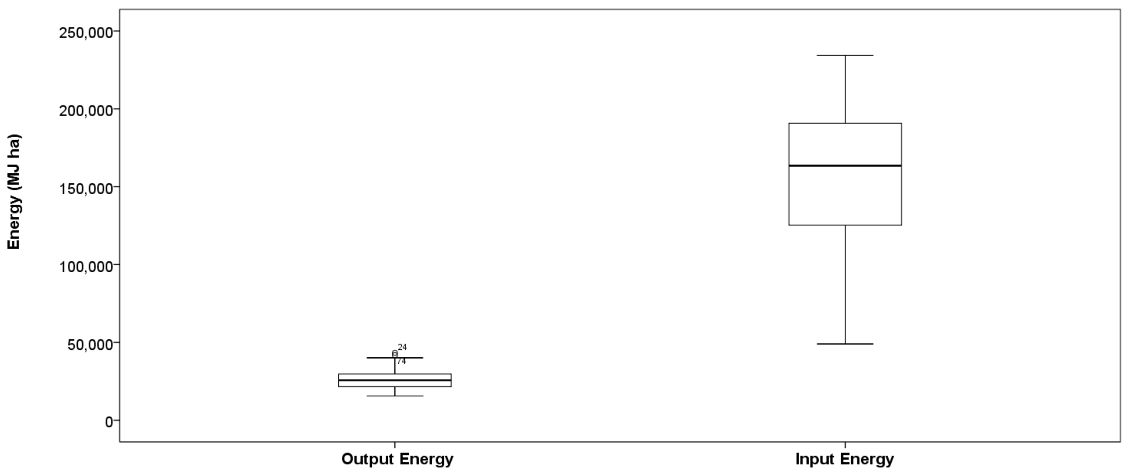

Figure 2. *Box plot of input and output energy for cotton production in Golestan province*

Beheshti-Tabar et al. (2010) examined the energy consumption in the agricultural sector from 1990 to 2006. Their results showed that the amount of input energy in 1990 increased from 32.4 GJ/ha to 37.2 GJ in 2007. The amount of output energy also increased from 30.85 GJ/ha to 43.68 GJ/ha. The increase in the use of chemical fertilizers, the field mechanization index and the consumption of agricultural toxins, which resulted in an increase in fertilizer use and high yielding cultivars, were considered as the reasons for increased use of input and output energies from 1990 to 2006. Haidari and Omid (2011) investigated energy consumption pattern for greenhouse cucumber and tomato production in Iran. The input energies for cucumbers and tomatoes were 141 and 131 GJ/ha, respectively. However, this amount was reported as 14 GJ/ha for input energy of the rapeseed in northern Iran (Azarpour, 2012). The main reasons for this difference might be the energy consumption of different products due to different conditions in cultivation, climate, and crop management in the production of each product.

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Greenhouse gas emissions

As presented in *Table 4,* the total GWP of different activities per cotton farm varied from 741 to 7790 kg equivalent to CO_2 per ha. In the next order, the highest GHG emissions were related to the chemical fertilizers and the amount of GHG emission in the inputs of manure and fuel (*Fig. 3*). Taheri-Rad et al. (2014) reported that GHG emissions from diesel fuel was 646.23 kg of CO_2 per ha that had 45% of GHG emissions of cotton production in Golestan province, followed by manure with 23% of GHG emissions. In this regard, Tzilivakis et al. (2005a) estimated the total GWP values per area for potato products, wheat, oilseed rape, barley, and chickpea as 3, 1.7, 1.2, 0.7, and 0.7 tons equivalent to CO_2 per ha.

Table 4. *The amounts of greenhouse gases in kilograms of carbon dioxide per hectare for cotton production inputs*

The type of operation	Average	Minimum	Maximum
Diesel fuel	983.00	586.22	1364.54
Fungicides	0.87	0.53	1.14
Chemical fertilizer	1019.15	9.20	6406.90
Machinery	144.59	58.22	300.08
Pesticides	23.20	0.00	58.65
Animal manure	946.26	0.00	6300.00
Herbicides	7.84	0.00	25.20
Total	2178.65	741.65	7790.81

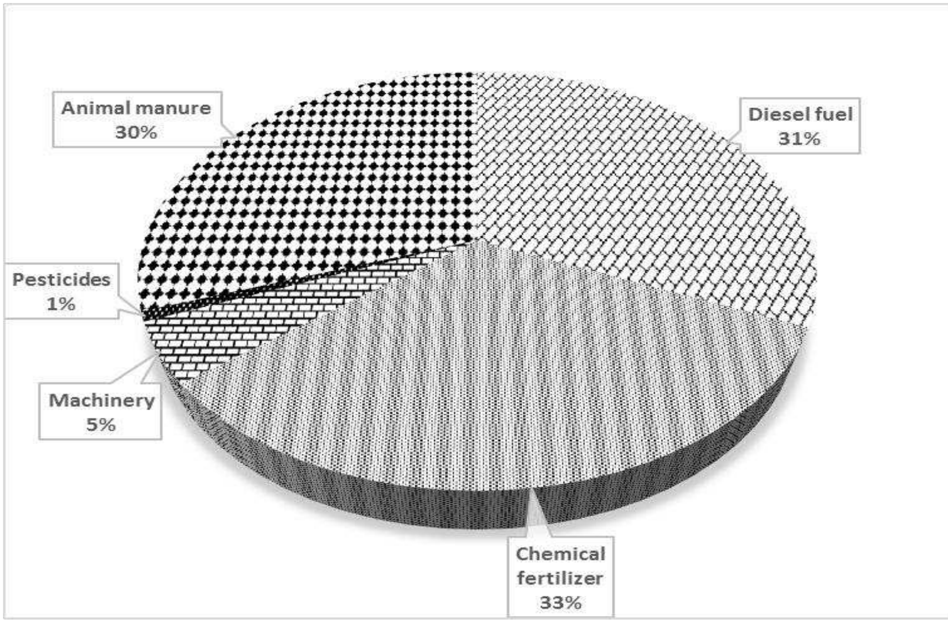

Figure 3. *The share of each crop inputs in terms of percent of total GWP*

The results of the present research showed that manure was not used in 60% of farms; hence, GHG emissions in these fields was zero (*Fig. 4*). The minimum amount of GHG emission with the least dispersion is related to the input of herbicides and insecticides (*Fig. 5*). Moreover, the comparison between the output energy and the GWP of the manure input showed that there was a direct and very significant relationship between the output energy in the cotton production and the GWP (*Table 5*).

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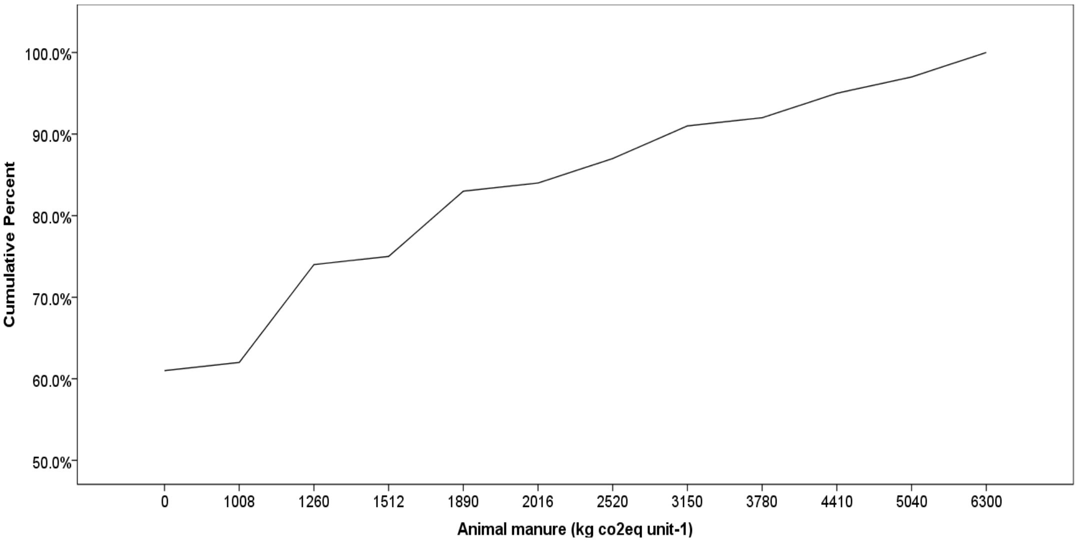

Figure 4. *Cumulative frequency graph of CO_2 produced from manure input on one ha of cotton fields*

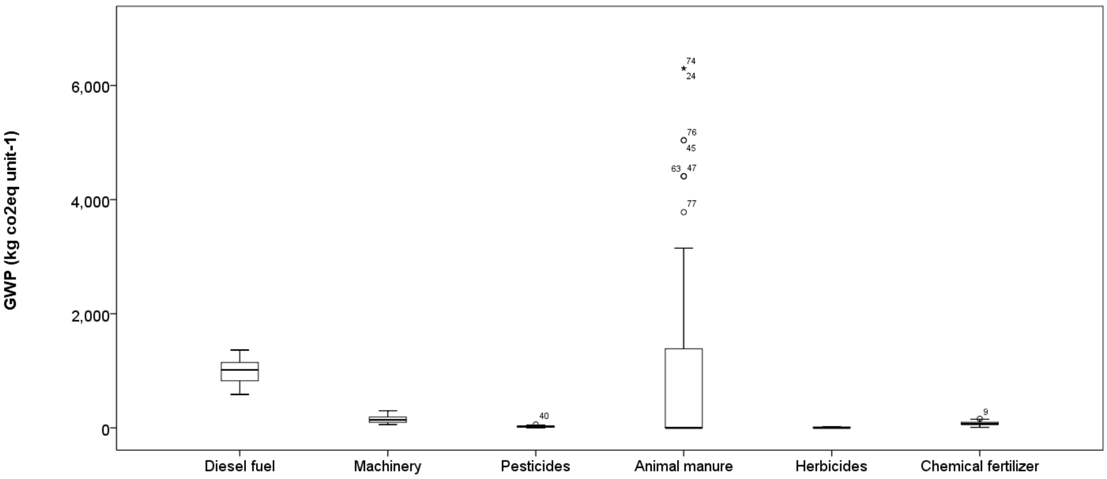

Figure 5. *Box plot of the GHG emissions resulting from the use of machinery, fuels, pesticides, manure, and herbicides in cotton production fields*

Table 5. *Pearson correlation coefficients between the emissions between field operations for cotton production*

The type of operation	Diesel fuel (kg co2eq unit-1)	Machinery (kg co2eq unit-1)	Pesticides (kg co2eq unit-1)	Animal manure (kg co2eq unit-1)	Herbicides (kg co2eq unit-1)	Chemical fertilizer (kg co2eq unit-1)	Energy input (MJ ha)	Yield	Total greenhouse gas emissions
Diesel fuel (kg co2eq unit-1)	1								
Machinery (kg co2eq unit-1)	.452**	1							
Pesticides (kg co2eq unit-1)	.367**	0.176	1						
Animal manure (kg co2eq unit-1)	1.050	.701**	-0.055	1					
Herbicides (kg co2eq unit-1)	-0.054	-0.095	-0.070	-0.064	1				
Chemical fertilizer (kg co2eq unit-1)	-0.142	-0.104	-0.082	-0.008	-0.163	1			
Energy input (MJ ha)	.560**	.827**	1.169	.798**	-0.108	0.177	1		
Yield	.384**	.531**	.238*	.408**	0.045	0.022	.593**	1	
Total greenhouse gas emissions	1.179	.762**	1.002	.990**	-0.078	-0.006	.868**	.457**	1

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

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The results showed that the highest rate of the greenhouse gases emissions was related to the chemical fertilizer input with an average of 1019 kg CO_2 per ha of global warming (*Fig. 4* and *Table 5*). The rate of GHG emission released from the chemical fertilizer in 20% of the farms under investigation was more than 1 ton per ha (*Fig. 6*). In addition, similar research results on other crops showed that the use of chemical fertilizers (especially nitrogen fertilizer) and fossil fuels have the highest impact on GHG emissions and GWP (Tzilivakis et al., 2005a, 2005b; Kaltsas et al., 2007; Lal, 2004). Rajabi et al. (2013) reported the amount of GHG emissions from nitrogen, phosphorus, and potassium fertilizers to produce wheat in Gorgan as 97, 67, and 64 kg, respectively, equivalent to CO_2-carbon per ha. Safa et al. (2011a) reported that GHG emissions from chemical fertilizers in wheat production in New Zealand were 52% and equal to 539 kg equivalent to CO_2 per ha, of which 48% was related to the nitrogen fertilizers.

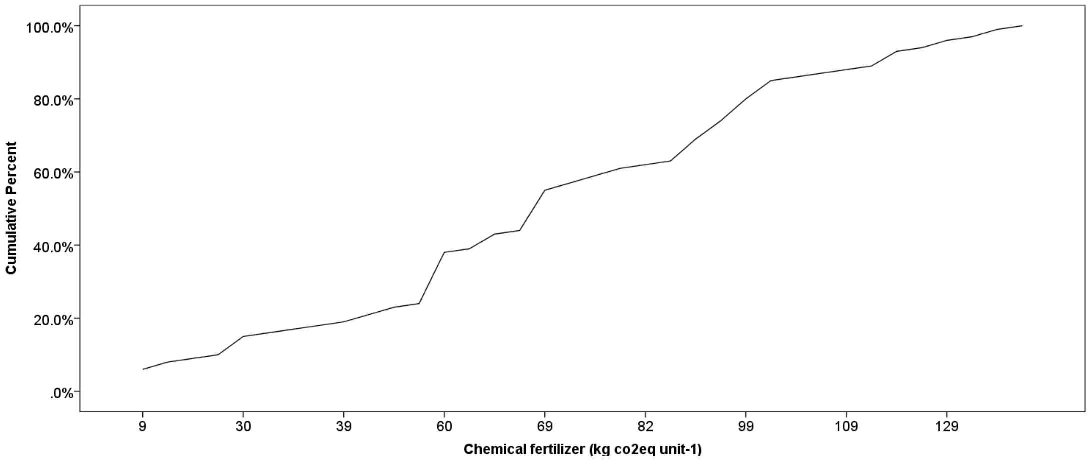

Figure 6. *The cumulative frequency graph of fertilizer (kg of CO_2 equivalent per ha) fields in cotton production*

In all fields studied in this work, the average GHG emissions from the consumption of fuel were 983 kg CO_2 per ha of global warming, of which 678 kg/ha was related to the irrigation, while tillage and spraying operations standing in the next ranks (*Fig. 7*).

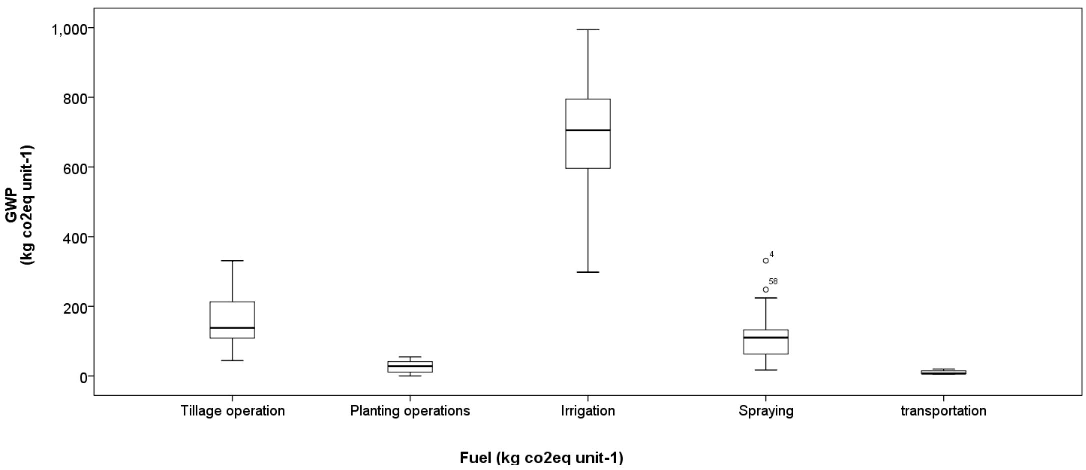

Figure 7. *Box plot of GHG emissions and fuel consumption of inputs in the production of cotton in the various crop operations*

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When using different irrigation methods, GHG emissions for the fuel use for this operation also changes in the farms.

In all fields studied in this work, GHG emissions in the use of machinery for irrigation operation had the highest rank as 49 kg of CO_2 dioxide per ha of global warming, while tillage, nutrition, and control of weeds and pests standing in the next ranks (*Fig. 8*).

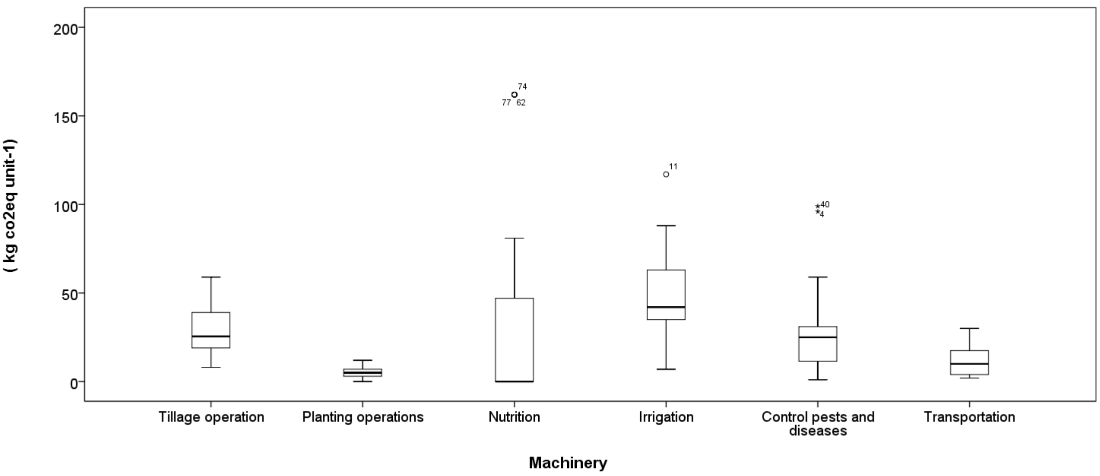

Figure 8. *Box plot of the production of GHG emissions from the use of machinery in the cotton fields*

Studying the CO_2 released by the pesticide input showed that the highest GHG emission is related to the Kauqueron and Lavrin pesticides (*Fig. 9*).

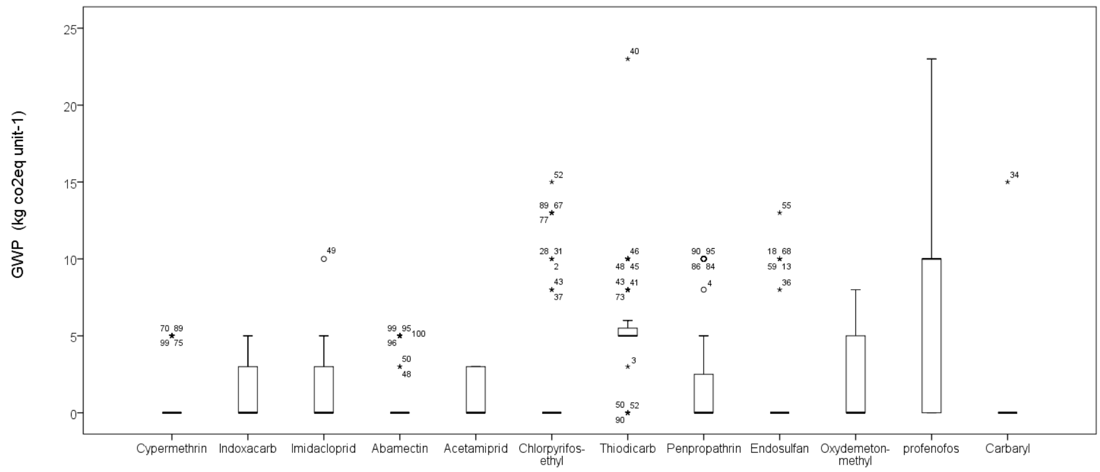

Figure 9. *Box plot of GHG emissions from inputs of pesticides in the cotton field*

The total average of the released gas was 23 kg CO_2 of the global warming. Safa et al. (2011a) estimated GHG emission as 55 kg, equivalent to CO_2 per ha. Some researchers reported that the use of natural methods of controlling pests and plant diseases could greatly reduce the use of agricultural pesticides, including. Among these natural methods are: increasing the resistance genes of the crops against the pests, diseases, and

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weeds, strengthening their natural enemies, using the correct crop rotation, combing the conservation tillage, and cultivating some forage plants and trees in the fields (Pimentel and Pimentel, 2008; Safa et al., 2011b; Kitani, 1999). The largest herbicide followed by the highest release of the gases released by Trafuralin herbicides is equivalent to 26.6 kg of CO_2 per ha of global warming (*Fig. 10*).

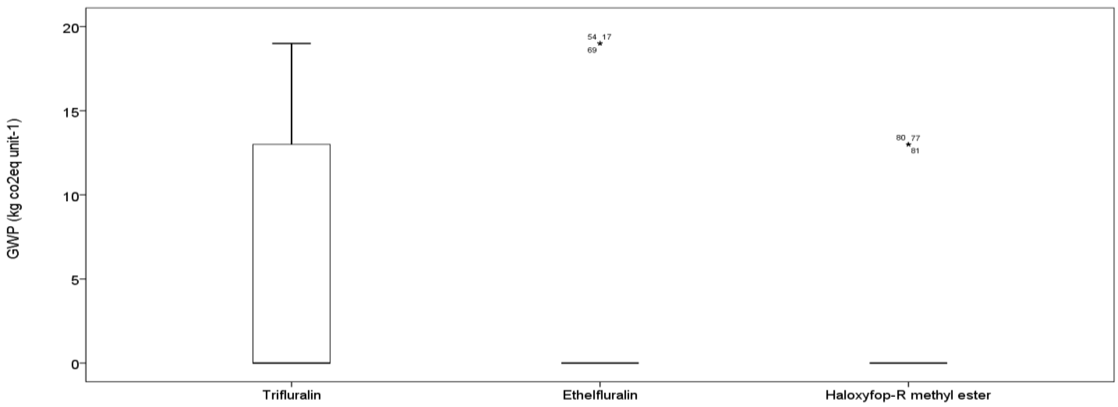

Figure 10. *Box plot of GHG emissions resulting from herbicide input in cotton fields*

The relationship between GWP and the amount of input energy production

The results of the comparison between the input energy and the GWP (*Fig. 11*) show that there is a direct and significant relationship between input energy in the cotton fields and the GWP; in other words, increasing energy consumption in the production of cotton will increase the emissions of greenhouse gases.

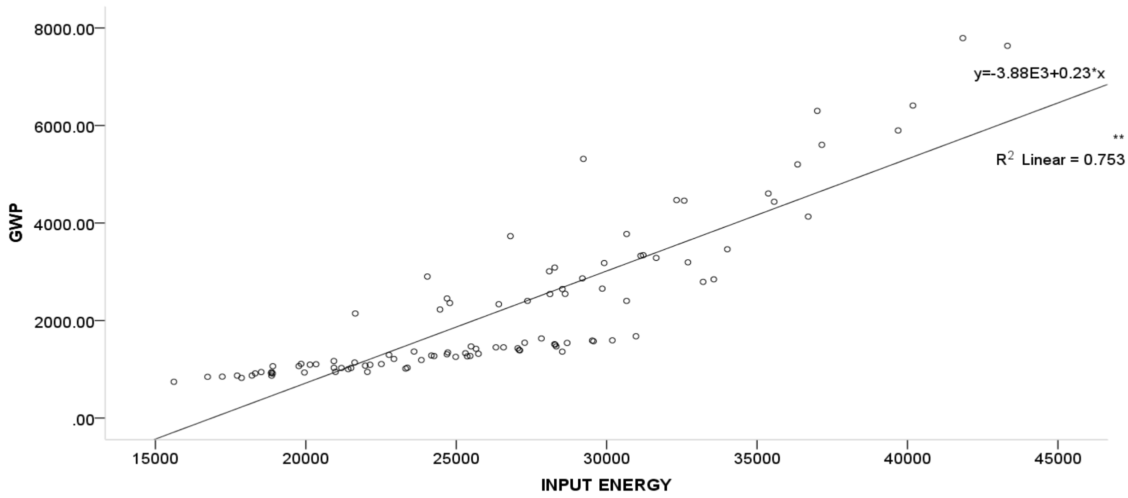

Figure 11. *The relationship between GWP and the amount of input energy production in cotton fields*

The concentration of the points around the line indicates that there is a complete and important relationship between input energy and GHG emissions. As shown in *Table 5,* the input energy with fuel gases emitted by fuel inputs, machinery, and manure is significant at 0.01%. Tzilivakis et al. (2005 b) also investigated GWP in beet production in England and stated that there is a direct relationship between the GWP values and

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amount of input energy in beet production. The findings of other studies were consistent with those of the present work in terms of assessing the input energy and the GWP obtained from it in various products (Kaltsas et al., 2007; Lal, 2004; Pathak and Wassmann, 2007). In this work, a direct relationship was found between the output energy and the input energy; in other words, the increase of input energy will increase the yield of the product and consequently the output energy and GHG emissions (*Table 5*). *Figure 12* shows a direct and significant relationship between the performance and the GHG emissions from cotton production. As the performance increases, the dispersion rate of the points in the line also increases. The high scattering of the points shown in *Figure 12* suggests that there is a high variation in energy consumption in high performances.

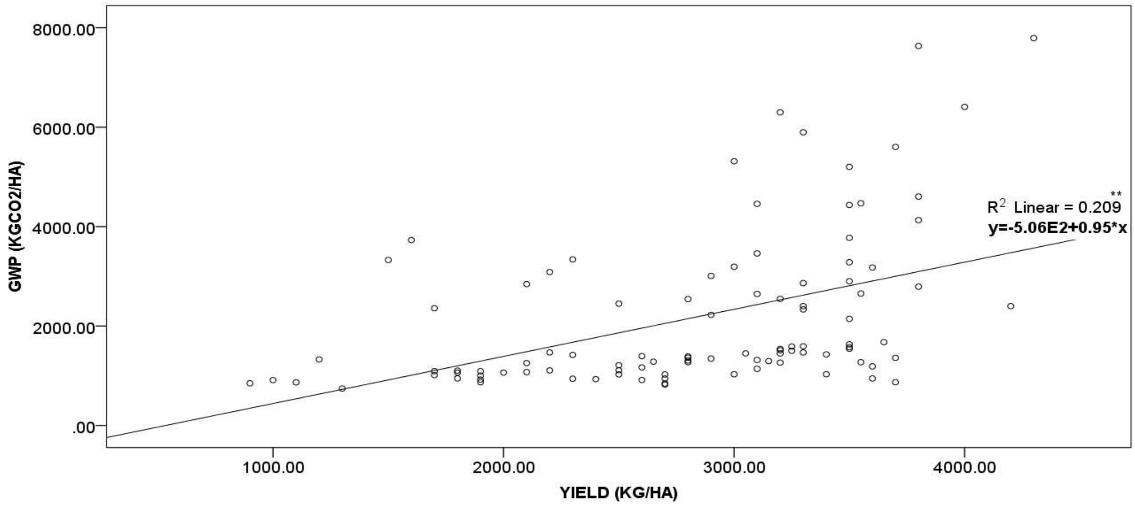

Figure 12. *The relationship between performance and GHG emissions in cotton production*

Global warming potential values for kg of CO_2 per unit area, weight, energy input and energy output

Table 6 presents the total GWP values in terms of area, weight, input energy, and output energy. GWP per weight represents CO_2 emissions per tons of product. Our results show that for 1 ton of cotton, 2178 kg of CO_2 per ton of cotton is produced. Gas emissions in terms of area, input energy, and output energy were estimated as 768 kg CO_2 per ha, 76 kg equivalent to CO_2 in GJ, and 14 kg equivalent to CO_2 in GJ, respectively.

Table 6. *Global warming potential values for kg of CO_2 per unit area, weight, energy input and energy output*

G W P	Unit (in ha)	Average	Minimum	Maximum
Global warming per unit weight	Kg CO2 equivalent per ton of cotton	2178.65	741.65	7790.81
Global warming per unit area	Kg CO2 equivalent per hectare	768.05	234.91	2331.44
Global Warming per unit energy input	Kg of CO2 equivalent in GJ	76.38	42.85	186.21
Global Warming energy per unit of output	Kg of CO2 equivalent in GJ	14.09	4.31	42.78

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Conclusion

In this research, we estimated the input and output energies of GHG emissions and the GWP due to agronomic activities in cotton fields in Golestan province. The results revealed that the average input and output energies are 26 and 154 GJ/ha, respectively, with an output energy being six times larger than the input energy. The average GHG emission for cotton production was estimated at 2181 kilograms of CO_2 per ha, with the highest value as 33% of the total GHG emissions for chemical fertilizer input, with fuel inputs and manure standing at the next ranks. Irrigation, nutrition, and preparation had the highest fuel consumption and resulted in the increased GHG emissions. Hence, it can be concluded that reducing fuel consumption and the use of chemical fertilizers reduced energy consumption and GHG emissions. Among the management methods to reduce fossil fuel consumption and chemical fertilizers that release greenhouse gases in agriculture we can name: protective tillage that reduces traffic in the field, which results in the lowered fuel consumption; using legumes in agriculture, which leads to the reduced nitrogen consumption; using the new irrigation methods and increasing water efficiency; using agronomic rotation and biological methods to control pests and weeds; using the nitrogen fertilizers based on soil test; adapting the time of fertilization with the needs of the plant; improving the fertilization methods such as placement in soil instead of manual propulsion and centrifugation; using the nitrification inhibitor compounds or coated fertilizers; and finally, using the green fertilizers. Further research are required with respect to above mentioned measures to decline GHG emissions.

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RELATIONSHIP BETWEEN OPERATIONAL CHARACTERISTICS OF SMALL NON-COMMUNITY DRINKING WATER SYSTEMS AND ADVERSE WATER QUALITY INCIDENTS IN SOUTHERN ONTARIO, CANADA

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Abstract. Ensuring that water sources are safe by protecting them from disease causing organisms is integral for the continued health of people as drinking contaminated water leads to waterborne diseases which can be life-threatening. The purpose of this study is to examine small non-community drinking water systems' (SDWSs) operational characteristics and their relationships with adverse water quality incidents (AWQIs) which is defined as presence of total coliforms and/or *Escherichia coli.* We explored the relationship between operational characteristics of SDWSs and the occurrence of adverse water quality outcomes using de-identified data provided by Wellington-Dufferin-Guelph Public Health, Ontario. We examined the associations between water system operational characteristics and the adverse water quality outcome using logistic regression models. The analyses results indicated that operator training was associated with a lower risk for AWQI. None of the other predictors were significantly associated with AWQI: treatment method, water source, operating period, or sampling frequency. Our research concluded that the presence of operator training, an upstream behavioural determinant, is related to the incidence of AWQIs in SDWSs in Ontario, Canada. The high percentage of SDWSs with no treatment and lack of interest in testing for chemicals are potential areas of concern for ensuring the provision of safe drinking water from these systems.
Keywords: *waterborne diseases, small drinking water systems, safety, total coliforms, Escherichia coli, operator training*

Introduction

Behavioral influences on multi-user, non-household, public water systems have been understudied in the developed world. The drinking water system, which includes water source, treatment, distribution, and discharge requires the good use of technology and well-trained people to operate it. Several disease-causing organisms and substances are transmitted by water. Ensuring that water sources are of good quality and water treatment is done effectively are fundamental to protect the public's health (Coleman et al., 2013). In simple terms, drinking water is considered safe when it does not contain pathogens or unsafe concentrations of toxic chemicals or radioactive substances (MOE, 2006). Although approximately 15% of Canadians use Small Non-Community Drinking Water Systems (SDWSs), more than 50% of the waterborne outbreaks in Canada are associated with these systems (Pons et al., 2015). This study aims to examine the SDWS operational characteristics and their relationships with Adverse Water Quality Incidents (AWQIs).

There are several environmental determinants of water source contamination. Water quality degrades during extreme weather events such as drought and heavy rainfall, which consequently increases the risk for adverse health outcomes in affected communities (Delpla et al., 2009). According to O'Dwyer et al. (2014), aquifer type and rainfall amount impact the vulnerability of groundwater sources. Collins et al. (2005) and Park et al. (2014) also identify a correlation between the increased rainfall amount and the presence of *Escherichia coli (*Migula, *1895)* in water sources (surface and/or ground water). Another significant cause of ground water contamination with total coliform and *E. coli* is industrial activities such as mining operations (Armah, 2014) and livestock or non-point sources. Total coliform bacteria include several soil bacteria and are not likely to cause illness, but their presence indicates that the water system may be prone to contamination, whilst E. coli is commonly found in the intestines of mammals, including humans (Armah, 2014). The genera that belongs to coliforms include several organisms including *Citrobacter, Enterobacter, Klebsiella* (Harwood et al., 1999).

Rizak et al. (2003) note that water source contamination should be addressed using a holistic approach. In addition to environmental effects, social and behavioral characteristics play significant roles in water contamination that cause waterborne human disease outbreaks (Heymann, 2005). Social and behavioral factors underpinned by complacency contributed to the Walkerton tragedy in Ontario, Canada in May 2000 when Escherichia coli O157:H7 entered the water system and led to the deaths of seven people and made over 2300 people ill (Huck et al., 2003). Hrudey and Hrudey (2007) analyzed the cause of 74 recent waterborne outbreaks across the world and identified the major contributing factors to these incidents as insufficient source water knowledge, lack of disinfection, and operational deficiencies, which suggests that adequate operator training could have potentially prevented these outbreaks. Ercumen et al. (2014) examine the correlation between water distribution systems and gastrointestinal illnesses and conclude that operational deficiencies result in significant increase of gastrointestinal illnesses among users. According to Craun et al. (2001) distribution system issues not addressed by the operators are the leading cause of waterborne outbreaks. In other words, 'the environment' or water source is not the major contributing factor, it is human and technological deficiencies. Pons et al. (2015) notes that etiology was not identified in more than half of the reported waterborne outbreaks in Canada and the United States between 1970 and 2014 whilst, *Giardia intestinalis* was the most commonly identified pathogen followed by Norovirus and *Campylobacter jejuni* respectively. Enhanced reporting and identification of waterborne outbreaks would contribute to the explanation of the region's general characteristics and initiate strategies to prevent future occurrences.

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Climate change will impact the operations of SDWSs significantly with reduced water quality and availability (Grover, 2012). Frequent extreme weather events will result in increased number of waterborne outbreaks (Thomas et al., 2006; Cann et al., 2013). The operators and users of SDWSs will be unjustly affected as these systems have lower adaptive capacity and high vulnerability than Community Drinking Water Systems (CDWSs) (Cann et al., 2013). Social dimensions of SDWS operation should be examined to address current and emerging issues for the provision of safe drinking water.

The operator training in SDWSs can be considered an upstream behavioural determinant (Gehlert et al., 2008; Williams et al., 2008) within the context of environmental and societal factors. Dreibelbis et al. (2013) argue that behaviour change underpins enhanced water safety practices at individual, community and structural levels.

Most of the research and regulatory attention has been placed on industrial and municipal water systems because of their size and potential health risks in the event of inadequate treatment. SDWSs are defined as systems that make drinking water available to the public and are not connected to a CDWS (MOHLTC, 2015). SDWSs potentially fall through the regulatory cracks in Ontario and elsewhere as either they are not regulated or their regulatory requirements are considerably less stringent. Relative to urban water users, these factors could contribute to a greater potential risk faced by users of SDWSs. Furthermore, the number of people experiencing waterborne illnesses from SDWSs is predicted to be significantly higher than the documented cases since there is no national waterborne illness surveillance system (Schuster et al., 2005; Wilson et al., 2009).

Therefore, there is a substantial need to better understand their weaknesses and strengths. With many of them facing significant challenges for the provision of safe drinking water, it is estimated that 20% of Ontarians use over 9000 SDWSs across the province (Pons et al., 2014; Pons et al., 2015). If we consider transient populations such as travelers, the number of SDWSs users is considerably higher than the initial estimates.

Pons et al. (2015) reviewed the waterborne disease outbreaks in SDWSs in the United States and Canada between 1970 and 2014, and concluded that untreated and inadequately treated water systems have been the leading cause of these outbreaks. Less is understood about the predictors of this inadequacy of treatment. Our study looks at a wider set of factors, so-called upstream behavioral determinants, that may be related to AWQIs. We seek to fill a knowledge gap concerning the relationship of SDWS operational characteristics and the provision of safe drinking water. The purpose of this study is to examine the SDWS operational characteristics and their relationships with AWQIs.

AWQIs are documented when a water sample test result does not meet the regulatory standards indicated for that test or the water system may not be able to supply safe drinking water (MOHLTC, 2009). Although the Ministry of Health and Long-Term Care (MOHLTC) identifies 11 conditions for an AWQI, the detection of total coliforms and/or *E. coli* constitutes the significant majority of these incidents (MOHLTC, 2009). Locas et al. (2008) examine the ground water quality in three Canadian provinces and conclude that sampling for total coliforms and *E. coli* is the best approach to assessing the bacteriological quality of drinking water.

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In Ontario, the detection of total coliforms or *E. coli* at any level in water sample constitutes an AWQI.

SDWSs are mandated by the MOHLTC to meet similar water safety standards with larger municipal CDWSs. The regulatory oversight of SDWSs was transferred from the Ministry of the Environment (MOE) to local health units under the MOHLTC in 2008. The Health Protection and Promotion Act (HPPA) regulates SDWSs, while the Safe Drinking Water Act provides legal oversight for CDWSs.

Five categories of SDWSs are: (1) Large municipal non-residential drinking water systems such as recreational facilities, (2) Small municipal non-residential drinking water systems, community centres and libraries, (3) Non-municipal seasonal residential drinking water systems such as privately owned cottages on communal system, (4) Large non-municipal non-residential drinking water systems such as motels, and (5) Small non-municipal non-residential drinking water systems such as restaurants and churches (MOHLTC, 2015). Ontario Regulation 319 (Small Drinking Water Systems) established under the HPPA regulates SDWSs making the owners of these systems legally responsible for complying with the requirements (MOHLTC, 2015).

There are 36 health units in Ontario, and 29 of these health units are located in Southern Ontario. The study region is the health unit of Wellington-Dufferin-Guelph Public Health (WDGPH) which includes Wellington and Dufferin Counties and the City of Guelph. This region, centrally located in Southern Ontario, has 98% rural, and 2% urban area with 229 SDWSs (WDGPH, 2016). We examined the operational characteristics of the 229 systems in this region with respect to experiencing AWQIs defined as an above guideline, positive test for total coliform and/or *E. coli.* The incidence of water-borne illness within the WDGPH jurisdiction has not been studied.

The objective of our study was to explore the relationship between characteristics of the water systems and the presence of the adverse outcome with total coliforms and/or E. coli between the years 2010 and 2015. We hypothesized that the presence or absence of AWQI can be predicted by whether the SDWS operator had received formal operator training or not after adjusting for water source (ground water, surface water or other), treatment method (UV, chlorination, combination of the two, or none), operating period (seasonal, year round) and sampling frequency.

Methods

Data

The data used for this study is a mixture of the outcome variable (presence of AWQIs with total coliforms and/or *E. coli* between 2010 and 2015), behavioral (operator training) and non-behavioral predictors (the location of the water system, water source, treatment method of the water system, operating period and sampling frequency) with 229 data points. As a result, the de-identified data employed in this study included information on characteristics of the water systems and operations as well as the presence of AWQIs with total coliforms and/or *E. coli.* Public Health Inspectors from WDGPH collected the data between January 2010 and December 2015. The information included the name, location and contact information of the water system, any positive total coliforms and/or *E. coli* water test results (AWQIs) between 2010 and 2015; water source (ground water or surface water); treatment method (Ultraviolet [UV], Chlorinator, UV and Chlorinator, or no treatment); operation period (seasonal or year round operation); operator training as present or absent (whether the

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SDWS operator had received formal operator training or not); and sampling frequency per calendar year (number of samples in a calendar year) from 229 SDWSs in the region. *Figure 1* depicts the AWQIs on the dot distribution map in Wellington-Dufferin-Guelph region.

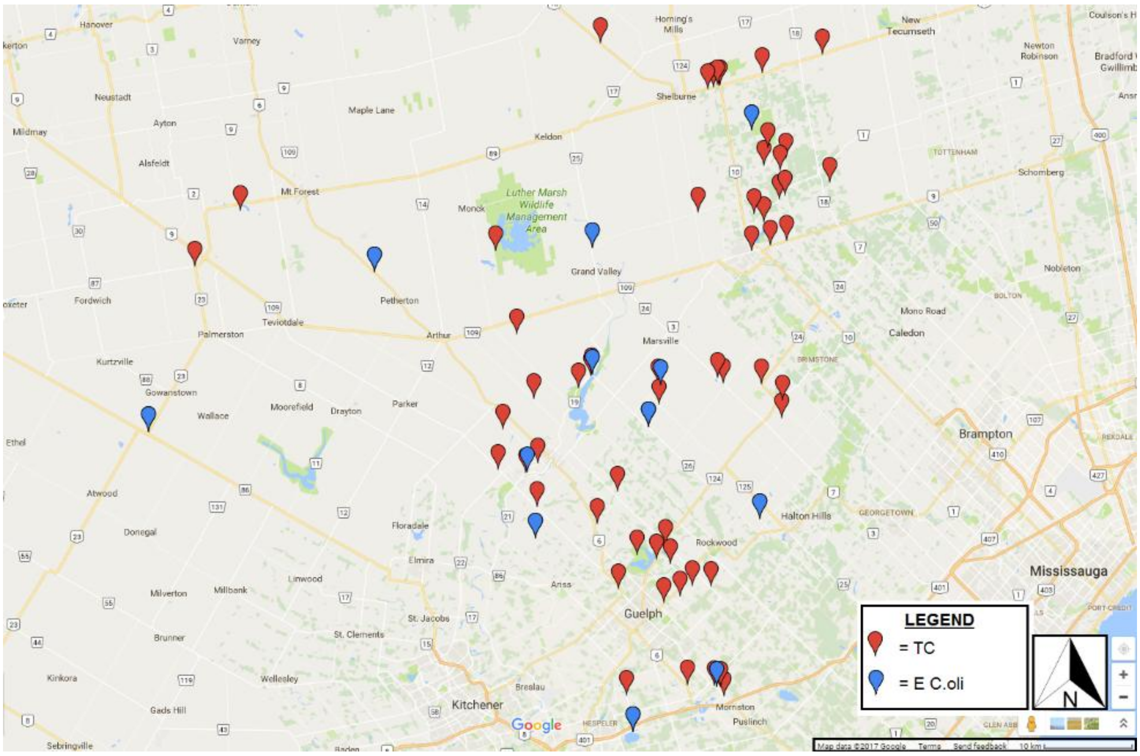

Figure 1. *Map of AWQIs in Wellington-Dufferin-Guelph Region*

Analysis Plan

The overall analysis strategy compared those with and without AWQIs. We planned to detect important patterns in all individual variables as well as the relationship between predictors and AWQI in both bivariate and more rigorous regression analyses. We started with descriptive statistics and provided a mean and standard deviation for our only discrete numerical variable (count data) which was sampling frequency. Frequencies and percentages were provided for categorical variables: water source (ground water or surface water, or other), treatment method (UV, chlorination, UV and chlorination, or none), operator training (present or absent) and operating period (seasonal or year around).

Two-sample t tests were used to compare mean differences between the groups (in those with and without an adverse water quality incident outcome) for sampling frequency with a student t-test. The Pearson chi-square test was employed to compare the distribution of categorical variables (water treatment, operating period, and operator training) in those with and without an AWQI. If the Pearson chi-square test assumption was violated (at least 80% of the expected counts are equal or greater than 5), we employed the Fisher's exact test as a substitute for the Pearson chi-square test when the expected counts were less.

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We tested pair-wise correlations between predictor variables using the Pearson correlation coefficient (r). We planned to remove the variable with lesser importance if *r* was greater than 0.80 for 2 predictors. The linear regression model was used to generate collinearity statistics. Tolerance and variance inflation factor (VIF) were used to test the assumption. Values less than 10 for VIF, and more than 0.1 for tolerance were considered violations. All the values were below the limits for *r* and VIF.

We also examined the associations between our outcome variable (AWQI) and all of the predictors (water source, treatment method, operating period, operator training and sampling frequency) using the logistic regression models in our inferential statistical analysis. We dichotomized the outcome into positive and negative adverse event which was defined by the MOHLTC (2009) guideline. Our logistic regression models explored the relationship between characteristics of the water systems (i.e. operator training, operating period, treatment, water source and sampling frequency) and the presence of the adverse outcome with total coliforms and/or *E. coli* in the past six years. The hypothesis of "the presence or absence of AWQI can be predicted by whether the SDWS operator had received formal operator training or not after adjusting for water source (ground water, surface water or other), treatment method (UV, chlorination, combination of the two, or none), operating period (seasonal, year round) and sampling frequency" was tested in the study sample. We reported odds ratios with 95% confidence intervals (CI).

Two-sided tests were employed with a significance level of 0.05 in our final model. All data analyses were performed using Stata (StataCorp., 2013). We also visually examined the map to verify the existence of clustering in data points.

Results

This study included 229 SDWSs from WDGPH. Two of the systems were eliminated from the data due to missing data (the sampling frequency was missing) and 18 SDWSs were posted[1]. As a result, we included a total of 209 water systems in our final analysis. The WDGPH data showed only two systems tested for chemical parameters.

Overall, a total of 165 water systems (79%) did not have operator training whereas 44 (21%) had operator training. *Table 1* shows the characteristics of water systems divided by the presence of AWQIs. The group which had AWQIs had lower frequency of operator training as compared to the group without an AWQI (P=0.02, *Table 1*). We also examined the associations between operating period and AWQIs using the Pearson Chi-square test. The frequency was not significantly different between the groups (P: 0.71). Likewise, the associations between presence of treatment, water source and sampling frequency with AWQIs were not statistically significant (P=0.47, P=0.32, P=0.48) (*Table 1*).

The distribution of water systems by treatment is depicted in *Figure 2*. A total of 59 (27%) water systems did not use any treatment systems while 128 (61%) employed UV to treat water. Sampling frequency ranged from 0 to 26 (*Figure 2*). The significant majority of the water systems (n=207, 99%) had ground water source while only two water systems had surface water.

[1] When a Small Drinking Water System is posted, the system owner is required to post signage regarding the public's access/consumption of water and the system is considered exempt from the operational requirements such as sampling, treatment and operator training (MOHLTC, 2015).

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Table 1. *Characteristics of water systems divided by the presence of AWQI*

Variables	AWQI (n=68, 33%)	No AWQI (n=141, 67%)	P value
Sampling frequency; mean (SD)	4.32 (3.3)	3.97 (3.3)	0.48
Presence of treatment			
Any treatment; n (%)	51 (75%)	99 (70%)	0.47
No treatment; n (%)	17 (25%)	42 (30%)	
Treatment method			
No treatment; n (%)	17 (25%)	42 (29%)	
Chlorinated; n (%)	2 (2%)	10 (7%)	0.26
UV; n (%)	47 (69%)	80 (56%)	
UV and chlorinated; n (%)	2 (2%)	9 (6%)	
Operating period			
Seasonal; n (%)	18	34	0.71
Year around; n (%)	50	107	
Operator training			
Positive; n (%)	8 (11%)	36 (25%)	**0.02**
Negative; n (%)	60 (88%)	105 (74%)	
Water source			
Ground water; n (%)	68 (100%)	139 (98%)	0.32
Surface water; n (%)	0 (0%)	2 (2%)	

Note: Significant values are in bold with significance level of 0.05; AWQI: Adverse Water Quality Incident; SD: standard deviation

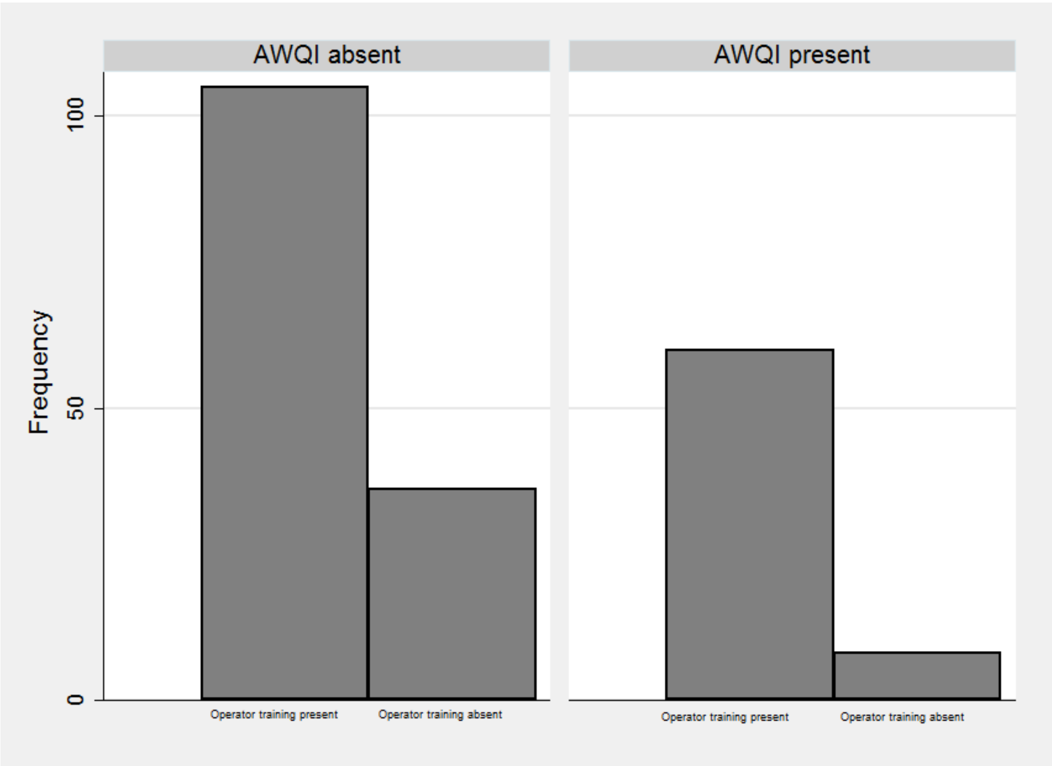

Figure 2. *The Distribution of Water System by Operator training and adverse water quality incident*

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To conclude, the results of the multivariate analyses indicated that operator training was associated with a lower risk for AWQI (OR= 0.38, 95% CI= 0.16 to 0.89, P= 0.02) (*Table 2*). The treatment method, operating period and sampling frequency did not indicate statistically significant results (*Table 2*).

Table 2. *Summary of our logistic models for AWQIs*

Variable	**Effect estimate OR (95% CI); p**
Seasonality	1.13 (0.58 to 2.19); 0.37
Sampling frequency	1.02 (0.94 to 1.11); 0.49
Treatment	1.27 (0.65 to 2.45); 0.72
Operator training	0.38 (0.16 to 0.89); 0.02
Treatment (3 categories)	1.12 (0.82 to 1.52); 0.73

CI: confidence interval; OR: odds ratio

Discussion

The findings support the idea that upstream behavioral determinants, specifically operator training, plays an integral role in the provision of safe drinking water in SDWSs. The summary of our findings are as follows: (1) the SDWSs with trained operators were significantly less likely to have an AWQI; (2) there was not a significant association between AWQIs and treatment method, operating period or sampling frequency. (3) the distribution of treatment methods was as follows: 61% of SDWSs used a UV treatment system (n=127); 28% of SDWSs did not use any treatment (n=59); 6% used chlorination (n=12); and 5% used a combination of chlorination and UV treatment system (n=11); (4) 1% of the SDWSs conducted chemical tests (n=2) while 99% of the SDWSs did not conduct chemical tests (n=207).

The findings about the operator training suggest the presence of trained operators in SDWSs significantly associated with the possibility of experiencing AWQIs. Review of the causes of recent waterborne disease outbreaks shows that meeting the regulatory water quality parameters alone is not sufficient to safely operate a drinking water system (Rizak et al., 2013). It is also fundamental to note that the lack of a robust surveillance system results in underreporting of waterborne disease outbreaks in SDWSs (Schuster et al., 2005; Wilson et al., 2009) which consequently hinders the development of interventions to increase the safety net for these systems. Xie et al. (1999) argue that small water systems face challenges to meet the regulatory requirements and operator training is essential to increase these systems' capacity to meet the regulations. According to Murphy et al. (2015), both owners and operators should receive water system training so that they can have a better understanding of the challenges for the provision of safe drinking water. Upstream determinants are fundamental parts of the social environment where individual differences in expression of feelings, thoughts and activities are shaped (Gehlert et al., 2008). The focus on operator training can be a viable intervention to address upstream behavioral determinants. Preventing illnesses by establishing mechanisms to increase the percentage of operators properly trained in SDSWs, supports the efforts to reduce health disparities (Williams et al. 2008).

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The Walkerton outbreak was a stark reminder the importance of operator training for the provision of safe drinking water. One of the major findings of the Walkerton tragedy was the complacency of the trained water system operators (Huck et al., 2003) where corrective action procedures were not diligently carried out prior to the outbreak. The operator training should be coupled with a better understanding of the consequences of not adequately responding to AWQIs. The Multiple-Barrier Approach (MBA) is an integrative risk management approach to water safety from source to tap. Baird et al. (2013) (as cited in Canadian Council of Ministers of the Environment, 2004, 16) explains the MBA as “an integrated system of procedures, processes and tools that collectively prevent or reduce the contamination of drinking water from source to tap in order to reduce risks to public health” (p.122). Water system operator training and establishing safety measures from source to tap are fundamental steps for the provision of safe drinking water.

Parr (2005) examined the societal effects and government approach to operator training just before the Walkerton outbreak and argues that the lack of consistency in training was a contributing factor to the outbreak. Training opportunities supported by the regulatory agencies assist SDWS owners and operators to enhance their capabilities for building and applying knowledge, which in return results in safer operations of these systems.

Over a quarter of the SDWSs in the Wellington-Dufferin-Guelph region operate with no treatment, yet a SDWS with no treatment system might be prone to contamination from external sources. Pons et al. (2015) reported that having no treatment system is one of the leading causes of outbreaks in SDWSs. Edwards et al. (2012) examined the safe operation characteristics of small commercial water systems in British Columbia, Canada, and concluded that the lack of a treatment system and water source vulnerability are among factors causing adverse conditions in SDWSs. Schuster et al. (2005) also identified treatment system failure along with inadequate operational practices as leading causes of waterborne outbreaks. The source water protection planning in Ontario does not include SDWSs therefore there is no enhanced safety net for these systems. Health risks of consuming water from an unprotected source are considerably high compared to a protected water source (Davies and Mazumder, 2003). The effects of climate change in the region, which include frequent extreme weather events, might put stress on the safety of the water sources. Thomas et al. (2006) and Cann et al. (2013) identify a correlation between increased amount of rainfall and waterborne disease outbreaks in Canada. SDWSs may not have sufficient resources and capacity to eliminate the adverse effects of extreme weather events which puts the safety of drinking water at risk. Dow et al. (2007) investigate the perceptions of water system managers about climate change effects and identify water quality, financial impact and scarcity of supply as major concerns. Source water protection is an integral step to protect SDWSs from impacts of climate change. Furthermore, complimentary strategies for source water protection, such as shoreline stewardship and groundwater sales policies, may become increasingly important in ensuring the safety of SDWS and CDWS alike.

The findings concerning treatment method were expected given that all of the technologies used are well understood. UV was the most commonly used water treatment method among SDWSs. UV treatment has been in the market for over 30 years but has gained popularity in the past decade (Corfield, 2015). In addition to treating microbiological contaminants, UV systems are effective on chlorine resistant species such as Giardia parasite and are therefore considered a viable option to enhance

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water quality (Corfield, 2015). Although UV treatment does not affect water properties such as chemistry and taste, the regular maintenance of the system is fundamental to maintain a safe operation (McClean, 2008). The widespread use of UV treatment in SDWSs reminds how essential the training component is for the provision of safe drinking water.

The findings about a lack of chemical testing are concerning. Chemical testing is an integral step to investigate potential threats to the water source which can be naturally occurring or human-made. Our dataset showed that only 1% of the SDWSs had conducted chemical tests to understand the chemical composition of their water sources. Chemical contaminants in drinking water might cause several illnesses with serious and long-term health effects (Barrett, 2014). A study examining chemicals in water from 6013 private wells over a 12 month-period concluded that over 25% of the wells exceeded the acceptable levels of chemical contaminants (Harrison et al., 2000). Davies and Mazumder (2003) discussed the negative effects of agricultural, industrial and domestic use of chemicals on water sources and advocated for the reduction of their use and environmentally friendly disposal practices to reduce chemical contamination. Our study recommends greater emphasis on monitoring the chemical composition of the source water to confirm drinking water meets the regulatory limits.

There were several limitations of our study that shouldn't however undermine our findings about training. That said; this study involved secondary analysis of the existing data set therefore the number of variables was limited by the existing database. A variable that would be useful to include in a model of AWQI was risk category. The definition of AWQI is narrow in that we defined as incidents with positive total coliforms and/or *E. coli* test result as our dataset did not have consistent information for other conditions that may be classified as an AWQI. That said; presence of total coliforms in water sources is considered as one of the best pathogen indicators (Locas et al., 2007).

Further research is needed to explore the determinants of adverse water quality events with total coliforms and/or *E. coli* as well as other AWQI events like treatment system failures, structural deficiencies, and exceeding chemical parameters. Examining other upstream behavioural determinants within the context of environmental and societal norms will provide a deeper understanding of the current challenges of SDWSs in the provision of safe drinking water. Exploration of the factors associated with the adverse events will require a prospective well-designed and well-conducted study with a larger dataset with a possibility of linking records from several databases to retrieve complete information about SDWSs.

Conclusion

In Ontario, there are 36 health units with over 9000 SDWSs in their respective jurisdictions. Our analysis using the data from 229 SDWSs located in the Wellington-Dufferin-Guelph region provided critical insight for operation and safety of these systems.

Our research concluded that the presence of operator training, an upstream behavioural determinant, significantly reduces the incidence of AWQIs in SDWSs. The high percentage of SDWSs with no treatment, lack of interest in testing for chemical parameters, and source water protection are potential areas of concern to ensure the provision of safe drinking water from these systems. Future research should attempt to flesh out the risk awareness and perceptions of SDWS owners to understand the challenges to operate these systems.

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ADAPTIVE CHANGES IN PHYSIOLOGICAL TRAITS OF WHEAT GENOTYPES UNDER WATER DEFICIT CONDITIONS

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Abstract. Two year (2013/14 and 2014/15 growing seasons) field experiments were carried out to study the effect of soil water deficit on some physiological traits and grain yield of durum (*Triticum durum* Desf.) and bread wheat (*Triticum aestivum* L.) genotypes. Drought stress led to adaptive changes in physiological traits of wheat genotypes. Gas exchange parameters decreased significantly in response to water deficit. A reduction in relative water content was not strong. Genotypic differences in reducing the content of pigments, the relative stability of carotenoids to water stress were revealed. The leaf assimilation area of durum wheat genotypes exceeds that of bread wheat genotypes. Water stress caused acceleration of leaf senescence, inhibited an increase in the area of stem and spike**.** Adaptive changes in the distribution of assimilation area between leaves and stem were revealed. Unlike leaf assimilation area, stem assimilation area increased to stage of kernel~~s~~ watery ripe. Water stress caused an increase in proline content. Generally, grain yield was higher in genotypes of bread wheat than that of durum wheat, but a decrease in grain yield was over expressed in durum wheat. Grain yield less correlated with physiological traits. The decrease in the physiological parameters, as well as grain yield, was more expressed in 2013/14 growing season.
Keywords: *Triticum durum Desf., Triticum aestivum L., drought, gas exchange, relative water content, chlorophyll, proline, grain yield*

Introduction

Water deficit during growth and development of plants results in adaptive changes in the physiological and biochemical processes and morphological traits that allow the survival of the plants and formation of the yield. Wheat grain yield is significantly reduced in the arid and semi-arid regions of the world, where lack of water is also accompanied by high temperatures and high radiation levels. Wheat (*Triticum* spp.) is one of the first domesticated food crops and for 8000 years has been the basic staple food of major civilizations of Europe, West Asia, and North Africa (Monneveux et al., 2012). In parts of northern Africa and in the Caucasus region, annual consumption of wheat products per person is highest being ~~at~~ around 200 kg (Pingali, 1999). About 33% of wheat fields in the world and about 55% in the developing countries suffer from drought stress (Aghanejad et al., 2015). Wheat is one of the most important crops in Azerbaijan, where almost half of wheat growing areas are under rainfed conditions. Increasing wheat productivity is a national target in Azerbaijan to fill the gap between consumption and production.

Over the years, many physiological, morphological, and developmental traits have been suggested to be useful in improving drought tolerance (Quarrie et al., 1999; Wright

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and Rachaputi, 2004). Recent studies suggest that selection of physiological traits has the potential to improve wheat grain yield under drought (Reynolds et al., 2002; Condon et al., 2004).

The limitation of plant growth imposed by low water availability is mainly due to reductions of plant carbon balance, which is largely dependent on photosynthesis (Flexas et al., 2009). Photosynthetic responses to water stress have been the subject of studies and debates for decades, in particular, concerning which are the most limiting factors (stomatal or mesophyll limitations, photochemical and/or biochemical reactions) for photosynthesis under water stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002). The foliar photosynthetic rate of higher plants is known to decrease as the relative water content and leaf water potential decrease (Lawlor and Cornic, 2002). The decline of transpiration rate was mainly affected by stomatal conductance and the decline in photosynthesis rate was affected by non-stomatal factors (Changhai et al., 2010). Water deficit reduced leaf gas exchange and relative water content of durum wheat genotypes at anthesis and grain filling (Bogale et al., 2011). Morphophysiological traits can be used as indirect selection criteria for grain yield; however, their effectiveness depends on correlations with grain yield under drought and the degree to which each trait is genetically controlled (Nachit and Elouafi, 2004). Identifying wheat genotypes with suitable agronomical, morphological and physiological traits under water stress conditions is a main goal of wheat researchers.

The relationships between physiological traits and grain yield have not been identified clearly yet. We aimed to study adaptive changes in some physiological traits of wheat genotypes under drought stress, identify relations between physiological traits and grain yield.

Materials and methods

Plant material and growth conditions

Field experiments were conducted during the 2013/14 and 2014/15 growing seasons at the Research Institute of Crop Husbandry, located in the Apsheron peninsula, Baku. Plant materials consisted of 8 durum wheat (*Triticum durum* Desf.) genotypes Garagylchyg 2, Vugar, Shiraslan 23, Barakatli 95, Alinja 84, Tartar, Sharg, Gyrmyzy bugda and 14 bread wheat (*Triticum aestivum* L.) genotypes Nurlu 99, Gobustan, Akinchi 84, Giymatli 2\17, Gyrmyzy gul 1, Azamatli 95, Tale 38, Ruzi 84, Pirshahin 1, 12ndFAWWON№97, 4thFEFWSN№50, Gunashli, Dagdash, Saratovskaya 29. Sowing was performed in the third decade of October, at an average density of 400 seeds/m^2 with mechanical planter in 1 x 10 m plots, consisting of 7 rows placed 15 cm apart. Genotypes were grown in irrigated and rainfed plots with three replications. Irrigated plots were watered after the appearance of seedlings, at the stem elongation, anthesis and grain filling stages. Rainfed plots were not watered during ontogeny. Soil moisture content was determined at 0-20, 20-40 and 40-60 cm depths and was an average 60% in irrigated and 30% in rainfed plots at the grain formation stage.

Measurements

Gas exchange parameters (photosynthesis rate- P_n, stomatal conductance- g_s, intercellular CO_2 concentration- C_i and transpiration rate- E) were measured using LI-COR 6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE,

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USA). Gas exchange measurements were conducted at the booting (Feekes Stage 10), anthesis (Feekes 10.5.1) and kernels watery ripe (Feekes Stage 10.5.4) stages. Measurements were carried out between 10:00 and 12:00 a.m. Data logging started after 45 s of the insertion of leaves into chamber. The measurements of pigment-contents and relative water content were conducted at the kernels watery ripe stage. Leaf Chl a, b and Car (x + c) contents were determined following the method of Lichtenthaler (1987), with little modifications. About 0.1 g of fresh leaves were ground in 96% ethanol for the extraction of chlorophyll and carotenoids. Absorbance of the supernatant was recorded at 664, 648, and 470 nm, spectrophotometrically (Genesys 20, Thermo Scientific, USA). Pigment contents were calculated using the following formulas.

$$\mathrm{Chla} = \left(13.36 \cdot \mathrm{A}_{664} - 5.19 \cdot \mathrm{A}_{648}\right) \cdot 25 / \mathrm{DW}; \; \mathrm{Chlb} = \left(27.43 \cdot \mathrm{A}_{648} - 8.12 \cdot \mathrm{A}_{664}\right) \cdot 25 / \mathrm{DW};$$

$$\mathrm{Chl} \cdot \left(\mathrm{a} + \mathrm{b}\right) = \left(5.24 \cdot \mathrm{A}_{664} + 22.24 \cdot \mathrm{A}_{648}\right) \cdot 25 / \mathrm{DW};$$

$$\mathrm{Car}\left(\mathrm{x} + \mathrm{c}\right) = \left(4.785 \cdot \mathrm{A}_{470} + 3.657 \cdot \mathrm{A}_{664} - 12.76 \cdot \mathrm{A}_{648}\right) \cdot 25 / \mathrm{DW}$$

Leaf area per stem (LAS), also projected area of stem multiplied by 3.14 according to Kvët and Marshall (1971), and spike multiplied by 2 according to Alvaro et al. (2008) were measured with an area meter (AAC-400, Hayashi Denkon Co, LTD, Japan). The flag leaf RWC was determined gravimetrically. RWC was calculated using the following formula: RWC(%)=(FW-DW)/(TW-DW) x 100, where FW-fresh mass, DW-dry mass, TW-turgid mass. Proline content was determined following the method of Bates et al. (1973), with little modifications. About 0.5 g of leaves were homogenized in a pre-chilled pestle and mortar with 5 ml of 3% sulphosalicylic acid. Then, the homogenate was centrifuged at 3500 g (HERMLEZ 400K, Germany) for 15 min at 4°C. The supernatant (0.2 ml) was transferred to a plastic tube containing 3% ninhydrin (0.4 ml), and 0.2 ml of 96% acetic acid and 0.2 ml of 3% sulphosalicylic acid were added. Tubes were incubated for 1 h at 96°C in a water-bath and 2 ml of toluene was added to each tube, then stirred, and centrifuged at 3500 g for 15 min at 4°C. The absorbance of the upper phase was measured at 520 nm. The determination of proline was carried out with a calibration curve, constructed for a concentration range of 0.01-0.2 mM proline.

Statistical analysis

Correlations between traits were calculated using SPSS 16 software.

Results

Water deficit caused a decline in P_n (*Figs. 1* and *2*), especially a big difference was detected between irrigated and exposed to drought plants at the anthesis stage. The P_n of the genotypes Garagylchyg 2, Vugar, Barakatli 95, Nurlu 99, Gobustan, Akinchi 84, Azamatli 95, 12ndFAWWON№97 and Saratovskaya 29 was susceptible to water deficit in both years, while the P_n of the genotypes Sharg, Gyrmyzy bugda, Gyrmyzy gul 1, Dagdash was resistant. The g_s of irrigated and rainfed plants was higher at the heading stage (*Figs. 3* and *4*), when the water status of leaves was relatively higher.

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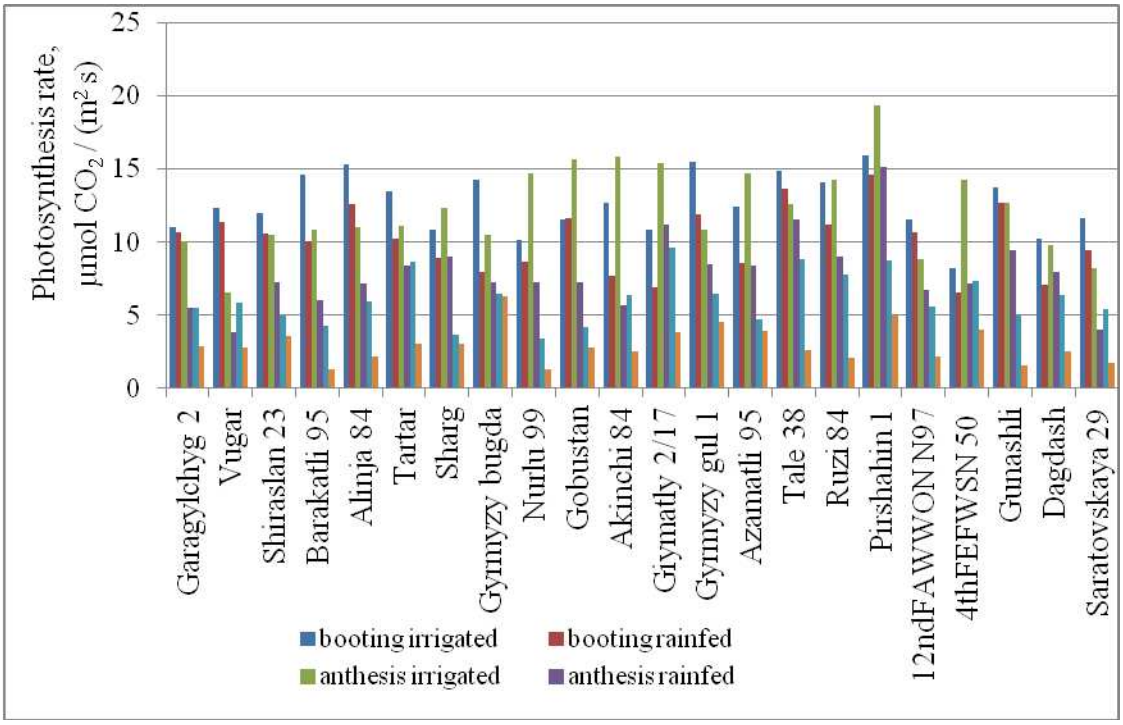

Figure 1. *Effect of drought stress on photosynthesis rate- P_n (2013/14). Each value represents mean of 7-9 replicates.*

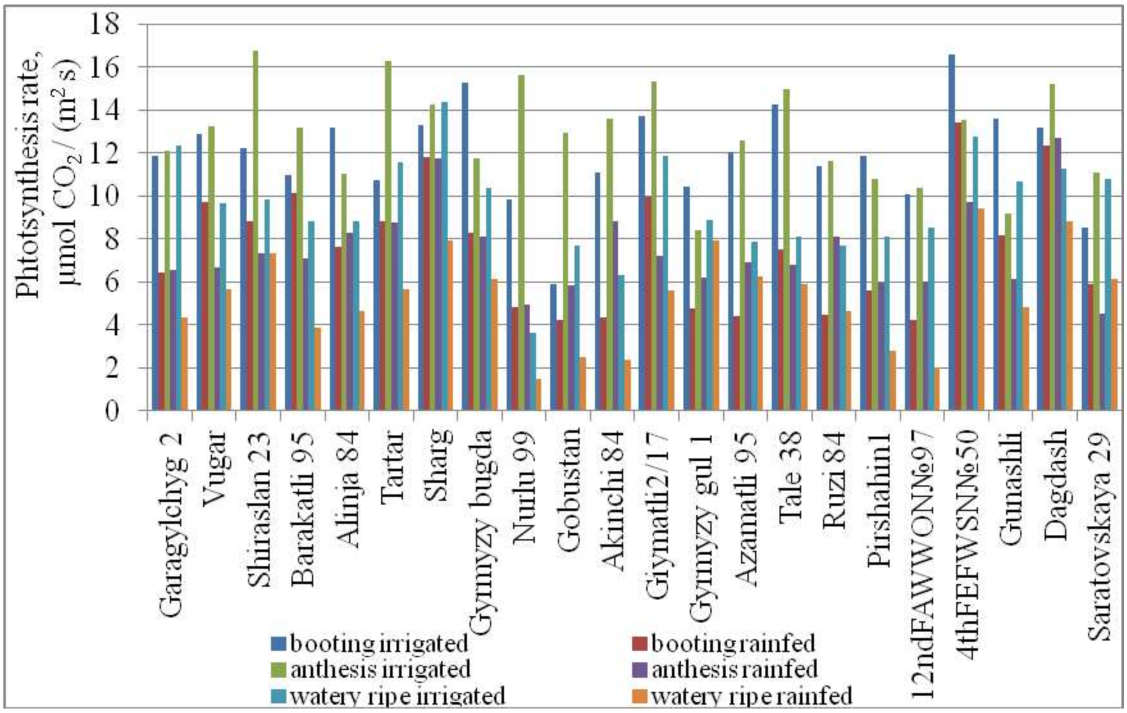

Figure 2. *Effect of drought stress on photosynthesis rate- P_n (2014/15). Each value represents mean of 7-9 replicates.*

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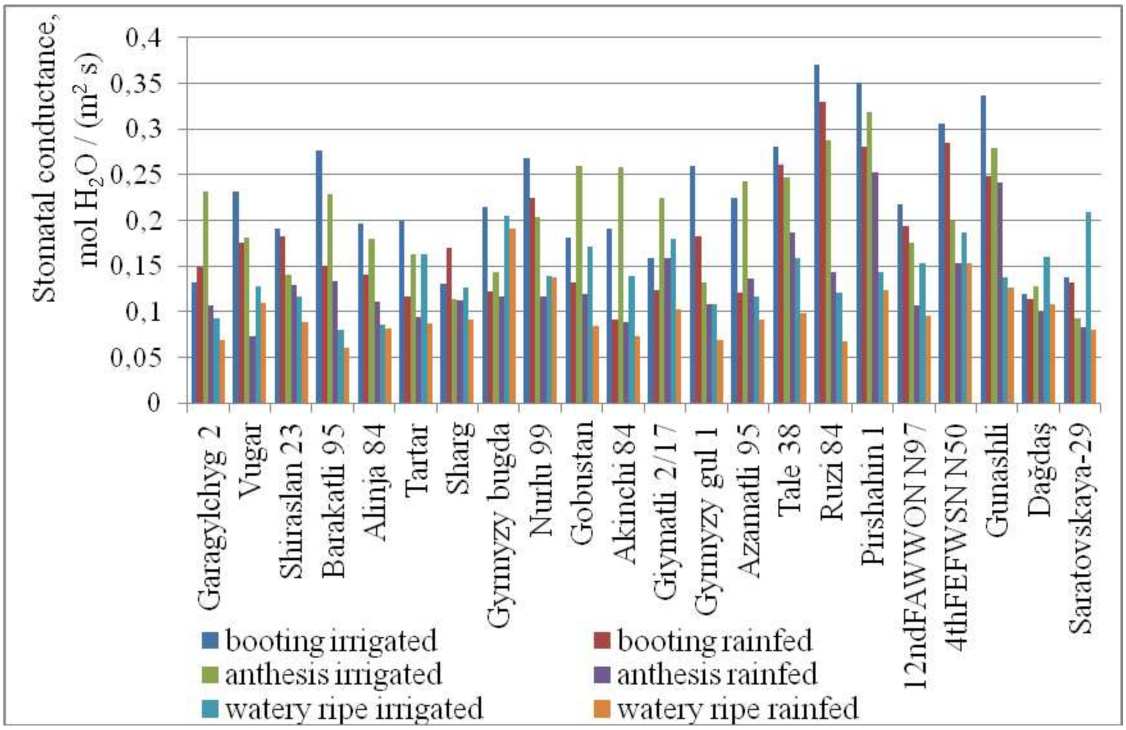

Figure 3. *Effect of drought stress on stomatal conductance-g_s (2013/14). Each value represents mean of 7-9 replicates.*

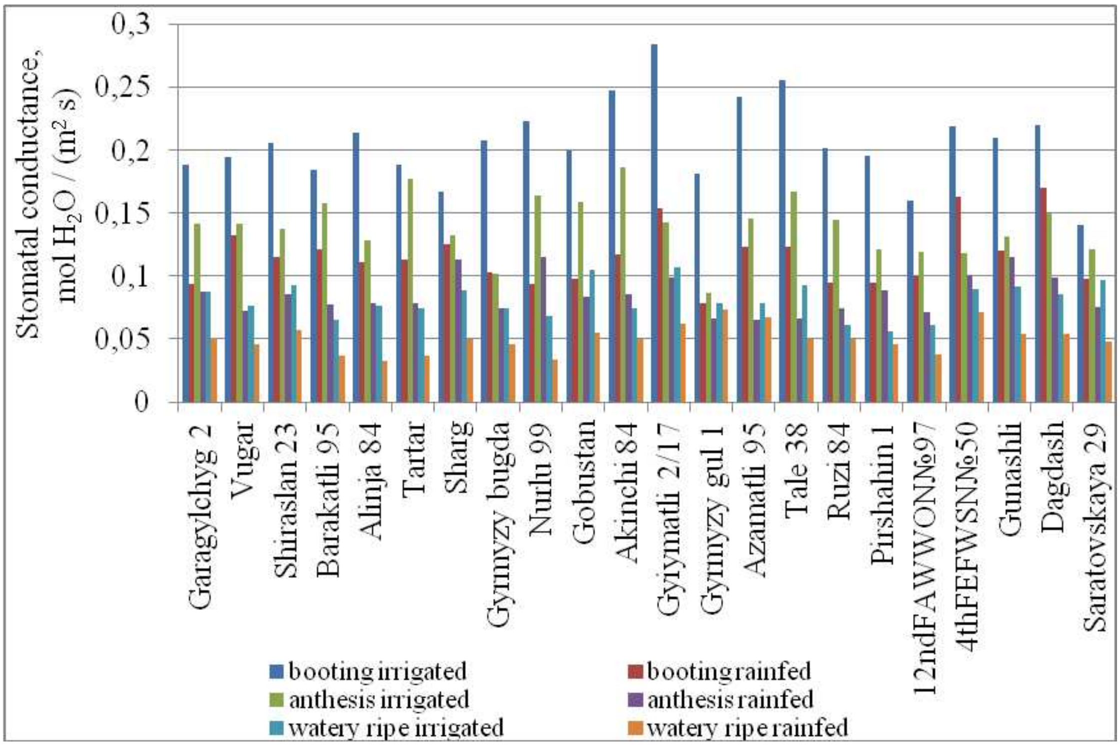

Figure 4. *Effect of drought stress on stomatal conductance-g_s (2014/15). Each value represents mean of 7-9 replicates.*

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The g_s of wheat genotypes declined sharply in response to water deficit. Drought stress caused a decline in C_i at the booting stage in most genotypes. However, as the drought stress strengthened at following stages, the C_i increased. The E was higher at the heading stage. Drought stress caused a strong reduction of E, especially in the genotypes Alinja 84, Tartar, Gobustan, Akinchi 84, Giymatli 2/17, and Tale 38.

The rate of photosynthesis and stomatal conductance were higher in 2014/15 growing season than that in 2013/14.

Genotypic variations in RWC of flag leaves were revealed (*Fig. 5*). It was also found that the RWC of the leaves was higher in the 2014/15 growing season. A decrease in RWC of flag leaf was not strict and was about 4-10% (2013/14) and 2-8% (2014/15) in most wheat genotypes. A significant decline in RWC was found in the genotypes Barakatli-95 (10%), Nurlu 99 (12%), and Gunashli (26%) in 2013/14, in the genotypes Gobustan (12%), Tale 38 (10%) and Azamatli 95 (26%) in 2014/15. A slight decrease in RWC was assumed to be associated with a profound reduction in g_s and accumulation of osmotically active compounds.

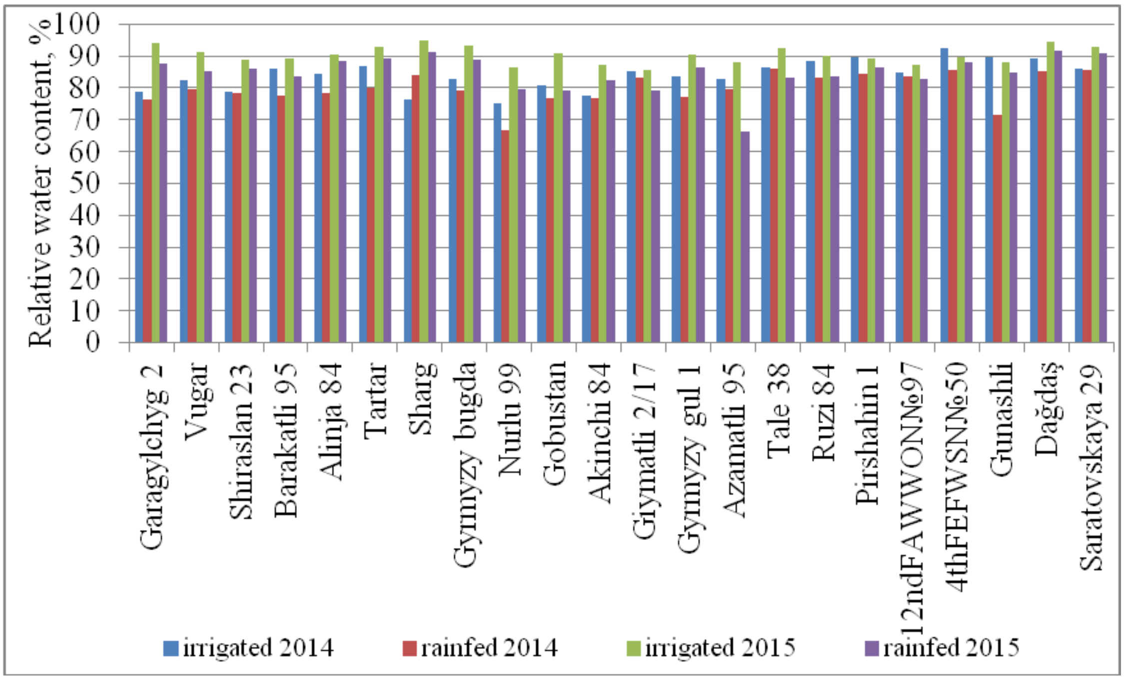

Figure 5. *Effect of drought stress on flag leaf relative water content. Each value represents mean of 3 replicates.*

The contents of Chl(a+b) and Car(x+c) was higher in the 2014/15 growing season than in the 2013/14 growing season (*Tables 1* and *2*). A higher content of pigments was found under irrigation in the genotypes Garagylchyg 2, Tartar, Barakatli 95, Gobustan, Giymatli 2/17, Gyrmyzy gul 1, Tale 38, 4thFEFWSN№50, Saratovskaya 29, whereas low levels of pigments were found in the genotypes Shiraslan 23, Nurlu 99, Azamatli 95. Water shortage greatly affected the pigment content of the genotypes Garagylchyg 2, Tartar, Gobustan, Akinchi 84, Gunashli, whereas a slight decrease was detected in genotypes Vugar, Gyrmyzy gul 1, Tale 38, Azamatli 95 and Saratovskaya 29. An increase in the Chl a/b ratio in half of all genotypes was identified. Reducing the

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Chl(a + b)/Car(x + c) ratio was identified in the majority of genotypes, indicating a relative resistance of carotenoids to water shortage.

Table 1. *Effect of drought stress on flag leaf photosynthetic pigments content (2013/14). Each value represents mean of 3 replicates.*

Genotypes	Growth condition	Chl a	Chl b	Chl (a+b)	Car (x+c)	Chl a/b	Chl (a+b)/Kar (x+c)
		mg g^{-1} dry mass					
Triticum durum Desf.							
Garagylchyg 2	Irrigated	6.34	2.56	8.9	1.48	2.47	6.01
	Rainfed	3.08	1.75	4.83	0.63	1.77	7.66
Vugar	Irrigated	6.29	2.70	8.98	1.33	2.33	6.75
	Rainfed	6.04	2.70	8.74	1.40	2.23	6.24
Shiraslan 23	Irrigated	5.54	2.82	8.36	1.02	1.96	8.23
	Rainfed	4.16	2.15	6.31	1.01	1.93	6.27
Barakatli 95	Irrigated	6.85	2.91	9.75	1.53	2.36	6.37
	Rainfed	5.36	2.40	7.76	1.15	2.23	6.75
Alinja 84	Irrigated	5.68	2.67	8.36	1.24	2.13	6.73
	Rainfed	4.72	2.35	7.06	0.98	2.01	7.16
Tartar	Irrigated	10.04	3.99	14.02	2.08	2.52	6.74
	Rainfed	4.57	1.83	6.40	1.09	2.50	5.87
Sharg	Irrigated	5.16	1.93	7.08	1.46	2.67	4.84
	Rainfed	3.97	1.47	5.44	1.36	2.69	4.01
Gyrmyzy bugda	Irrigated	5.84	2.07	7.91	1.47	2.81	5.38
	Rainfed	5.04	1.79	6.83	1.49	2.81	4.57
Triticum aestivum L.							
Nurlu 99	Irrigated	6.06	2.42	8.48	1.45	2.51	5.83
	Rainfed	3.12	1.21	4.33	1.03	2.59	4.22
Gobustan	Irrigated	7.82	3.04	10.85	1.65	2.57	6.57
	Rainfed	3.23	1.35	4.58	0.82	2.40	5.56
Akinchi 84	Irrigated	7.98	3.12	11.10	1.87	2.55	5.92
	Rainfed	4.77	1.90	6.67	1.43	2.51	4.67
Giymatli 2/17	Irrigated	6.63	2.87	9.50	1.37	2.31	6.93
	Rainfed	4.97	1.78	6.75	1.18	2.78	5.74
Gyrmyzy gul1	Irrigated	8.02	3.10	11.12	1.70	2.59	6.54
	Rainfed	5.99	2.32	8.31	1.33	2.59	6.24
Azamatli 95	Irrigated	5.88	2.37	8.25	1.29	2.48	6.39
	Rainfed	5.17	2.17	7.34	1.34	2.39	5.48
Tale 38	Irrigated	7.52	3.01	10.53	1.58	2.50	6.65
	Rainfed	5.86	2.26	8.12	1.44	2.59	5.62
Ruzi 84	Irrigated	5.92	2.23	8.15	1.60	2.66	5.09
	Rainfed	4.23	1.72	5.95	1.25	2.47	4.75
Pirshahin 1	Irrigated	5.31	2.56	7.87	1.21	2.08	6.50
	Rainfed	3.58	1.49	5.07	1.31	2.41	4.48

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12ndFAWWON№97	Irrigated	6.48	2.86	9.34	1.40	2.27	6.68
	Rainfed	5.25	2.24	7.49	1.41	2.34	5.30
4thFEFWSN№50	Irrigated	6.97	2.94	9.90	1.61	2.37	6.16
	Rainfed	5.42	2.18	7.60	1.41	2.48	5.40
Günashli	Irrigated	6.46	2.44	8.90	1.59	2.64	5.60
	Rainfed	3.13	1.16	4.28	1.25	2.70	3.42
Dagdash	Irrigated	7.34	2.77	10.11	1.73	2.65	5.83
	Rainfed	6.35	2.38	8.73	1.87	2.66	4.66
Saratovskaya 29	Irrigated	7.20	2.73	9.93	1.71	2.64	5.82
	Rainfed	6.79	2.59	9.38	1.65	2.62	5.67

Table 2. *Effect of drought stress on flag leaf photosynthetic pigments content (2014/15). Each value represents mean of 3 replicates.*

Genotypes	**Growth condition**	**Chl a**	**Chl b**	**Chl (a+b)**	**Car (x+c)**	**Chl a/b**	**Chl (a+b)/Kar (x+c)**
		mg g^{-1} dry mass					
Triticum durum Desf.							
Garagylchyg 2	Irrigated	7.51	2.65	10.16	2.29	2.84	4.43
	Rainfed	4.89	1.88	6.77	1.73	2.60	3.92
Vugar	Irrigated	6.82	2.38	9.20	2.16	2.87	4.26
	Rainfed	5.87	2.04	7.91	1.71	2.88	4.63
Shiraslan 23	Irrigated	5.23	1.92	7.15	1.47	2.73	4.86
	Rainfed	5.15	1.77	6.92	1.65	2.91	4.19
Barakatli 95	Irrigated	7.16	2.65	9.81	2.02	2.70	4.85
	Rainfed	6.60	2.52	9.12	1.87	2.62	4.87
Alinja 84	Irrigated	6.59	2.47	9.06	1,87	2,66	4,85
	Rainfed	5.41	1.89	7.29	1.65	2.86	4.43
Tartar	Irrigated	7.48	2.93	10.41	2.16	2.55	4.83
	Rainfed	5.98	2.38	8.36	1.78	2.52	4.69
Sharg	Irrigated	6.52	2.34	8.86	1.90	2.78	4.66
	Rainfed	5.09	1.66	6.75	1.63	3.07	4.15
Gyrmyzy bugda	Irrigated	7.63	2.54	10.17	2.14	3.01	4.76
	Rainfed	6.06	2.05	8.11	1.77	2.96	4.57
Triticum aestivum L.							
Nurlu 99	Irrigated	4.32	1.62	5.94	1.27	2.67	4.69
	Rainfed	4.14	1.53	5.67	1.16	2.70	4.91
Gobustan	Irrigated	7.82	2.80	10.62	2.15	2.79	4.94
	Rainfed	4.85	1.61	6.45	1.41	3.01	4.58
Akinchi 84	Irrigated	6.51	2.43	8.95	1.95	2.68	4.60
	Rainfed	4.78	1.81	6.59	0.74	2.65	8.92
Giymatli 2/17	Irrigated	7.78	2.72	10.50	2.24	2.86	4.70
	Rainfed	6.18	2.19	8.37	1.72	2.82	4.87

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Gyrmyzy gul1	Irrigated	7.96	2.96	10.92	2.26	2.69	4.84
	Rainfed	7.71	2.84	10.55	2.25	2.72	4.67
Azamatli 95	Irrigated	4.87	2.09	6.95	1.34	2.33	5.20
	Rainfed	5.23	1.95	7.18	1.46	2.67	4.92
Tale 38	Irrigated	7.22	2.57	9.79	2.07	2.81	4.73
	Rainfed	6.56	2.48	9.03	1.90	2.65	4.75
Ruzi 84	Irrigated	6.76	2.36	9.12	1.93	2.86	4.73
	Rainfed	5.58	1.95	7.54	1.58	2.86	4.77
Pirshahin 1	Irrigated	7.01	2.54	9.55	2.01	2.76	4.76
	Rainfed	6.31	2.25	8.56	1.88	2.80	4.55
12ndFAWWON№97	Irrigated	6.97	2.51	9.48	2.04	2.77	4.64
	Rainfed	6.12	2.43	8.55	1.72	2.52	4.97
4thFEFWSN№50	Irrigated	7.68	2.78	10.46	2.23	2.76	4.70
	Rainfed	6.17	2.29	8.45	1.88	2.70	4.50
Günashli	Irrigated	7.26	2.72	9.98	1.92	2.67	5.20
	Rainfed	5.08	1.95	7.03	1.44	2.61	4.87
Dagdash	Irrigated	6.39	2.14	8.52	1.99	2.99	4.28
	Rainfed	5.66	2.00	7.66	1.93	2.83	3.97
Saratovskaya 29	Irrigated	7.80	2.97	10.78	2.06	2.62	5.24
	Rainfed	7.00	2.53	9.53	2.05	2.77	4.66

Durum wheat genotypes formed a greater leaf area per stem compared to bread wheat genotypes until the heading stage (*Figs. 6* and *7*). The largest area of leaves per stem was formed in genotypes of durum wheat Vugar, Alinja 84, Tartar and in bread wheat Giymatli 2/17. The genotypes Giymatli 2/17, Gyrmyzy gul 1 and Tale 38 are also characterized by higher leaf area index (data are not shown). Water shortage caused a reduction in assimilation area of leaves. A decrease in leaf area at following growth stages occurred both in irrigated and rainfed plants, but water deficit accelerated leaf senescence. An increase in stem assimilation area of stem was detected in some genotypes under water deficit, which can be explained as a compensation of leaf area reduction (*Fig. 8*). In most genotypes an increase in stem assimilation area continued until the ~~kernels~~ watery ripe stage in the favorable 2014/15 growing season (*Fig. 9*). A high assimilation area of stem was revealed in the taller genotypes Sharg, Gyrmyzy bugda, and in the genotypes Akinchi 84 and Giymatli 2/17 whereas a low assimilation area of stem was revealed in genotypes Gyrmyzy gul 1, 12ndFAWWON№97 and Gunashli. Water deficit slowed an increase in stem assimilation area. A large decrease in the assimilation area of stem was found to kernels watery ripe stage in the genotypes Tartar, Gyrmyzy bugda, Akinchi 84, Ruzi-84, 4thFEFWSN№50 and Dagdash. The assimilation area of spike was relatively greater in most genotypes of durum wheat than that of bread wheat (data are not shown). Under the condition of water scarcity a great delay in the formation of the assimilation area of spike was observed in the genotypes Gyrmyzy gul 1, Azamatli 95, Tale 38, 12ndFAWWON№97 and Dagdash.

Proline content increased under drought conditions, especially in the genotypes Tartar, Giymatli 2/17, Azamatli 95, Tale 38 (*Fig. 10*). The substantial content of proline in irrigated plants is probably associated with the senescense of leaves.

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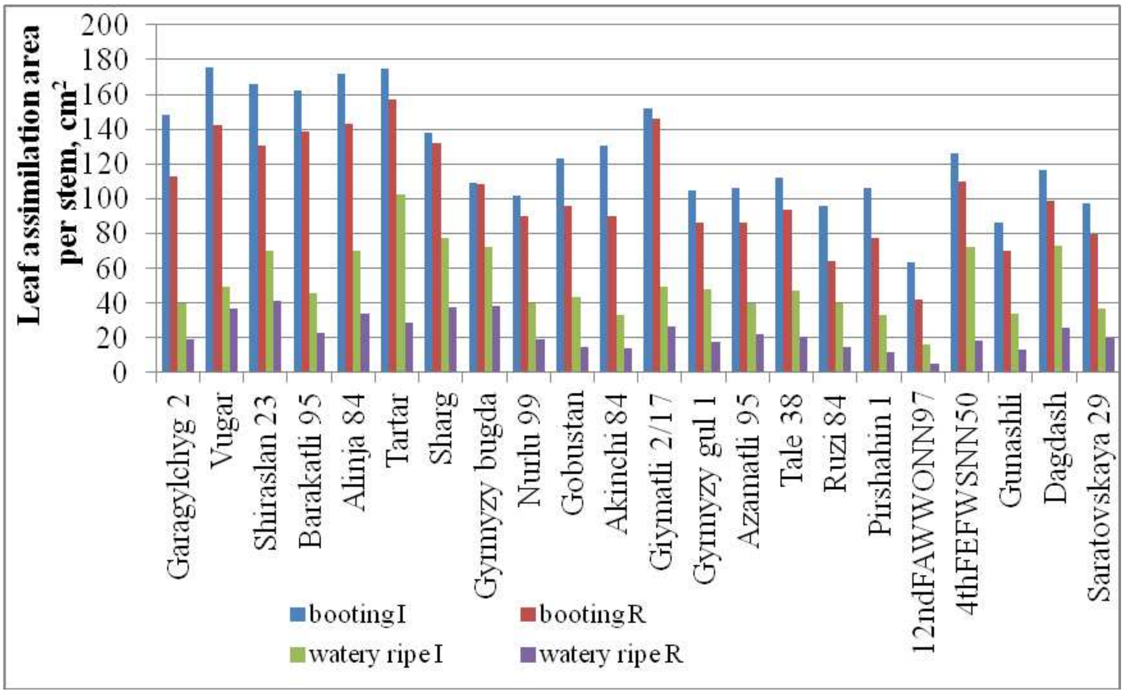

Figure 6. *Effect of drought stress on leaf area per stem (2013/14). Each value represent mean of 7 replicates. I-irrigated, R-rainfed.*

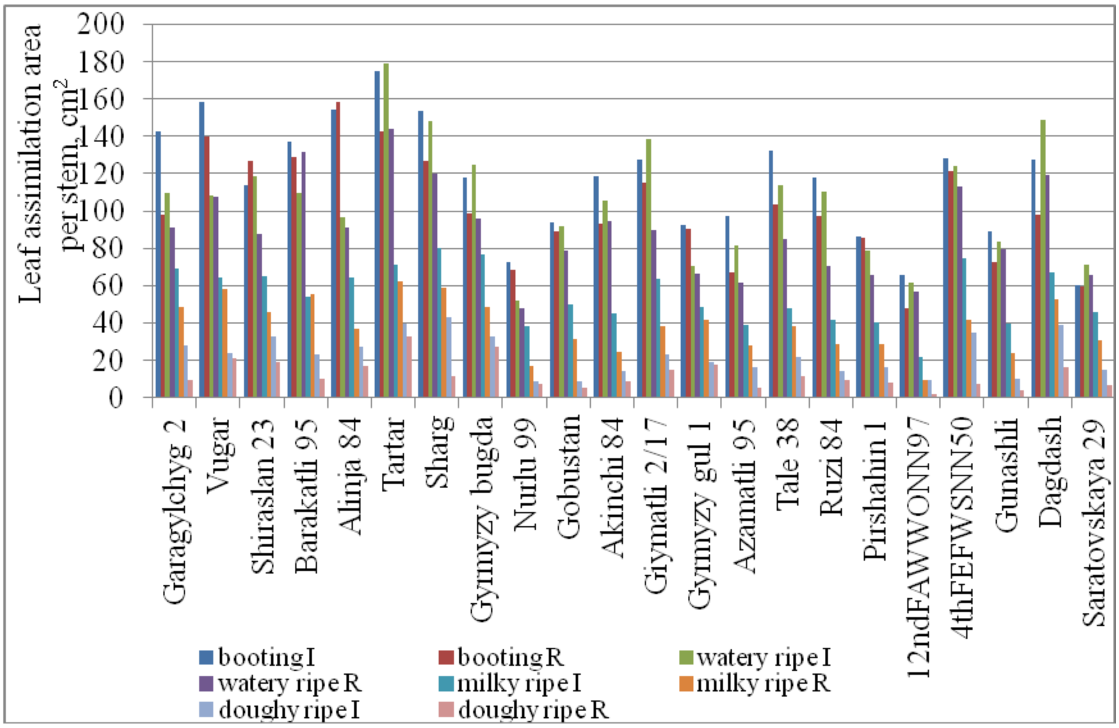

Figure 7. *Effect of drought stress on leaf area per stem (2014/15). Each value represent mean of 7 replicates. I-irrigated, R-rainfed.*

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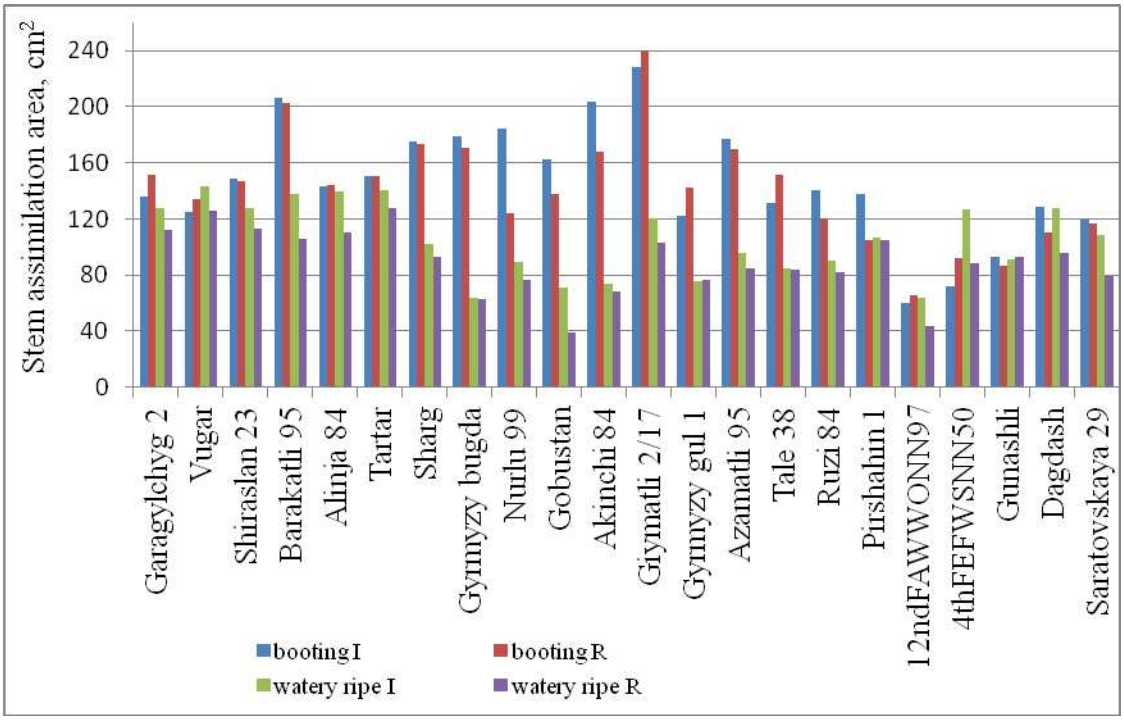

Figure 8. *Effect of drought stress on stem assimilation area (2013/14). Each value represent mean of 7 replicates.*

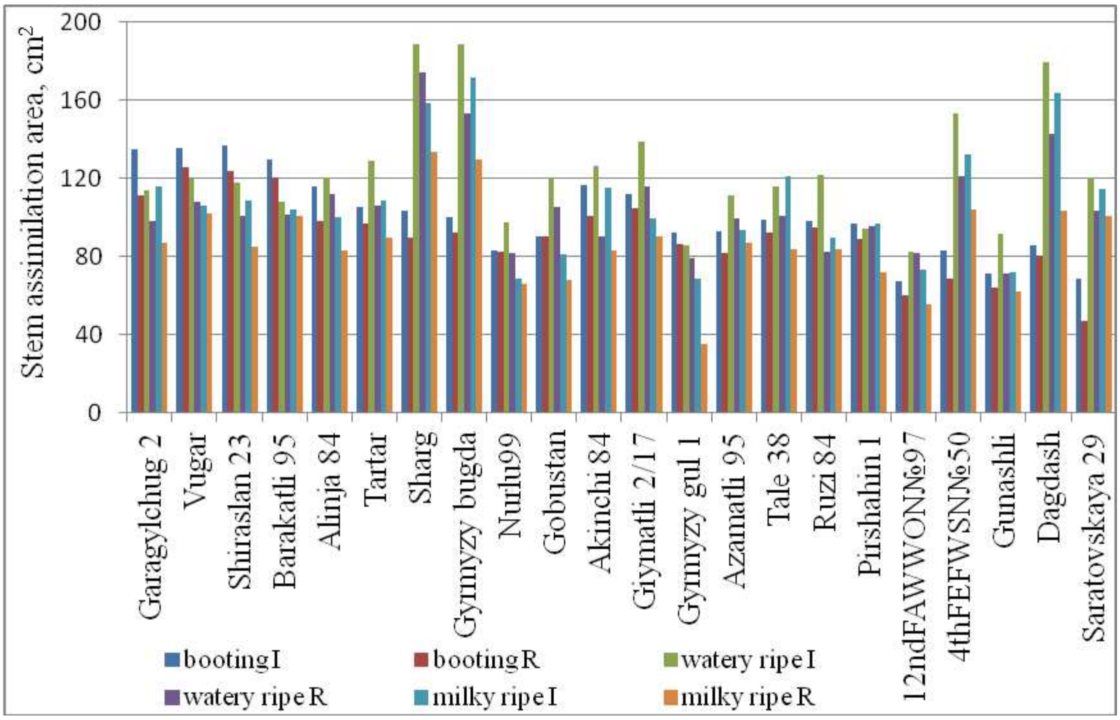

Figure 9. *Effect of drought stress on stem assimilation area (2014/15). Each value represent mean of 7 replicates.*

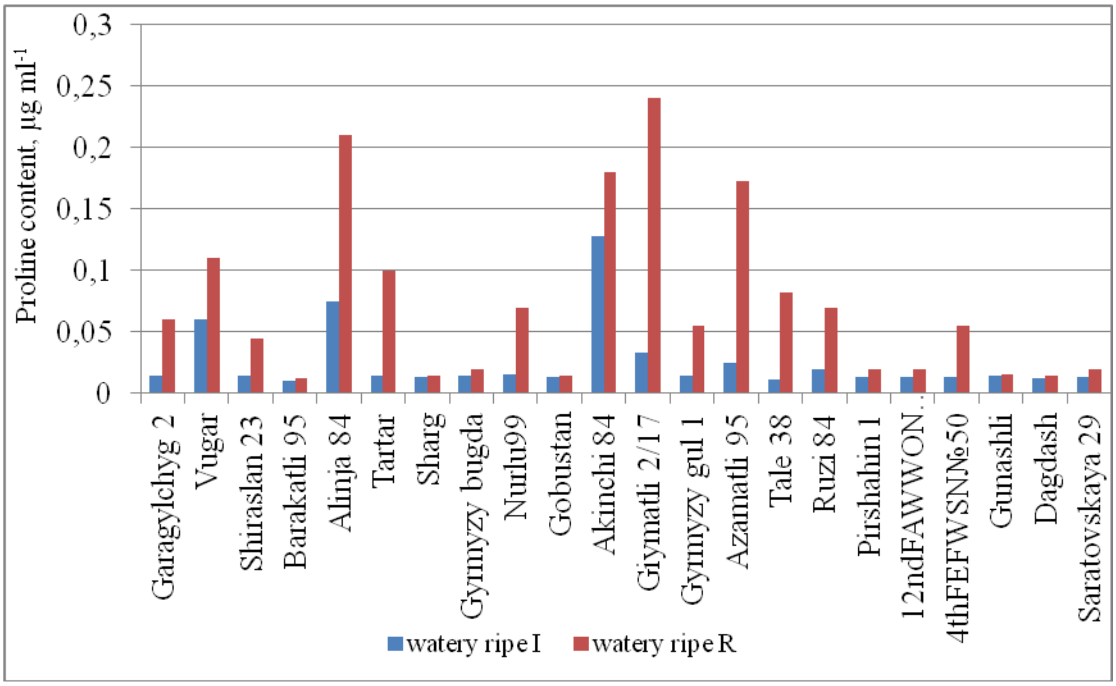

Figure 10. *Effect of drought stress on proline content (2014/15). Each value represent mean of 3 replicates.*

The grain yield of the majority of genotypes was higher in the 2013/14 growing season than that in the 2014-2015 growing season under irrigation (*Fig. 11*). However the grain yield of most genotypes was relatively higher in the 2014/15 growing season under rainfed condition. A relatively strong reduction in grain yield of genotypes occurred in the 2013/14 growing season. An average grain yield of durum and bread wheat genotypes was 588.6 g/m^2 and 670 g/m^2 under irrigation, 357.4 g/m^2 and 451.5 g/m^2 under rainfed condition in the 2013/14 growing season. An average grain yield of durum and bread wheat genotypes was 539.3 and 558.4 g/m^2 under irrigation, and 382.8 and 443.2 g/m^2 under rainfed condition in the 2014/15 growing season. Reduction of grain yield was 39% for durum wheat, 33% for bread wheat in the 2013/14 growing season and 29% for durum wheat, 21% for bread wheat in the 2014/15 growing season, respectively. A potential productivity of genotypes Garagylchyg 2, Barakatli 95, Tartar, Giymatli 2/17, Gyrmyzy gul 1, Tale 38, Pirshahin 1, 4thFEFWSN№50 were higher under irrigation in the studied years. Genotypes Vugar, Sharg, Gyrmyzy bugda, Nurlu 99, Gobustan, Ruzi 84, 12ndFAWWON№97, Gunashli, Saratovskaya 29 formed stable grain yield under irrigated and rainfed conditions in the studied years. P_n was found to correlate positively and significantly with g_s, E and assimilation area of leaf, stem and spike and leaf area index under rainfed (*Table 3*). The RWC positively correlated with Chl(a + b) and Car(x + c) contents. The highest positive correlations were detected between P_n and g_s, as well as between Chl(a + b) and Car(x+c) contents. Correlations between grain yield and physiological traits (P_n, RWC, LAA, SAA, SPAA) were negative under both irrigated and rainfed conditions. These correlations mean that grain yield is less associated with physiological parameters.

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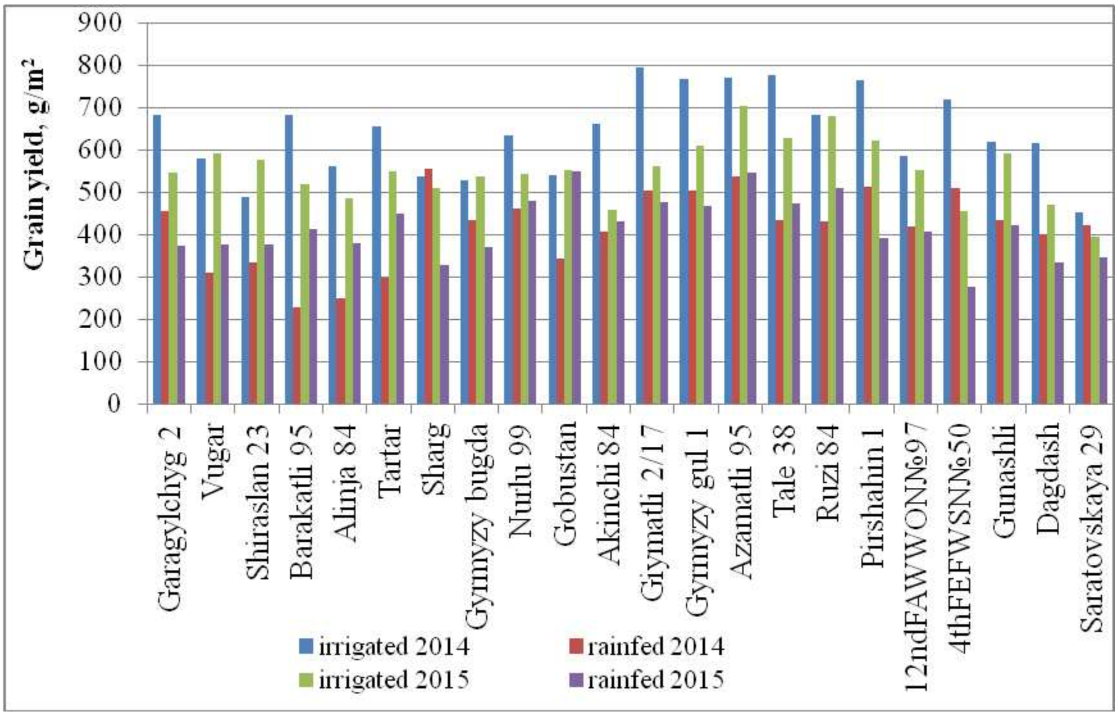

Figure 11. *Effect of drought stress on grain yield. Each value represent mean of 3 replicates*

Table 3. *Correlations between grain yield and physiological traits (2014/15 growing season).*

	Traits	Pn	g_s	E	RWC	Chl(a+b)	Car(x+c)	LAA	LAI	SAA	SPAA	Proline	GY	
	Pn	1	0.284	0.270	0.394	-0.153	0.008	0.677**	0.444*	0.708**	0.584**	0.029	-0.436*	
	g_s	0.653**	1	0.965**	0.202	-0.525*	-0.322	0.122	-0.337	0.214	0.333	-0.154	-0.309	
	E	0.660**	0.951**	1	0.122	-0.532*	-0.371	0.081	-0.314	0.200	0.216	-0.024	-0.186	
	RWC	0.049	-0.123	-0.275	1	0.247	0.444*	0.469*	0.372	0.410	0.389	-0.429*	-0.768**	
	Chl(a+b)	-0.408	-0.310	-0.291	0.305	1	0.803**	0.069	0.381	-0.075	-0.176	-0.136	-0.137	
IRRIGATED	**Car(x+c)**	-0.277	-0.277	-0.232	0.378	0.929**	1	0.158	0.347	0.193	0.005	-0.345	-0.330	**RAIN-FED**
	LAA	0.597**	0.297	0.272	0.484*	0.198	0.324	1	0.734**	0.561**	0.790**	-0.019	-0.428*	
	LAI	0.394	0.155	0.076	0.450*	0.262	0.383	0.736**	1	0.251	0.264	0.005	-0.146	
	SAA	0.406	-0.009	0.021	0.561**	0.053	0.167	0.717**	0.473*	1	0.526*	-0.101	-0.499*	
	SPAA	0.419	0.204	0.151	0.416	0.039	0.142	0.728**	0.292	0.548**	1	-0.076	-0.496*	
	Proline	0.007	0.330	0.410	-0.285	-0.123	-0.033	-0.014	-0.161	-0.005	-0.059	1	0.309	
	GY	-0.134	-0.009	-0.124	-0.241	-0.235	-0.249	-0.162	0.171	-0.392	-0.135	-0.226	1	

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level. P_n- photosynthesis rate, g_s-stomatal conductance, E-transpiration rate, RWC-relative water content, Chl- chlorophyl, Car-carotenoids, LAA-leaf assimilation area per stem, LAI-leaf area index, SAA-stem assimilation area, SPAA-spike assimilation area, GY-grain yield

Discussion

It was reported that water stress decreased P_n, g_s, E values of wheat genotypes at different growth stages (Hassan, 2006; Shan et al., 2012). In the present study, soil water deficit caused a decrease in P_n, g_s, and E values. Limitation of P_n is related to the decrease in g_s and mesophyll conductance (g_m) (Allahverdiyev et al., 2015). The P_n had a positive and significant correlation with g_m, negative and significant correlation with C_i under both irrigated and rainfed field conditions. C_i had a positive, significant correlation with g_s, negative and significant correlation with g_m. Flexas et al. (2009)

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showed that P_n and C_i had a strong correlation with g_s in both field-grown and potted grape wine plants. An increase in C_i indicates predominance of limitation through mesophyll conductance. Under drought condition, the C_i/C_aratio (C_a-ambient CO_2 concentration) was also increased (unpublished dates). After watering of irrigated plots there was a competition between CO_2 and water vapor in the passage through stomata in favor of transpiration. Our results showed that the wheat genotypes Shiraslan 23, Tartar, Sharg, Giymatli 2/17, Tale 38, 4thFEFWSN№50, Dagdash revealed a higher P_n both under irrigated and drought stress conditions. There are inconsistent reports about the relationship between gas exchange characteristics and growth or yield of different cultivars (Ashraf and Harris, 2013). Our results showed that high gas exchange characteristics (P_n, g_s, E) of the genotypes Tartar, Sharg, Giymatli 2/17, Tale 38, 4thFEFWSN№50, Dagdash positively associated with assimilation area formation and dry mass accumulation. A positive association of gas exchange characteristics with grain yield was not strongly fixed. No significant correlation was found between leaf gas exchange parameters and grain yield (Bogale et al., 2011). The absence of correlation between photosynthetic parameters and grain yield is caused by biotic and abiotic factros whose limiting effect can be noticed during the phase of assimilate transport from the source (green photosynthetic organ) to the sink (spike, grain) (Sharkey et al., 1995).

Despite the fact that the gas exchange parameters were strongly influenced by drought, RWC remained relatively higher, especially in the taller genotypes Sharg, Gyrmyzy bugda, Dagdash, and Saratovskaya 29. A decrease in the RWC in response to drought stress was noted in wheat genotypes (Wu et al., 2014). In fact, although components of plant water relations are affected by reduced availability of water, stomatal opening and closing are more strongly affected (Anjum et al., 2011). In our opinion, a strong reduction of g_s allows keeping the RWC at a high level. Under drought stress proline content increased about sevenfold in some genotypes. Proline is osmotically active and contributes to membrane stability, also acts as a signaling regulatory molecule able to activate multiple responses that are components of the adaptation process (Maggio et al., 2002). We revealed a positive correlation between proline content and grain yield under drought condition. Water deficit caused a decrease in Chl a, b and Car(x + c) contents of wheat genotypes, that is in consistent with results of other authors (Chandrasekar et al., 2000). Saving large leaf area per stem under conditions of water scarcity is a good indicator, allowing greater accumulation of photoassimilates transported to the ear. In addition, relatively higher tillering capacity of bread wheat genotypes increased the leaf area index, contributed to greater accumulation of photoassimilates in a unit of assimilating area. Nonleaf organs (stem, spike, leaf sheath) are resistant to water deficit, and are important source of photosynthetic carbon assimilation under drought condition during the grain filling phase (Inoue et al., 2004) A large assimilation area of stem is linked with higher plant height or larger diameter of stem of the studied wheat genotypes. Grain yield is a result of many physiological processes (photosynthesis, respiration etc.), agronomical and yield traits that are under influence of environment. Our results showed that genotypes of bread wheat are more productive than that of durum wheat. Drought stress more affected on grain yield of durum wheat genotypes. Our results showed that grain yield of wheat genotypes is more related to yield components: spike number per unit area, biological yield, and harvest index under rainfed condition (Allahverdiyev, 2016).

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PRODUCTION OF ALKALINE PHOSPHATASE FROM A FACULTATIVE PSYCHROPHILIC *PSEUDOMONAS SP.* MRLBA1 ISOLATED FROM PASSU GLACIER, PAKISTAN

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Abstract. Among our collection of psychrophilic bacteria from various non-polar glaciers, the present study involves the study of the ability of the psychrophilic bacterial strains, isolated from Passu Glacier, Pakistan, to produce thermolabile alkaline phosphatase extracellularly as well as intracellularly. The biophysio-chemical characteristics and 16S rRNA signatures of the isolate MRLBA1 were similar to the members of genus *Pseudomonas.* This rod shaped MRLBA1 isolated from glacial ice exhibited aerobic growth cycle with cardinal limits of pH; 4-11 and temperature; 2-30°C. The strain showed highest extracellular alkaline phosphatase activity at pH 8.0 and 18°C when inoculated with 24 h old inoculum (5%), after 48 h of incubation in shake flask at 150 rpm. The strain's targeting mechanism of alkaline phosphatase at cell membrane as well as in the extracellular medium is interesting. The results demonstrated that alkaline phosphatase being thermolabile, acts as a stress protein also in addition to harvesting the energy directly in carbon deficient ice.
Keywords: *psychrotrophs, thermolabile activity, mountain glaciers, phosphomonoesterase*

Introduction

Non-polar glaciers are analogous to other frozen bodies of the crust and harbor simple life entombed since ages. Facultatively psychrophilic bacteria have been reported in normal flora of glaciers which play major role in harvesting energy through re-designing their metabolic machinery (Morita and Moyer, 2001). Glacial ice represents a unique niche with variant aspects for physiology of bacteria including scarce nutrients, pH, temperature, properties of water and other physico-chemical parameters. A long term exposure and adaptability of these bacteria provide with modified pattern of physiology though with similar biochemistry. Psychrophilic microorganisms produce enzymes which are adapted to be active at low temperatures. As compared to their mesophilic counterparts, they display an increased catalytic efficiency over a temperature range of about 0-30°C and a high thermosensitivity. These enzymes have a great potential to be used in biotechnology industry, e.g. in the detergent and food industries, production of fine chemicals and in bioremediation processes (Gerday et al., 1997). Contrary to primary metabolites (proteases, lipases, nucleases), phosphate cannot be synthesized and must be obtained from nucleic acids, phosphorylated sugars and proteins etc. The nutrient deficient ecosystems stipulate the

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release of Pi through hydrolysis of phosphate esters using phosphatises (De Prada et al., 1996).

In an intensive competition for phosphate, bacteria express enhanced activities of phosphatases. We propose that the same phosphatase under various inductions like stress of temperature is processed and targeted differently, as suggested in previous reports (De Prada et al., 1996; Vincent et al., 1992). There is wide variation among phosphatases depending on specificity of substrate, temperature and pH range, and/or requirement of metal ions (Hong et al., 2007). Periplasmic membrane bound (Baoudene-Assali et al., 1993) and extracellular (Glew and Health, 1971) isozymes of phosphoesterase activity have been reported previously. Phosphatases play major role in ability of a psychrophile to compete at low temperature and attribute in its various commercial applications. Alkaline phosphatase (EC 3.1.3.1) is a cellular metabolite widely distributed in nature from microbes to complex animals. In vitro applications of these enzymes span diagnostics (markers, biosensors), molecular biology (dephosphorylation) and immunology (ELISA), Western blot, nucleic acid hybridization and In-situ hybridization. A heat labile phosphatase will be beneficial in terms of time and shortening of protocols; as robust enzyme without prior thawing procedure before use, or activity silencing steps using heat inactivation (Col et al., 2010).

Lee et al. (2015) reported mAP enzyme as the first thermolabile alkaline phosphatase found in cold-adapted marine metagenomes and expected to be useful for efficient dephosphorylation of linearized DNA. The gene encoding a novel AP was isolated from a metagenomic library constructed with ocean-tidal flat sediments from the west coast of Korea. The amino acid sequence of mAP showed a high degree of similarity to other members of the AP family. Phylogenetic analysis showed mAP to be a member of a recently identified family of PhoX that is different from the classical PhoA (Lewenza et al., 2005) family. The crystal structure of a cold-active alkaline phosphatase from a psychrophile, *Shewanella sp.* (SCAP), have been determined by Tsuruta et al. (2010). Psychrophiles synthesize cold-active enzymes to sustain their cell cycle, and these enzymes are already used in many biotechnological applications requiring high activity at mild temperatures or fast heat-inactivation rate. Most psychrophilic enzymes optimize a high activity at low temperature at the expense of substrate affinity, therefore reducing the free energy barrier of the transition state (Struvay and Feller, 2012).

Isolate MRLBA1 exhibited robust phosphatase activity which was inactivated completely at 56°C, suggesting it as a heat labile enzyme. A comprehensive biophysio-chemical characterization and identification of *Pseudomonas sp.* MRLBA1 is presented here. With emphasis on physiological adaptations of this strain under stress conditions, alkaline phosphatase is proposed to be expressed differently and functioning under various inductions, as described here.

Materials and methods

Sample collection

Samples of deep ice and melt water were collected in sterile polypropylene bags and bottles aseptically from Passu Glacier (temperature 0°C, pressure 794 mb, located about 2830 m above sea level) situated at 36° 27'21.9E and 074° 52'32.8N in Karakoram Range of mountains, Northern Areas of Pakistan. The samples were transported to the laboratory in their intact physical conditions and stored at -80°C.

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Isolation and characterization of isolate MRLBA1

Samples of molten ice and water were serially diluted up to ten fold in normal saline. About 100 µl of each dilution was spread on Nutrient agar plates (Oxoid, Basingstoke, UK), which were then, incubated aerobically at 4, 10 and 20°C for 7 days. A few discrete colonies were inoculated in Nutrient broth and incubated at 20°C. The isolates were screened for alkaline phosphatase activity intra and extracellularly and the isolate MRLBA1 was selected for further studies.

For morpho-physiological studies, colony morphology (color, shape, elevation, margins, and odor), Gram's staining, optimum temperature, pH, respiration using Gas Generating Kit (Oxoid, UK), growth rate and carbohydrate assimilation using Analytical Profile Index (API® 50CHB, BioMérieux® France) was examined. For biochemical characteristics, the tests were performed following protocols described by Lanyi (1987) and Simbert and Kreig (1994).

Electron microscopy

Cells grown at log phase under conditions given above were fixed with 3% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.3). After dehydration, the samples were coated with gold in a sputter-coater (SPI-Module, USA) and examined with a scanning electron microscope (JSM5910, JEOL, Japan).

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA extraction and amplification of the 16S rRNA gene were done as mentioned previously (Janarthanan and Vincent, 2007) using the set of universal primers; 27F 5′AGAGTTTGATCCTGGCTCAG3′ and 1492R 5′TACGGTTACCTTGTTACGACTT3′ according to Lane (1991) and Reysenbach et al. (1995). PCR products were sequenced according to the manufacturer's instructions using the ABI PRISM® BigDye™ Terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom). The nucleotide homology search was performed against the partial 16S rRNA sequences of 1047 base pairs using the nucleotide BLAST program "BLASTn" (Altschul et al., 1997) in non-redundant (nr) data base. The sequences having equal or more than 97% sequence homology but validated by IJSEM previously were retrieved from the Gene Bank and aligned using Clustal W program (Thompson et al., 1994) in the Molecular Evolutionary Genetics Analysis Program (MEGA) version 4.0.1 (Tamura et al., 2007). The regions in the sequences corresponding to the isolate MRLBA1 sequence were retained and all non-aligned sequence parts were trimmed. This alignment was used to construct a neighbor joining (NJ) tree and finally the maximum parsimony (MP) tree (Eck and Dayhoff, 1966) using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with random addition of 10 replicates.

Culture conditions for production of phosphatase enzyme

The cultures were routinely maintained on nutrient agar slants at 4°C. The production of enzyme was carried out in modified production medium (0.5% peptone, 0.2% glucose, 0.08 M NaCl, 0.2 mM $CaCl_2$, 0.02 M NH_4Cl, 0.02 M KCl, 1 mM $MgSO_4$ and 0.004 mM $ZnCl_2$). Sodium phosphate (0-200 µM) and calcium phosphate (0-50 mM) were added in the basal medium to study the regulation of alkaline phosphatase production as given below. The effect of pH on growth and enzyme production was studied by growing cells in basal medium. The pH of the medium was adjusted to 5-13 using appropriate buffers (10 mM; Dhaked et al., 2005). Growth was measured spectrophotometrically at 600 nm (Agilent 8354).

Enzyme assay conditions

Activity of alkaline phosphatase was measured by the method of Dhaked et al. (2005) by measuring the absorbance (OD_{405}) to monitor the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP) as mentioned. Reaction mixture contained 300 µl of enzyme diluted in 1 M diethanolamine buffer (pH 9.8), 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 150 mM *p*-nitrophenyl phosphate (pNPP), in a final volume of 3 ml.

The reaction performed at 37°C for 30 min was stopped by addition of 50 µl of 4 M sodium hydroxide. One unit was defined as the amount of alkaline phosphatase which hydrolysed 1 µmol of *p*-nitrophenyl phosphate to *p*-nitrophenol in 1 min at pH 9.8 and 37°C. The quantification of enzyme activity (OD_{405}/OD_{600}) was done by standard curve of *p*-nitrophenol (0-500 µM) at 405 nm.

Results and discussion

Isolation and characterization of isolate MRLBA1

The bacterial colonies appeared on Nutrient agar (pH 8.0) after incubation at 4°C for 16 days. The isolate MRLBA1 formed white, smooth circular colonies. The cells were Gram negative short rods (*Fig. 1*).

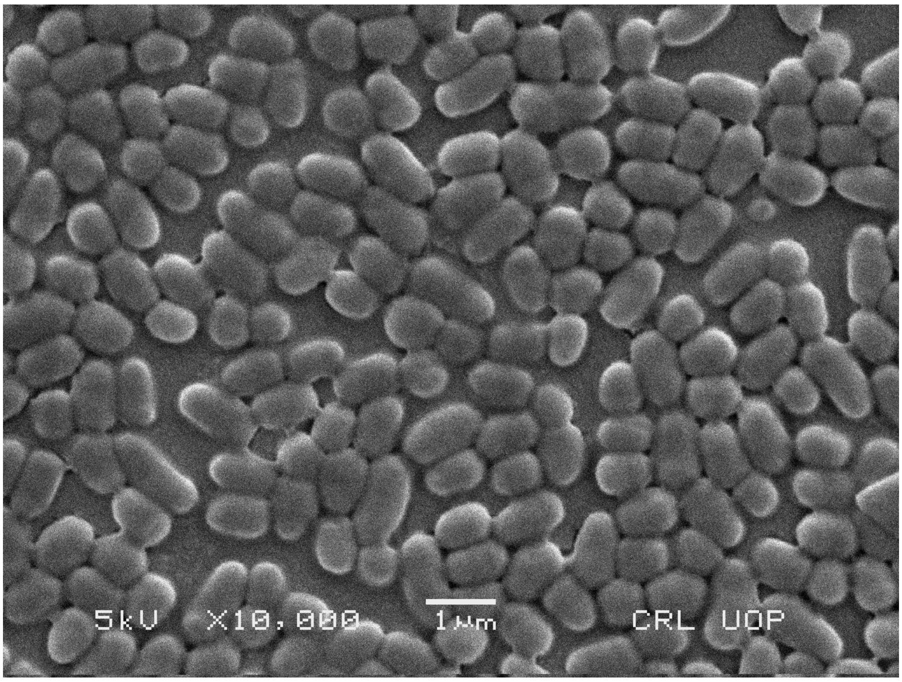

Figure 1. *Scanning electron micrograph of cells of Pseudomonas sp. MRLBA1 (10000× at 5 kV). The bacteria were harvested in log phase and i). Fixed: (3% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.3), incubated at 4°C), Rinsed: (0.1 M phosphate pH 7.3), Dehydrated: (50-90% gradients of acetone for 10 min each), and dried using $CaCl_2$ (Stadtländer, 2007)*

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Obligatory aerobic isolate MRLBA1 was positive for catalase, urease, and Voges Proskauer tests. The isolate exhibited facultatively psychrophilic adjustment of temperature and exhibited respective cardinal and optimum limits of temperatures as: 2-37°C; 25°C, and pH: 4-11; 9 (*Fig. 2*). The physiological pattern of metabolism and carbon assimilation is given in *Tables 1* and *2,* respectively.

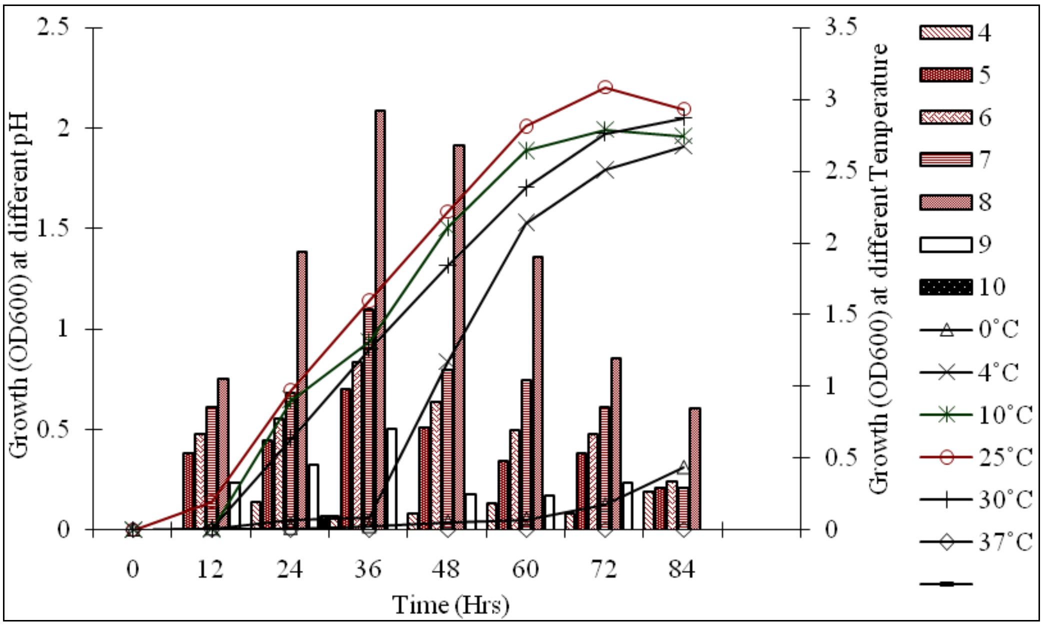

Figure 2. *Effect of pH and temperature on the growth of Pseudomonas sp. MRLBA1. The isolate exhibited maximum growth at pH 8 and 25°C*

Table 1. *Morpho-physiological and biochemical characteristics of the MRLBA1 isolate*

Colony	
Color	White
Margin	Smooth
Gram's staining	-
Cells	
Shape	Rod
Spore	-
Motility	-
Oxygen utilization	Aerobic
Growth Cardinals	
Lower limit	4
Upper limit	11
Lower limit (°C)	2
Upper limit (°C)	30
Biochemical	
Alk. Phosphatase	+
Amylase	+
Catalase	+
Gelatinase	-

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Methyl Red	-
Nitrate Reductase	-
Simmon citrate	-
Triple sugar Iron	-
Urease	+
Voges-Proskauer	+

Table 2. *Carbon source utilization of the isolate MRLBA1*

Carbon source	Growth	Carbon source	Growth	Carbon source	Growth	Carbon source	Growth
Glycerol	+	D-Fructose	+	N-Acetyl Glucosamine	-	Glycogen	-
Erythritol	-	D-Mannose	+	Asculin	-	Xylitol	-
D-Arabinose	+	Methyl α-D Glucopyranoside	-	Salicin	-	Gentiobiose	+
L-Arabinose	+	L-Sarbose	-	D-Cellobiose	+	D-Turanose	-
D-Ribose	+	L-Ramnose	-	D-Maltose	+	D-Lyrose	-
D-Xylose	+	Dulcitol	-	D-Lactose	+	D-Tagatose	-
L-Xylose	-	Inositol	+	D-Mallobiose	+	D-Fucose	+
D-Addonitol	-	D-Mannitol	+	D-Sucrose	+	L-Fucose	+
Methyl β-Dxylpyranoside	-	D-Sorbitol	-	D-Trehalose	+	D-Arabitol	+
D-Glactose	+	Methyl α-D Mannpyrunoside	-	Inuline	-	L-Arabitol	+
D-Glucose	+	Amygdaline	-	D-Melezitose	-	Potassium Gluconate	+
Glycerol	+	Arabutin	-	D-Rafinose	+	Potassium 2-Ketoglutarate	+
Erythritol	-	L-Arabinose	+	Amidon	-	Potassium 5-Ketogluconate	+
D-Arabinose	+	D-Ribose	+	D-Addonitol	-	D-Xylose	+
D-Xylose	+	Methyl β-Dxylpyranoside	-	L-Arabinose	+	L-Xylose	-
L-Xylose	-	D-Glactose	+	D-Ribose	+	D-Addonitol	-
Methyl β-Dxylpyranoside	-	D-Melezitose	-	D-Turanose	-		
D-Glactose	+	D-Rafinose	+	D-Lyrose	-		

Expression of proteins from stressed microorganisms entombed away from natural exposure of communities may provide clues of resistance mechanism other than acquired through exposure to antibiotics. Out of seven β-lactam antibiotics (cell wall synthesis inhibitors and protein synthesis inhibitors), isolate MRLBA1 exhibited sensitivity to neomycin (18 mm), streptomycin (22 mm); vancomycin (11 mm), aztreonam (24 mm) and tetracycline (25 mm) but was resistant to penicillin and fosphomycin (*Table 3*). The additional antibiotic resistance mechanisms regulated through Mg^{++} (Lewenza, 2005), also regulate the secretion and/or activity of alkaline phosphatase.

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Table 3. *Antibiotic sensitivity of Pseudomonas sp. MRLBA1*

Class	**Antibiotics (groups)**	**Zone of inhibition (MRLBA1)**
Protein synthesis inhibitors	Streptomycin (aminoglycoside)	S (22)
	Neomycin (aminoglycoside)	S (18)
Cell wall synthesis inhibitors	Vancomycin (glycopeptide)	S (11)
	Tetracycline (glycopeptide)	S (25)
	Penicillin (β-lactam)	R
	Aztreonam (monocyclicβ-lactam)	S (24)
	Fosphomycin (phosphonomycin)	R

R = resistant, S = susceptible

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequence and relevant phylogenetic analysis revealed that bacterium MRLBA1 closely resembles genus *Pseudomonas* (*Fig. 3*).

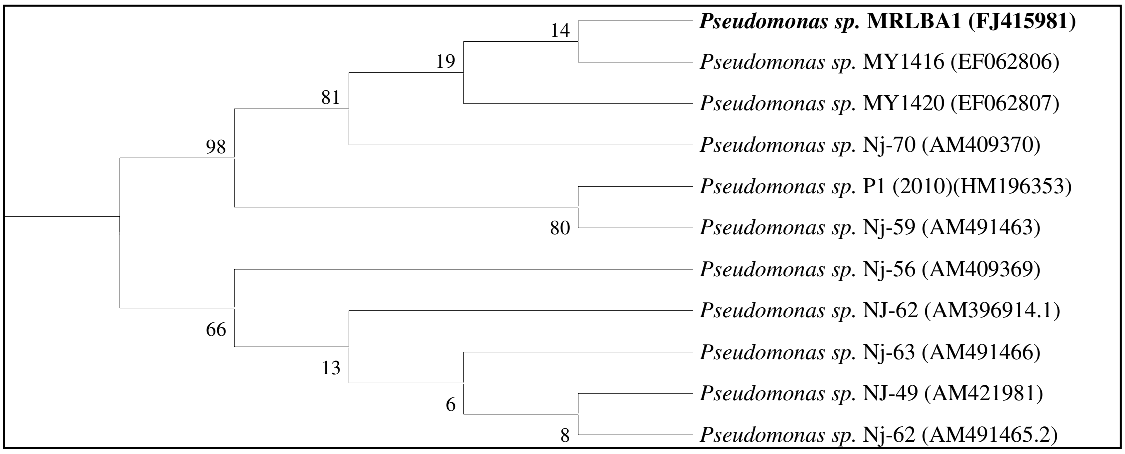

Figure 3. *Maximum parsimony phenogram; showing phylogenetic relationship between Pseudomonas sp. MRLBA1 and other related species of the genus Pseudomonas and related reference microorganisms based on the 16s rRNA gene sequence analysis. Bootstrap values (500 replicates) are given at the nods. Tree was generated with 10 replicates using MEGA4 (Close-Neighbor-Interchange algorithm)*

The closest relative appears to be *Pseudomonas sp.* MY1416 with a similarity of 97% in BLAST analysis. The isolates from polar (Antarctica) and non- polar glaciers like mountain glaciers of Pakistan-China border mostly show novel characteristics, since most of the flora has not been studied in these ecosystems. A small number of similar isolates have been characterized as psychrophilic (Miteva, 2008) including some species

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from *Methanococcoides*, *M. burtonii* (Franzmann et al., 1992). Along with other nearby glaciers in the region, like Batura Glacier in Hindu Kush (West Pakistan), Mingyong Glacier in Tibet (North China), Siachen in Himalaya (North-East Kashmir) and the Karakoram glaciers present a huge frozen ancient ecosystem out of pole harboring psychrophilic mode of life. From this isolated cold niche, contrary to other *Pseudomonas* species under study, the isolate MRLBA1 showed resistance to certain cell wall synthesis inhibitors like penicillin (β-lactam) and fosphomycin (phosphonomycin). The finding points out the strain's spatial induction of genetic assembly and/or cell wall divergence.

Growth and enzyme production

Psychrotolerants with scant energy express alkaline phosphatase as a stress protein to release energy from phosphoanhydrides – the high energy bonds (Seufferheld et al., 2008). Various cold active bacterial species including *Arthrobacter sp.* (De Prada et al., 1996), *Bacillus sphaericus* P9 (Dhaked et al., 2005), *Pseudomonas aeruginosa* (Cheng et al., 1970) and *Pseudomonas fluorescens* E2 and *Pseudomonas sp.* 8E3 (Pratt-Lowe et al., 1988) have been used for the production of alkaline phosphatase. The cell mass was observed as 1.81 cells per hour during exponential phase of the bacterium. A plot of growth (O.D) versus time (hours), yielded a typical growth curve with a prolonged lag phase of about 10 h. The exponential phase appeared as a steep curve after 24 h and lasted up to 36 h followed by a long stationary phase that lasted up to 120 h (*Fig. 4*). The bacterial physiology is not dependent upon the growth rates observed at various temperatures. Also the viable counts have been considered as the crucial parameter to distinguish between psychrophilic and psychrotrophic beings (Feller et al., 1994). A very little activity of alkaline phosphatase (0.396 U/ml) was found associated with periplasmic space during growth curve.

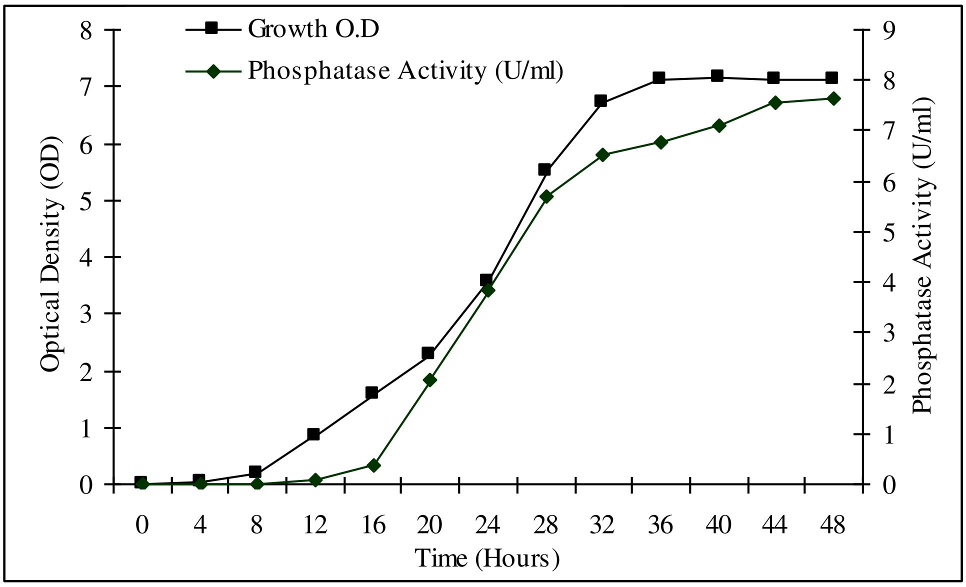

Figure 4. *Production of alkaline phosphatase at different stages of bacterial growth. Cells were grown in the basal medium described in the text and growth was monitored by measuring optical density at 600 nm. Phosphatase activity was estimated in the culture supernatant at different stages of growth*

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Batch culturing in shake flask

Pseudomonas sp. MRLBA1 yielded 16 g of wet cells after 72 h from 1.6 l biomass under optimized conditions.

Effect of pH on the production of alkaline phosphatase

The *Pseudomonas sp.* MRLBA1 was capable of growing in the pH range of 4-11 with maximum growth at pH 9.0. However, the strain produced alkaline phosphatase optimally at pH 8.0 after 72 h of incubation at 18^{o}C and 150 rpm with specific activity of 57.56 U/mg protein ($P < 0.0001$). The production of enzyme (specific activity) was reported as 33.05, 44.32, 47.14, 56.28 and 35.39 U/mg at pH 5.0, 6.0, 7.0, 9.0 and 10.0, respectively (*Fig. 5a*).

The production of enzyme significantly decreased on pH other than optimal value. The production of enzyme was targeted in late stationary and death phase of the growth curve. A very little activity was found attributed to the cell bound during growth cycle of the bacterium (*Fig. 5a*).

Effect of temperature on the production of alkaline phosphatase

Optimum alkaline phosphatase was produced (22.41 U/ml) with specific activity (53.72 U/mg) at 18°C after 48 h of incubation ($P < 0.0001$). At lower temperatures i.e. 4, 10 and 15°C, the production of enzyme (6.73, 11.13 and 14.57 U/ml) with specific activities as 28.56, 41.17 and 45.81 U/mg, respectively (*Fig. 5b*). The yield of enzyme was enhanced when harvested cells were temperature shocked at -70°C for 10 min and harvested again after resuspension in 20 mM Tris HCl (pH 8.0; *Fig. 5b*). The same practice has been adapted by Hong et al. (2007) for *E. coli* and Cheng et al. (1970) for *Pseudomonas aeruginosa* to collect the alkaline phosphatase from periplasm.

Effect of incubation period

The inoculated production medium revealed specific activities of 4.42 U/mg (24 h), 54.01 U/mg (48 h) and 59.43 U/mg after 72 h of incubation at pH 8, 18°C for 72 h and 150 rpm (*p* 0.07; *Fig. 5c*).

Effect of size of inoculum on the production of alkaline phosphatase

Optimum alkaline phosphatase (specific activity; 77.02 U/mg) was produced in case of 5% inoculum, whereas, in case of 1, 10, 15 and 20% inoculum size, the specific activities were 38.71, 32.37, 18.85 and 12.7 U/mg, respectively, with significant value ($p > 0.001$; *Fig. 5d*). The pH of the medium increased up to 8.3 starting from initial pH 8.0.

Effect of age of inoculum on the production of alkaline phosphatase

Optimum production of alkaline phosphatase (56.42 U/mg) was observed in production medium inoculated with a 24 h old inoculum after 48 h of incubation at pH 8.0, 18°C, 5% inoculum and 150 rpm ($p < .001$). When 12, 48 and 72 h old inocula were used, specific activities of alkaline phosphatase were recorded as 34.19, 39.53.12 and 16.8 U/mg respectively (*Fig. 5e*).

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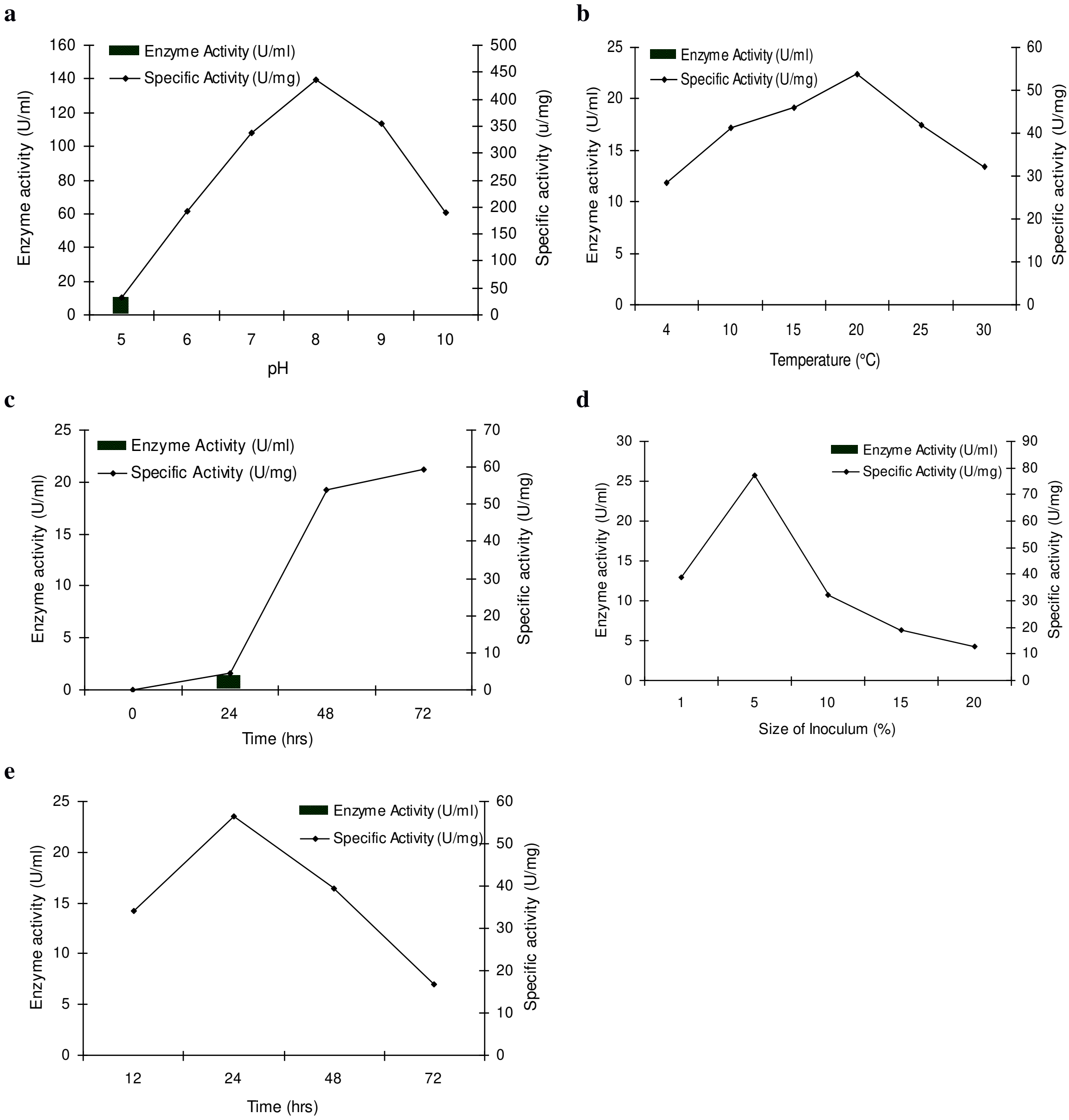

Figure 5. *Effect of various parameters (a-e) on the production of alkaline phosphatase. a Effect of pH on the production of alkaline phosphatase. b Effect of temperature on the production of alkaline phosphatase. c Effect of incubation period on the production of alkaline phosphatase. d Effect of size of inoculum on production of alkaline phosphatase. e Effect of age of inoculum on the production of alkaline phosphatase*

Conclusion

Out of pole, the mountain glaciers are a big resource of psychrophilic microbial diversity and hence and hence for industrial biotechnology. Cold active bacteria from depth of glacial ice could be potential source of enzymes capable of catalysis at lower temperatures. Bacterial metabolism at psychrophilic and/or psychrotrophic range of temperatures depends on the adjustment of their enzymes to work in cold conditions. Hence their enzymes drive the evolutionary adaptations. Psychrophilic enzymes produced by cold-adapted microorganisms display a high catalytic efficiency and may

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be associated with high thermosensitivity. The alkaline phosphatase from *Pseudomonas sp.* MRLBA1 was produced in acceptable range of physiological parameters in accordance with previous reports and implies an uncomplicated commercial source. The characterized production in this investigation is a valuable distinguished information to proceed for pilot scale production of low temperature active alkaline phosphatase.

We want to further this study for further purification and characterization of the heat sensitive activity.

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EFFECT OF PHOSPHORUS ON N, P, K, Mg ACCUMULATION AND PLANT GROWTH OF DIFFERENT CITRUS ROOTSTOCKS

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Abstract. Phosphorus is an essential nutrient for the growth of citrus plants. A pot experiment was conducted in greenhouse to investigate the citrus cultivars grafted on 10 rootstocks (Cuningmeng, Suanju, Suancheng, Honglimeng, Zhike, Goutoucheng, Xiangyuan, Hongju, Xiangcheng, and Zhicheng) in response to the different phosphorus treatments viz, low P (LP) = 0.01 mmol L^{-1}, moderate P (MP) = 0.5 mmol L^{-1}, high P (HP) = 1 mmol L^{-1}) concentrations. All P concentrations were employed in pots containing Hoagland solution. The leaves, branch, root, and total dry weight, and nitrogen, phosphorus, potassium and magnesium concentrations in the different plant parts were determined. Results showed that P application increased N, P, K and Mg content in different plant parts. With increasing P application, the root dry weight, branch dry weight, leaves dry weight, and total dry weight increased whilst low P treatment affected the P, K, and Mg accumulation in root and ultimately plant growth. Moreover, low P concentrations also affected Mg content in leaves, that can lead to low plant photosynthesis and finally decreased the plant dry biomass

Keywords: *citrus rootstocks, phosphorus treatment, plant growth, plant nutrients accumulation*

Introduction

The rootstocks have a critical role in the fruit cultivation (Shafieizargar et al., 2012; Cantuarias-Avilés et al., 2010, 2011; Tazima et al., 2013). Rootstock affects the horticultural characteristics in citrus, such nutritional quality (Zhang et al., 2011), tree performance (Cantuarias-Avilés et al., 2010), stress resistance (Simpson et al., 2014), fruit yield (Hussain et al., 2013), and quality (Benjamin et al., 2013). Thus, it is meaningful to choose various rootstocks for investigating citrus plant growth, elements absorption, yield, quality; however, studies are still needed to evaluate the adopted rootstocks for citrus trees.

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Phosphorus is important for plant growth, development and reproduction for it is constituent of phospholipids, nucleic acid and many proteins. Low P availability in soil is one of the most critical aspects that limit the productivity of many crops (Sanchez and Salinas, 1981; Liao and Yan, 2003). P deficiency or excess could cause nutritional imbalance in citrus tree, then may reduce the yield and fruit quality (Fan and Wang, 2012; Fan and Luo, 2015). Too low or too high P concentrations may disturb the uptake of N, K, Fe, Zn, and B in plants (Fan and Wang, 2012). P also affects the root morphological and physiological characteristics of different root stocks (Fan and Luo, 2015). On the other hand, Syvertsen (1987) did not find any relationship between the P level in leaves and photosynthetic capacity of orange and pomelo seedlings. Moreover, Bernardi et al. (2015) reported that P had little effects on photosynthesis. Zambrosi et al. (2013a) indicated that P uptake by young citrus plants in low P soil depends on rootstock varieties and nutrient management. Significant variations in the ability of citrus rootstocks to acquire P from the soil was differs with the nature of the rootstock (Wutscher, 1989; Mattos et al., 2006). Additionally, P application could mitigate the effects of hostile environment (Pestana et al., 2005; Gimeno et al., 2010). Zambrosi et al. (2013b) stated that sufficient P might contribute to increase the ability of young citrus trees to cope with Cu toxicity. Hence, P application could alter morpho-physiological characteristics, yield and quality and nutrient uptake in plants, however, there are still some specific mechanisms that explain the differential responses of cultivated citrus to P fertilization are not yet fully explored. The specific objective of this study was to evaluate the effect of the P on nutritional accumulation and citrus tree growth.

Materials and Methods

Experimental design

A pot experiment was conducted in greenhouse in South China Agricultural University. Citrus cultivars Cuningmeng (*Citrus jambhiri* Lush), Suanju (*Citrus reticulata* Blanco), Suancheng, Honglimeng (*Citrus limonia* Osbeck), Zhike [*Poncirus trifoliate* (L.) Raf.], Goutoucheng (*Citrus aurantium* L.), Xiangyuan (*Citrus medica* var . *ethrog* Engl.), Hongju (*Citrus reticulata* Blanco cv. Red tangerine), Xiangcheng (*Citrus junons* Sieb.), and Zhicheng (hybird of *Citrus sinensis* Osbeck. ×*Poncirus trifoliate* Raf) were used as rootstocks in this study. Five leaf old seedlings were transferred to the different P treatments in Hoagland solution. Three P treatments *viz,* low P treatment (LP) = 0.01 mmol L^{-1}, moderate P treatment (MP) = 0.5 mmol L^{-1}, high P treatment (HP) = 1 mmol L^{-1}) were employed in Hoagland solution with four replications. The nutrient solution was renewed once every three days.

Sampling and measurement

Dry weight measurement

After 45 days of treatment, three representative plants in each treatment and each cultivar were harvested and separated into root, branch and leaves. The plant samples were then oven dried at 105°C for 30min and then at 75°C to a constant weight for measurement of dry weight.

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N, P, K and Mg content measurements

The plant root, branch and leaves sample were then ground into powder for measurement of N, P, K and Mg content and calculation of N, P, K and Mg accumulation.

The N, P, and K content in plant root, branch and leaves were determined as described by Lu (1999). For N, P and K contents, the dried samples (~0.3 g) were digested using the H_2SO_4-$HClO_4$ method. The digestion mixtures were then used to determine the total N content by the Kjeldahl method with a 2300 Kjeltec Analyzer Unit (Foss Tecator AB, Sweden), total P concentration by using a spectrophotometer (Shimadzu UV-2550), and total K concentration by atomic absorption spectrometry (SHIMADZU AA- 6300C AA spectrometer).

Dry sample of plant root, branch and leaves were ground, and approximately 0.100 g of the plant samples were dry-digested in a muffle furnace at 500°C for 6 h, and then 10 ml of HNO_3: H_2O (1:1) was added to extract the ions. The contents of concentration were detected by using an atomic absorption spectrometry (SHIMADZU AA- 6300C AA spectrometer). The Mg content in plant root, branch and leaves was recorded and expressed in mg g^{-1}.

Statistical Analysis

Analyses of variances (ANOVA) and correlation analyses were performed by Statistix version 8 (Statistix 8, Analystical, Tallahassee, FL, USA). Comparisons of means among different P treatments were made according to the least significant difference (LSD) test at 5% probability level.

Results

Citrus plant growth

Significant impacts of cultivars, phosphorus and C×P on citrus plant growth i.e., root, branch, leaves and total plant dry weight were observed. Compared with LP treatment, middle level phosphorus supplement (MP) significantly increased root dry weight, branch dry weight, leaves dry weight, and total dry weight by 18.41%, 79.84%, 91.65%,and 67.72%, respectively; high level phosphorus supplement (HP) remarkably improved root dry weight, branch dry weight, leaves dry weight, and total dry weight by 18.18%, 85.05%, 103.21%, and 74.15%, respectively. For MP treatment, higher root dry weight (73.79% and 50.28%), leaves dry weight (200.78 % and 154.76%), total dry weight (166.43 % and 108.77%) in Cuningmeng and Suanju; while higher branch dry weight (190.57%, 115.32% and 116.16%) was investigated in Cuningmeng, Suanju and Suncheng. However, the root dry weight in Hongju and Xiangcheng was inhibited by 34.53% and 30.63%, respectively. Zhike gained lower branch, leaves and total dry weight improvement, Goutoucheng had lower branch dry weight improvement, and lower leaves dry weight and total dry weight improvement in Hongju was observed. For HP treatment, Cuningmeng and Suanju produced higher root dry weight (85.24 % and 115.08%), branch dry weight (116.60 % and 246.77%), leaves dry weight (167.03% and 279.21%), and total dry weight (133.40% and 214.10%). However, the root dry weight in Suancheng, Goutoucheng, Hongju, Xiangcheng, Zhicheng was inhibited. Zhike and Zhicheng had lower branch dry weight, leaves dry weight and total dry weight gained.

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Therefore, it can be stated that Cuningmeng and Suanju were high P efficiency cultivars but Zhike, Zhicheng or Hongju were low phosphorus efficiency cultivars (*Table 1*).

Table 1. *Effects of phosphorus treatment on citrus plant growth.*

Phosphorus	Cultivar	Root dry weight (g)	Branch dry weight (g)	Leaves dry weight (g)	Total dry weight (g)
LP	Cuningmeng	1.31±0.14	1.38±0.09	2.56±0.25	5.25±0.32
	Suanju	0.60±0.03	0.41±0.05	0.70±0.09	1.71±0.15
	Suancheng	1.31±0.12	1.00±0.14	2.39±0.27	4.70±0.47
	Honglimeng	1.53±0.10	2.09±0.17	2.74±0.17	6.36±0.43
	Zhike	0.76±0.08	0.79±0.02	0.89±0.05	2.44±0.13
	Goutoucheng	0.70±0.03	0.86±0.07	1.26±0.17	2.83±0.27
	Xiangyuan	1.61±0.13	2.52±0.16	3.21±0.23	7.34±0.48
	Hongju	1.02±0.06	0.38±0.04	0.88±0.07	2.29±0.15
	Xiangcheng	1.07±0.06	1.02±0.06	1.83±0.08	3.92±0.19
	Zhicheng	1.67±0.12	1.34±0.06	1.44±0.10	4.44±0.16
	Mean	*1.1587 b*	*1.1781 b*	*1.7902 b*	*4.1269 b*
MP	Cuningmeng	2.28±0.25	4.01±0.22	7.71±0.47	14.00±0.89
	Suanju	0.90±0.10	0.89±0.03	1.78±0.16	3.57±0.29
	Suancheng	1.69±0.06	2.15±0.11	4.44±0.22	8.28±0.38
	Honglimeng	1.92±0.10	3.44±0.23	4.93±0.32	10.29±0.46
	Zhike	0.98±0.09	0.97±0.08	1.14±0.13	3.09±0.29
	Goutoucheng	0.82±0.13	1.17±0.06	2.06±0.17	4.04±0.32
	Xiangyuan	1.94±0.12	4.29±0.16	5.93±0.09	12.16±0.37
	Hongju	0.67±0.03	0.71±0.06	1.32±0.09	2.70±0.12
	Xiangcheng	0.74±0.08	1.60±0.17	2.82±0.20	5.16±0.42
	Zhicheng	1.78±0.14	1.95±0.03	2.18±0.05	5.91±0.20
	Mean	*1.3720 a*	*2.1187 a*	*3.4308 a*	*6.9215 a*
HP	Cuningmeng	2.43±0.18	2.99±0.16	6.85±0.61	12.26±0.76
	Suanju	1.28±0.16	1.43±0.10	2.65±0.23	5.37±0.43
	Suancheng	1.08±0.09	1.46±0.21	3.57±0.21	6.12±0.44
	Honglimeng	1.78±0.24	3.66±0.21	5.72±0.23	11.15±0.60
	Zhike	0.86±0.09	1.01±0.05	1.30±0.09	3.17±0.23
	Goutoucheng	0.63±0.04	1.33±0.08	2.13±0.11	4.09±0.20
	Xiangyuan	2.50±0.31	5.16±0.39	6.90±0.61	14.55±1.3
	Hongju	0.82±0.10	0.91±0.04	1.77±0.1	3.51±0.18
	Xiangcheng	1.00±0.08	2.37±0.13	3.82±0.19	7.19±0.30
	Zhicheng	1.31±0.09	1.48±0.10	1.67±0.12	4.45±0.28
	Mean	*1.3693 a*	*2.1801 a*	*3.6378 a*	*7.1872 a*
F value					
	Cultivar(C)	53.07**	186.02**	143.66**	150.66**
	Phosphorus (P)	8.11**	154.92**	174.77**	140.75**
	C×P	4.91**	13.33**	13.29**	12.98**

Note: different lowercase letters are used to indicate values that are significantly different at $p < 0.05$ among P treatments.

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N content and N accumulation

Significant effects of cultivars, phosphorus and C×P on citrus plant N content and accumulation in different plant part were observed, except for the C×P effect on N content in branch. MP treatment significantly increased N content in root, branch, leaves, N accumulation in root, N accumulation in branch, N accumulation in leaves, and total N accumulation by 34.52%, 24.58%, 24.43%, 56.24%, 112.35%, 124.73%, and 103.25%, respectively. HP treatment significantly increased the N content in root, branch, leaves, N accumulation in root, N accumulation in branch, N accumulation in leaves, and total N accumulation by 31.82%, 29.35%, 25.87%, 52.09%, 126.56%, 142.72%, and 114.33%, respectively. Highest N content increment were observed in Goutoucheng for MP and HP treatment, while lowest increment in N content in branch and leaves were investigated in Cuningmeng for MP and HP. The highest increment in N content in branch and leaves were found in Quenching for MP and in Goutoucheng for HP. For the accumulation of N in citrus plant, Hongju and Xiangcheng had lower N accumulation in root for MP treatment, the highest N accumulation in branch was observed in Suancheng but the lowest N accumulation in branch was investigated in Zhike for MP. Higher N accumulation in leaves and total N accumulation were detected in Suancheng and lower N accumulation in leaves and total N accumulation were found in Zhike for MP. Suancheng had the highest N accumulation in plant while Zhicheng had the highest N accumulation in plant for HP (*Table 2*).

P content and P accumulation

Cultivars, phosphorus and C×P significantly affected P accumulation in citrus plants. MP treatment significantly increased P content in root, branch, and leaves. P accumulation in root, P accumulation in branch, P accumulation in leaves, and total P accumulation increased by 182.05%, 189.96%, 193.30%, 231.51%, 420.40%, 474.22%, and 388.03%, respectively. HP treatment dramatically increased P content in root, P content in branch, P content in leaves, P accumulation in root, P accumulation in branch, P accumulation in leaves, and total P accumulation by 211.06%, 197.08%, 205.49%, 257.99%, 455.24%, 547.75%, and 437.76%, respectively. For MP treatment, the increment in P content in root ranged from 129.46% to 298.85%, P content in branch ranged from 72.85% to 251.95%, P content in leaves ranged from 73.82% to 272.43% and P accumulation in root ranged from 73.08% to 586.10%, P accumulation in branch ranged from 113.59% to 847.09%, P accumulation in leaves ranged from 124.77% to 901.49%, and total P accumulation ranged from 145.38% to 812.48%. The data showed that Cuningmeng had higher increment in P content and accumulation, but that of Zhike was lower. For HP treatment, the increment in P content in root ranged from 134.58% to 347.99%, P content in branch ranged from 83.32% to 291.78%, P content in leaves ranged from 80.36% to 304.84% and P accumulation in root ranged from 107.59% to 725.85%, P accumulation in branch ranged from 134.27% to 834.43%, P accumulation in leaves ranged from 164.64% to 920.37%, and total P accumulation ranged from 162.06% to 961.33%. Among the cultivars, Cuningmeng showed higher P content and accumulation, but lower P content and accumulation was observed for Zhike (*Table 3*).

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Table 2. *Effects of phosphorus treatment on N content and N accumulation in citrus plants.*

Phosphorus	Cultivar	N content in root (mg g^{-1})	N content in branch (mg g^{-1})	N content in leaves (mg g^{-1})	N accumulation in root (mg)	N accumulation in branch (mg)	N accumulation in leaves (mg)	Total N accumulation (mg)
LP	Cuningmeng	19.73±2.13	8.60±0.52	25.47±1.54	26.00±4.21	11.95±1.47	66.07±10.45	104.02±8.82
	Suanju	16.17±0.81	11.42±0.85	24.48±2.33	9.68±0.94	4.65±0.26	16.74±1.13	31.06±1.32
	Suancheng	20.37±0.41	10.02±0.32	18.39±1.07	26.67±1.99	9.98±1.41	43.30±2.57	79.94±5.27
	Honglimeng	18.97±0.54	8.63±0.07	22.96±1.16	29.12±2.70	18.02±1.53	62.60±1.89	109.74±4.86
	Zhike	18.27±0.83	12.38±1.18	26.48±2.27	13.75±0.87	9.73±0.70	23.63±2.77	47.11±4.16
	Goutoucheng	23.13±2.33	11.10±0.99	24.93±1.48	16.40±2.37	9.43±0.42	31.08±2.41	56.91±4.58
	Xiangyuan	16.67±0.82	9.57±1.01	26.46±2.35	27.00±3.20	24.39±4.15	85.97±14.12	137.36±21.14
	Hongju	18.97±1.08	11.60±0.71	24.72±0.86	19.52±2.22	4.47±0.70	21.95±2.42	45.94±5.11
	Xiangcheng	28.80±1.27	11.24±0.23	24.45±0.35	30.87±3.09	11.49±0.83	44.75±2.39	87.12±6.25
	Zhicheng	15.27±0.69	13.36±0.98	30.15±2.32	25.56±2.49	17.97±2.04	43.83±6.10	87.35±8.58
	Mean	*19.633 b*	*10.793 b*	*24.850 b*	*22.457 b*	*12.208 b*	*43.99 c*	*78.66 b*
MP	Cuningmeng	26.4±1.72	8.31±0.53	23.53±0.41	59.68±5.66	33.47±3.80	181.16±8.26	274.31±17.69
	Suanju	20.70±0.93	13.76±1.03	29.58±1.45	18.60±2.33	12.22±0.88	52.88±6.00	83.71±9.06
	Suancheng	29.73±1.53	14.25±0.33	26.45±0.95	50.21±2.77	30.75±2.14	117.49±7.75	198.45±11.00
	Honglimeng	21.53±1.77	9.77±0.75	25.49±2.07	41.37±4.20	33.51±2.59	125.79±13.29	200.68±11.88
	Zhike	22.40±1.07	14.32±1.60	34.42±1.53	21.87±1.34	13.89±1.95	39.51±5.70	75.28±8.42
	Goutoucheng	34.70±0.64	14.61±0.65	35.10±1.32	28.52±4.70	17.01±0.63	71.70±3.48	117.23±6.61
	Xiangyuan	25.70±0.31	11.72±0.66	30.76±1.06	49.81±2.69	50.45±4.38	182.53±9.14	282.78±15.48
	Hongju	25.87±0.93	15.96±0.59	33.70±1.22	17.36±1.14	11.30±0.85	44.36±1.73	73.02±1.54
	Xiangcheng	36.70±0.31	14.75±0.58	32.14±1.15	27.16±2.99	23.42±1.97	90.30±4.18	140.88±8.97
	Zhicheng	20.37±0.13	17.02±0.21	38.04±0.59	36.29±2.56	33.20±0.36	82.89±1.19	152.37±4.07
	Mean	*25.880 a*	*13.446 a*	*30.921 a*	*35.087 a*	*25.923 a*	*98.86 b*	*159.87 a*
HP	Cuningmeng	24.07±2.29	9.30±1.02	25.57±2.26	58.97±9.42	27.56±2.55	172.35±1.08	258.87±9.40
	Suanju	22.67±1.90	15.20±0.67	31.24±1.07	29.69±6.30	21.68±0.83	82.76±6.75	134.13±10.58

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	Suancheng	28.17±2.87	14.57±1.00	29.01±1.53	30.76±4.95	20.93±1.43	103.02±0.68	154.72±4.52
	Honglimeng	22.73±1.63	11.57±1.69	27.68±1.16	40.91±7.40	41.73±4.22	158.32±9.88	240.97±19.34
	Zhike	23.63±0.50	15.13±1.06	31.40±2.18	20.29±2.11	15.17±0.51	40.47±0.73	75.93±2.17
	Goutoucheng	37.53±1.34	16.57±0.47	38.14±0.42	23.56±0.79	21.96±0.94	81.29±3.50	126.80±4.01
	Xiangyuan	23.40±2.29	11.12±1.64	28.22±3.04	60.02±12.87	56.41±5.65	192.00±13.55	308.43±19.53
	Hongju	25.47±0.60	15.97±0.47	33.56±0.48	20.90±2.71	14.58±0.49	59.47±2.96	94.96±5.62
	Xiangcheng	32.73±0.99	13.07±0.88	29.96±1.05	32.59±1.52	31.19±3.82	114.77±9.86	178.54±13.53
	Zhicheng	18.40±1.85	17.10±0.20	37.99±0.78	23.86±1.90	25.36±1.78	63.31±4.96	112.54±8.53
	Mean	*26.410 a*	*13.960 a*	*31.279 a*	*34.155 a*	*27.657 a*	*106.78 a*	*168.59 a*
F value								
	Cultivar(C)	36.05**	29.16**	15.94**	22.81**	119.82**	114.83**	98.11**
	Phosphorus (P)	70.17**	33.05**	56.86**	23.16**	109.86**	276.30**	251.27**
	C×P	1.91*	1.10ns	2.46**	3.01**	4.33**	9.62**	10.26**

Note: different lowercase letters are used to indicate values that are significantly different at p < 0.05 among P treatments.

Table 3. *Effects of phosphorus treatment on P content and P accumulation in citrus plants.*

Phosphorus	Cultivar	P content in root (mg g^{-1})	P content in branch (mg g^{-1})	P content in leaves (mg g^{-1})	P accumulation in root (mg)	P accumulation in branch (mg)	P accumulation in leaves (mg)	Total P accumulation (mg)
LP	Cuningmeng	0.64±0.03	0.54±0.02	0.72±0.01	0.84±0.13	0.75±0.07	1.85±0.19	3.45±0.25
	Suanju	1.22±0.08	1.02±0.11	1.06±0.05	0.72±0.02	0.43±0.09	0.74±0.11	1.90±0.19
	Suancheng	0.84±0.06	0.83±0.09	0.72±0.06	1.10±0.12	0.80±0.05	1.73±0.28	3.63±0.35
	Honglimeng	0.82±0.06	0.73±0.01	0.88±0.07	1.26±0.15	1.52±0.13	2.38±0.09	5.16±0.23
	Zhike	1.08±0.11	1.19±0.08	1.32±0.13	0.82±0.12	0.94±0.04	1.17±0.11	2.93±0.16
	Goutoucheng	0.99±0.00	0.83±0.02	0.81±0.05	0.70±0.03	0.72±0.08	1.03±0.18	2.45±0.28
	Xiangyuan	0.91±0.06	0.51±0.04	1.01±0.04	1.48±0.22	1.28±0.17	3.25±0.38	6.01±0.73
	Hongju	1.17±0.10	0.89±0.02	0.90±0.06	1.21±0.16	0.34±0.04	0.80±0.11	2.35±0.30
	Xiangcheng	0.87±0.04	0.84±0.02	0.79±0.02	0.92±0.01	0.85±0.05	1.45±0.08	3.23±0.14

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	Zhicheng	0.96±0.09	0.96±0.09	1.09±0.09	1.61±0.22	1.30±0.17	1.58±0.23	4.49±0.25
	Mean	*0.9497 c*	*0.8337 b*	*0.9307 c*	*1.0657 b*	*0.8936 c*	*1.600 c*	*3.559 c*
MP	Cuningmeng	2.54±0.17	1.77±0.07	2.41±0.08	5.79±0.75	7.09±0.45	18.58±0.90	31.45±1.93
	Suanju	3.03±0.10	3.04±0.19	2.98±0.12	2.72±0.34	2.72±0.27	5.36±0.73	10.80±1.33
	Suancheng	2.63±0.07	2.69±0.05	2.38±0.05	4.45±0.26	5.81±0.34	10.60±0.72	20.85±1.30
	Honglimeng	2.39±0.10	2.41±0.13	3.13±0.21	4.58±0.26	8.25±0.38	15.27±0.25	28.10±0.35
	Zhike	2.58±0.06	2.07±0.01	2.30±0.07	2.55±0.28	2.01±0.18	2.63±0.35	7.19±0.80
	Goutoucheng	3.25±0.05	2.75±0.04	2.89±0.05	2.67±0.44	3.21±0.15	5.91±0.39	11.79±0.87
	Xiangyuan	2.39±0.23	1.61±0.08	2.80±0.14	4.64±0.51	6.91±0.59	16.61±1.08	28.17±1.95
	Hongju	3.12±0.04	3.12±0.19	3.36±0.17	2.09±0.06	2.19±0.06	4.41±0.11	8.69±0.06
	Xiangcheng	2.65±0.07	2.51±0.05	2.40±0.05	1.97±0.26	4.00±0.39	6.78±0.52	12.75±1.10
	Zhicheng	2.2±0.14	2.21±0.03	2.62±0.07	3.88±0.03	4.32±0.05	5.71±0.16	13.91±0.22
	Mean	*2.6773 b*	*2.4173 a*	*2.7263 b*	*3.5331 a*	*4.6501 b*	*9.187 b*	*17.370 b*
HP	Cuningmeng	2.86±0.17	2.12±0.15	2.92±0.13	6.97±0.85	6.29±0.29	19.88±0.96	33.14±1.11
	Suanju	3.63±0.12	2.83±0.05	2.86±0.04	4.64±0.56	4.05±0.20	7.56±0.57	16.26±1.22
	Suancheng	3.41±0.06	2.96±0.1	2.59±0.11	3.70±0.36	4.31±0.53	9.23±0.33	17.24±1.08
	Honglimeng	2.08±0.09	2.32±0.18	3.10±0.22	3.66±0.37	8.55±1.11	17.81±2.02	30.02±3.34
	Zhike	2.75±0.10	2.19±0.08	2.38±0.06	2.38±0.33	2.20±0.06	3.09±0.19	7.67±0.54
	Goutoucheng	4.05±0.07	2.74±0.02	2.99±0.04	2.56±0.19	3.63±0.2	6.38±0.40	12.57±0.62
	Xiangyuan	2.13±0.18	1.67±0.15	2.95±0.20	5.44±1.10	8.50±0.37	20.11±0.59	34.05±1.53
	Hongju	3.32±0.10	3.19±0.07	3.50±0.05	2.73±0.38	2.92±0.18	6.21±0.40	11.86±0.68
	Xiangcheng	2.74±0.02	2.35±0.07	2.23±0.04	2.74±0.19	5.58±0.42	8.52±0.57	16.83±0.95
	Zhicheng	2.56±0.14	2.4±0.16	2.89±0.16	3.34±0.2	3.58±0.43	4.83±0.55	11.75±0.88
	Mean	*2.9527 a*	*2.4760 a*	*2.8393 a*	*3.8152 a*	*4.9614 a*	*10.363 a*	*19.140 a*
F value	Cultivar(C)	43.04**	31.11**	16.32**	16.41**	45.48**	119.62**	85.80**
	Phosphorus (P)	955.26**	1072.93**	964.39**	147.22**	514.50**	668.34**	667.66**
	C×P	7.82**	8.18**	5.61**	4.93**	15.35**	30.53**	23.49**

Note: different lowercase letters are used to indicate values that are significantly different at $p < 0.05$ among P treatments.

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K content and K accumulation

The cultivars, phosphorus and their interaction C×P significantly affected the K content and accumulation in citrus plant. MP treatment significantly increased K content in root, K content in branch, K content in leaves, K accumulation in root, K accumulation in branch, K accumulation in leaves, and total K accumulation by 51.99%, 21.46%, 21.27%, 87.18%, 122.44%, 138.65%, and 124.73%, respectively. HP treatment dramatically increased K content in root, K content in branch, K content in leaves, K accumulation in root, K accumulation in branch, K accumulation in leaves, and total K accumulation by 41.09%, 17.01%, 16.64%, 69.81%, 120.57%, 142.22%, and 122.91%, respectively. For MP treatment, Xiangyuan had the highest increment in K content in root, K content in branch, K content in leaves and K accumulation in root; while Cuningmeng had the highest increment in K accumulation in branch, K accumulation in leaves, and total K accumulation. The lowest increment in K content in branch, K content in leaves, K accumulation in branch, K accumulation in leaves, and total K accumulation was observed in Zhike for MP treatment. The lowest increment in K content in root and K accumulation in root was investigated in Suanju and Hongju for MP, respectively. For HP treatment, Cuningmeng had the highest increment in K content in root and K accumulation in root when compared to LP. Suanju decreased K content in branch and K content in leaves but had the highest increment in K accumulation in branch, K accumulation in leaves, and total K accumulation for HP treatment. Hongju had the highest increment in K content in branch and K content in leaves. Zhicheng showed lowest highest increment in K accumulation (*Table 4*).

Mg content and Mg accumulation

The cultivars, phosphorus and C×P on Mg content and Mg accumulation in different plant part were observed, except for the P treatment effect on Mg content in leaves and C×P effect on Mg content in branch and leaves. MP treatment significantly increased Mg content in root, Mg accumulation in root, Mg accumulation in branch, Mg accumulation in leaves, and total Mg accumulation by 26.58%, 52.06%, 81.35%, 95.35% and 84.81%, respectively. HP treatment dramatically increased Mg content in root, Mg content in branch, Mg accumulation in root, Mg accumulation in branch, Mg accumulation in leaves, and total Mg accumulation by 27.88%, 7.65%, 52.30%, 101.68%, 111.60% and 99.33%, respectively. MP treatment increased Mg content in root in range of 7.03 ~76.54 %. However, Zhike and Hongju decreased Mg content in root for HP treatment was detected. There observed decrease in Mg content in branch and leaves for Cuningmeng, Suanju, Zhike, Xiangcheng for MP treatment. Mg content in branch and leaves in Suanju, Goutoucheng and xiangcheng was decreased for MP treatment, and decrement in Mg content in branch for Honglimeng and Mg content in leaves for Zhike was investigated. Decrement in Mg accumulation in root in Hongju was observed for MP and HP treatment and in Xiangcheng for MP treatment (*Table 5*).

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***Table 4.** Effects of phosphorus treatment on K content and K accumulation in citrus plants.*

Phosphorus	Cultivar	K content in root (mg g^{-1})	K content in branch (mg g^{-1})	K content in leaves (mg g^{-1})	K accumulation in root (mg)	K accumulation in branch (mg)	K accumulation in leaves (mg)	Total K accumulation (mg)
LP	Cuningmeng	8.17±0.11	10.33±0.34	17.75±1.21	10.70±1.14	14.28±1.24	46.02±7.17	71.00±8.10
	Suanju	14.40±1.07	14.05±0.35	21.23±0.80	8.52±0.15	5.80±0.65	14.75±1.60	29.07±1.76
	Suancheng	11.39±0.86	11.12±0.31	17.43±0.59	14.82±1.08	11.04±1.38	41.60±5.07	67.46±6.67
	Honglimeng	7.52±0.09	10.74±0.07	21.83±0.99	11.49±0.66	22.4±1.81	59.53±1.61	93.42±3.58
	Zhike	14.43±0.68	8.89±0.64	18.30±1.73	11.05±1.60	7.00±0.35	16.26±1.73	34.32±3.28
	Goutoucheng	10.58±0.47	11.89±0.51	20.37±0.56	7.46±0.58	10.18±0.57	25.60±2.75	43.25±3.81
	Xiangyuan	7.46±0.60	11.82±1.17	17.74±1.88	12.05±1.56	30.09±4.78	57.77±10.32	99.91±16.5
	Hongju	10.20±0.21	10.96±0.73	18.37±0.81	10.43±0.55	4.24±0.72	16.33±1.98	31.01±3.09
	Xiangcheng	9.15±0.21	13.50±0.35	15.44±0.61	9.78±0.76	13.78±0.85	28.31±2.20	51.87±3.72
	Zhicheng	11.01±1.2	13.64±0.27	18.32±0.49	18.63±3.08	18.21±0.57	26.34±1.91	63.18±3.45
	Mean	*10.431 c*	*11.694 b*	*18.680 b*	*11.493 b*	*13.703 b*	*33.252 b*	*58.45 b*
MP	Cuningmeng	12.57±1.29	12.90±0.30	21.32±0.52	28.92±5.4	51.70±3.26	164.16±8.36	244.78±16.95
	Suanju	17.33±0.3	15.02±0.53	23.67±0.91	15.50±1.46	13.40±0.96	42.25±4.52	71.16±6.84
	Suancheng	18.30±1.11	14.75±0.32	23.16±0.62	31.02±2.75	31.86±2.25	103.00±7.49	165.87±12.21
	Honglimeng	13.70±0.40	13.35±1.25	26.70±1.61	26.34±1.76	45.48±2.44	130.55±0.29	202.37±3.42
	Zhike	18.87±0.64	9.05±0.47	17.54±0.48	18.48±1.34	8.86±1.13	20.08±2.65	47.41±5.10
	Goutoucheng	13.27±0.39	14.50±0.59	23.96±1.05	10.95±1.93	16.93±1.03	48.98±3.01	76.85±5.58
	Xiangyuan	19.11±0.44	16.81±0.50	26.24±0.86	36.99±1.67	72.33±4.86	155.72±7.49	265.04±13.98
	Hongju	17.00±0.97	15.18±0.42	25.91±0.53	11.44±1.10	10.82±1.21	34.26±2.46	56.52±2.81
	Xiangcheng	14.43±0.99	16.48±0.68	18.85±1.09	10.76±1.74	26.16±2.2	52.78±1.87	89.70±5.42
	Zhicheng	13.97±0.99	14±0.75	19.19±0.96	24.74±1.55	27.26±0.97	41.78±1.70	93.78±1.34
	Mean	*15.854 a*	*14.204 a*	*22.654 a*	*21.512 a*	*30.480 a*	*79.356 a*	*131.35 a*
HP	Cuningmeng	14.67±1.43	13.21±1.06	22.86±2.03	35.95±5.83	39.15±1.74	154.11±2.88	229.2±8.79
	Suanju	18.27±1.15	13.32±0.15	20.64±0.39	23.59±4.02	19.12±1.49	54.78±4.91	97.49±10.05

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	Suancheng	19.13±1.24	15.16±0.23	23.99±0.49	20.72±2.26	22.10±2.75	85.90±6.47	128.73±7.74
	Honglimeng	10.65±0.45	12.45±0.53	23.37±1.19	18.93±2.57	45.72±4.45	134.12±12.16	198.76±17.80
	Zhike	16.00±2.81	9.85±0.18	18.89±0.22	14.19±3.51	9.95±0.64	24.59±1.82	48.73±5.79
	Goutoucheng	13.73±0.43	12.50±0.37	22.47±0.89	8.68±0.78	16.56±0.60	47.76±1.51	73.00±2.29
	Xiangyuan	10.41±1.35	15.42±0.87	24.05±1.74	26.87±6.65	78.85±1.89	163.69±2.00	269.41±10.32
	Hongju	15.17±0.27	15.12±0.09	26.13±0.69	12.49±1.77	13.82±0.61	46.43±3.53	72.75±5.00
	Xiangcheng	13.50±0.23	14.57±0.63	16.27±0.65	13.48±0.91	34.39±0.75	61.87±1.06	109.74±2.39
	Zhicheng	15.63±0.92	15.24±0.81	19.22±0.98	20.26±0.29	22.60±1.96	32.19±3.63	75.05±5.40
	Mean	*14.716 b*	*13.684 a*	*21.788 a*	*19.516 a*	*30.225 a*	*80.544 a*	*130.29 a*
F value	Cultivar(C)	15.55**	20.11**	13.72**	12.01**	90.62**	121.95**	86.44**
	Phosphorus (P)	86.52**	51.97**	42.32**	46.95**	355.88**	447.71**	401.89**
	C×P	3.68**	3.29**	3.32**	4.77**	27.40**	30.59**	27.00**

Note: different lowercase letters are used to indicate values that are significantly different at $p < 0.05$ among P treatments.

Table 5. *Effects of phosphorus treatment on Mg content and Mg accumulation in citrus plants.*

Phosphorus	Cultivar	Mg content in root (mg g^{-1})	Mg content in branch (mg g^{-1})	Mg content in leaves (mg g^{-1})	Mg accumulation in root(mg)	Mg accumulation in branch (mg)	Mg accumulation in leaves(mg)	Total Mg accumulation (mg)
LP	Cuningmeng	0.72±0.06	0.77±0.07	2.06±0.16	0.94±0.12	1.07±0.15	5.36±0.93	7.38±1.11
	Suanju	1.26±0.08	1.92±0.03	2.74±0.09	0.75±0.01	0.79±0.10	1.91±0.23	3.45±0.28
	Suancheng	1.23±0.04	1.14±0.08	2.27±0.14	1.61±0.12	1.12±0.07	5.43±0.79	8.16±0.93
	Honglimeng	0.80±0.02	0.70±0.04	2.07±0.09	1.23±0.07	1.46±0.11	5.67±0.42	8.36±0.59
	Zhike	0.98±0.04	1.57±0.08	3.32±0.20	0.74±0.10	1.24±0.09	2.97±0.35	4.96±0.53
	Goutoucheng	1.51±0.03	0.90±0.05	2.32±0.17	1.06±0.03	0.77±0.09	2.95±0.47	4.79±0.58
	Xiangyuan	0.87±0.05	1.77±0.11	1.74±0.09	1.41±0.17	4.47±0.52	5.60±0.58	11.48±1.21
	Hongju	1.21±0.06	1.05±0.07	2.17±0.19	1.24±0.11	0.41±0.06	1.93±0.28	3.58±0.41
	Xiangcheng	0.98±0.03	1.81±0.10	1.86±0.10	1.04±0.07	1.84±0.03	3.39±0.05	6.27±0.11

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	Zhicheng	0.49±0.08	1.54±0.22	2.50±0.25	0.84±0.17	2.05±0.28	3.57±0.40	6.46±0.48
	Mean	*1.0057 b*	*1.3173 b*	*2.3050 a*	*1.0872 b*	*1.5219 c*	*3.8787 c*	*6.488 c*
MP	Cuningmeng	0.97±0.03	0.74±0.03	1.85±0.04	2.21±0.32	2.96±0.16	14.31±0.97	19.49±1.34
	Suanju	1.54±0.09	1.89±0.09	2.72±0.10	1.37±0.09	1.68±0.13	4.87±0.62	7.93±0.83
	Suancheng	1.32±0.06	1.31±0.06	2.41±0.13	2.23±0.15	2.84±0.23	10.74±1.03	15.81±1.41
	Honglimeng	1.12±0.04	0.71±0.09	2.37±0.16	2.14±0.07	2.43±0.28	11.57±0.22	16.14±0.55
	Zhike	1.05±0.03	1.46±0.17	3.08±0.38	1.03±0.07	1.42±0.23	3.55±0.67	6.00±0.96
	Goutoucheng	2.09±0.13	0.97±0.08	2.47±0.13	1.69±0.23	1.13±0.05	5.03±0.20	7.85±0.41
	Xiangyuan	1.54±0.22	1.88±0.17	1.88±0.21	2.98±0.42	8.05±0.55	11.1±1.09	22.13±1.97
	Hongju	1.33±0.05	1.12±0.05	2.44±0.18	0.89±0.06	0.80±0.10	3.25±0.45	4.95±0.49
	Xiangcheng	1.14±0.07	1.78±0.11	1.83±0.12	0.84±0.12	2.82±0.29	5.17±0.52	8.84±0.75
	Zhicheng	0.64±0.02	1.77±0.10	2.83±0.15	1.14±0.08	3.47±0.26	6.17±0.41	10.77±0.65
	Mean	*1.2730 a*	*1.3647 ab*	*2.3877 a*	*1.6531 a*	*2.7599 b*	*7.5773 b*	*11.990 b*
HP	Cuningmeng	1.09±0.04	0.91±0.08	2.36±0.31	2.67±0.29	2.70±0.09	15.84±1.27	21.21±1.60
	Suanju	1.72±0.03	1.74±0.01	2.54±0.02	2.20±0.26	2.49±0.19	6.75±0.63	11.44±0.97
	Suancheng	1.62±0.18	1.39±0.02	2.66±0.08	1.78±0.32	2.04±0.32	9.51±0.67	13.33±0.8
	Honglimeng	1.06±0.06	0.68±0.06	2.21±0.10	1.87±0.19	2.49±0.22	12.67±1.06	17.02±1.38
	Zhike	0.96±0.13	1.98±0.09	3.26±0.45	0.85±0.19	2.00±0.16	4.18±0.39	7.03±0.38
	Goutoucheng	2.19±0.10	0.88±0.05	2.13±0.06	1.39±0.14	1.17±0.09	4.55±0.35	7.11±0.52
	Xiangyuan	1.02±0.06	1.99±0.06	1.92±0.05	2.59±0.46	10.19±0.42	13.2±0.80	25.99±1.65
	Hongju	1.19±0.01	1.20±0.06	2.41±0.11	0.97±0.11	1.09±0.06	4.29±0.38	6.35±0.52
	Xiangcheng	1.29±0.06	1.63±0.03	1.66±0.08	1.30±0.15	3.87±0.19	6.36±0.53	11.52±0.78
	Zhicheng	0.73±0.05	1.77±0.08	2.83±0.17	0.95±0.08	2.64±0.24	4.74±0.54	8.33±0.71
	Mean	*1.2860 a*	*1.4177 a*	*2.3983 a*	*1.6557 a*	*3.0693 a*	*8.2072 a*	*12.932 a*
F value	Cultivar(C)	48.73**	116.82**	12.72**	23.13**	327.60**	47.56**	66.01**
	Phosphorus (P)	41.59**	2.54*	1.16ns	25.41**	105.48**	188.53**	159.23**
	C×P	3.88**	1.48ns	1.17ns	3.88**	11.13**	10.52**	9.24**

Note: different lowercase letters are used to indicate values that are significantly different at $p < 0.05$ among P treatments.

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Correlation analysis

For all treatments, total dry weight showed significant correlation relationship with root dry weight (r=0.8950, P<0.001), branch dry weight (r=0.9720, P<0.001), leaves dry weight (r=0.9846, P<0.001), N accumulation in root (r=0.9475, P<0.001), N accumulation in branch (r=0.8796, P<0.001), N accumulation in leaves (r=0.9684, P<0.001), total N accumulation (r=0.9724, P<0.001), P accumulation in root (0.7906, P<0.001), P accumulation in branch (0.8581, P<0.001), P accumulation in leaves (0.9244 P<0.001), total P accumulation (0.9070, P<0.001), K accumulation in root (0.7764, P<0.001), K accumulation in branch (0.9414, P<0.001), accumulation in leaves (0.9717, P<0.001), total K accumulation (0.9790, P<0.001), Mg accumulation in root (0.8365, P<0.001), Mg accumulation in branch (0.7293, P<0.001), Mg accumulation in leaves (0.9488, P<0.001), total mg accumulation (0.9699, P<0.001). Similar correlation relationships were observed for MP and HP treatment. However, for LP, there investigated no significant correlation relationship between total dry weight and P, K, Mg P accumulation in root. Significant negative correlation relationship between total dry weight and N content in branch was found. Significant negative correlation relationship between total dry weight and N content in leaves was observed for MP and HP only. Significant negative correlation relationship between total dry weight and P content in root was observed for LP. There investigated significant negative correlation relationship between total dry weight and P content in branch for LP, MP and HP. Significant negative correlation relationship between total dry weight and K content in root was observed for LP and HP. Total dry weight showed significant negative correlation with Mg content in leaves for all treatment, LP and MP (*Table 6*).

Discussion

In this study, we observed significant effects of rootstock on root dry weight, branch dry weight, leaves dry weight, and total dry weigh (*Table 1*). Similar to many previous reports (Li and Zhang, 2008; Shafieizargar et al., 2012; Cantuarias-Avilés et al., 2010; 2011; Tazima et al., 2013). Rootstock affects the horticultural characteristics in citrus (Cantuarias-Avilés et al., 2010; Zhang et al., 2011; Hussain et al., 2013; Benjamin et al., 2013; Simpson et al., 2014). The P efficiency is defined as the ability of the plant growth under low effective P concentration condition (Zhang, 1993). Researcher used the plant dry weight to evaluate the plant resistance to low phosphorus stress (Cao et al., 2000; Zhang et al., 2005; Zhou et al., 2005). Here, we found significant effects of phosphorus on N, P, K and Mg content, and their accumulation in plant (*Table 2-5*). It confirmed that it could have significant differences in responses of citrus trees to P fertilization (Wutscher, 1989; Mattos et al., 2006; Fan and Luo, 2015). Previously it was reported that different citrus trees respond to P differently due to the difference in the acid phosphatase activity of rhizosphere soil, for various factors affecting the acid phosphatase activity (Bonmati et al., 1991; Oberson et al., 1993; Luo and Fan, 2014).

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Table 6. *Correlation relationship between total dry weight and the investigated parameters.*

Investigated parameters	All treatment			LP			MP			HP	
	Correlation coefficient	*P* value		Correlation coefficient	*P* value		Correlation coefficient	*P* value		Correlation coefficient	*P* value
Root dry weight	0.8950	0.0000		0.8650	0.0012		0.9101	0.0003		0.9319	0.0001
Branch dry weight	0.9720	0.0000		0.9584	0.0000		0.9790	0.0000		0.9591	0.0000
Leaves dry weight	0.9846	0.0000		0.9658	0.0000		0.9857	0.0000		0.9841	0.0000
N content in root	0.1028	0.5888		-0.0982	0.7873		-0.0712	0.8451		-0.2058	0.5685
N content in Branch	-0.4494	0.0127		-0.6534	0.0405		-0.8189	0.0038		-0.9109	0.0002
N content in leaves	-0.2151	0.2536		-0.0400	0.9127		-0.7052	0.0227		-0.7310	0.0163
N accumulation in root	0.9475	0.0000		0.7889	0.0067		0.9423	0.0000		0.9721	0.0000
N accumulation in branch	0.8796	0.0000		0.8917	0.0005		0.8538	0.0017		0.8728	0.0010
N accumulation in leaves	0.9674	0.0000		0.9723	0.0000		0.9790	0.0000		0.9846	0.0000
Total N accumulation	0.9724	0.0000		0.9815	0.0000		0.9769	0.0000		0.9927	0.0000
P content in root	0.1221	0.5203		-0.7387	0.0147		-0.5794	0.0792		-0.6262	0.0527
P content in Branch	0.0446	0.8148		-0.7919	0.0063		-0.7098	0.0215		-0.7050	0.0228
P content in leaves	0.3448	0.0621		-0.2859	0.4232		-0.1991	0.5813		0.0998	0.7837
P accumulation in root	0.7906	0.0000		0.6066	0.0630		0.9293	0.0001		0.7646	0.0100
P accumulation in branch	0.8581	0.0000		0.7685	0.0094		0.9303	0.0001		0.9410	0.0000
P accumulation in leaves	0.9244	0.0000		0.9719	0.0000		0.9816	0.0000		0.9771	0.0000
Total P accumulation	0.9070	0.0000		0.9396	0.0001		0.9857	0.0000		0.9834	0.0000
K content in root	-0.0701	0.7129		-0.8036	0.0051		-0.2148	0.5513		-0.6357	0.0482

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K content in Branch	0.2894	0.1208		-0.1185	0.7444		0.1328	0.7145		0.2188	0.5437
K content in leaves	0.3703	0.0440		-0.1351	0.7097		0.2725	0.4462		0.2653	0.4588
K accumulation in root	0.7764	0.0000		0.3851	0.2718		0.8299	0.0030		0.7066	0.0223
K accumulation in branch	0.9414	0.0000		0.9342	0.0001		0.9316	0.0001		0.9332	0.0001
K accumulation in leaves	0.9717	0.0000		0.9587	0.0000		0.9788	0.0000		0.9721	0.0000
Total K accumulation	0.9790	0.0000		0.9934	0.0000		0.9811	0.0000		0.9903	0.0000
Mg content in root	-0.1220	0.5206		-0.5616	0.0911		-0.2087	0.5628		-0.2887	0.4185
Mg content in Branch	-0.1617	0.3934		-0.2026	0.5746		-0.3008	0.3983		-0.1497	0.6798
Mg content in leaves	-0.4858	0.0065		-0.6563	0.0393		-0.6525	0.0408		-0.5292	0.1157
Mg accumulation in root	0.8365	0.0000		0.5384	0.1084		0.8343	0.0027		0.8284	0.0031
Mg accumulation in branch	0.7293	0.0000		0.7295	0.0166		0.6554	0.0396		0.7234	0.0180
Mg accumulation in leaves	0.9488	0.0000		0.9224	0.0001		0.9730	0.0000		0.9304	0.0001
Total Mg accumulation	0.9699	0.0000		0.9643	0.0000		0.9683	0.0000		0.9785	0.0000

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P is an important for plant growth, development and reproduction as it is an essential constituent of phospholipids, nucleic acid and many proteins. With increasing P application, the root, branch and leaves dry weight, and total dry weight increased, however different cultivars responded differently (*Table 1-5*). Low P availability in soil is one of the most critical aspects that limit the productivity of many crops (Sanchez and Salinas, 1981). P deficiency or excess phosphate fertilization could cause nutrient imbalance in citrus tree and reduce the yield and fruit quality (Fan and Wang, 2012; Fan and Luo, 2015). When the phosphorus nutrition level was extremely low or high, P, Mg, Mn and Cu content was deficient or excessively high and inhibited absorption of N, K, Fe, Zn, and B (Fan and Wang, 2012). In our study, we found that low P treatment affected the P, K, and Mg accumulation in root (*Table 3-6*). Moreover, Syvertsen (1987) reported that P level in leaves showed no relationship with photosynthetic in orange and pomelo seedlings. Bernardi et al. (2015) reported that P had little interference on photosynthesis. Zambrosi et al. (2013a) indicated that phosphorus uptake by young citrus plants in low-P soil depends on rootstock varieties and nutrient management. Therefore in our work, low P treatment may affected Mg content in leaves may lead to low plant photosynthesis and finally decreased total dry weight (*Tables 5* and *6*). Further study to investigate the improvement citrus rootstocks growth under low P condition by Mg application is need.

Conclusion

In crux, P application increased N, P, K and Mg accumulation in citrus plants. With increasing P application, the plant total plant dry biomass was increased. Low P treatment affected P, K, and Mg accumulation in root and ultimately plant growth. Moreover, low P treatment may affect Mg content in leaves that may lead to low plant photosynthesis and finally decreased total dry weight.

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ASSESSING THE EFFECTIVENESS OF PROTECTED AREAS ON FLORISTIC DIVERSITY IN TROPICAL FORESTS

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Abstract. Understanding major drivers of tree species distribution and tropical forest composition is imperative for biodiversity conservation. This study aimed to assessing the floristic diversity and structure of Lesio-louna forest in southern Republic of Congo and its implications for sustainable development. The measurements were made in six plots: three plots located in the Iboubikro forest and three in Blue Lake Forest (circular plots of 1,256 m^2 or 40 m of plot diameter). 85 trees of DBH ≥ 10 cm, divided into 25 species and 14 families have been recorded. Biomass data was collected using the tree-ring or floristic inventory method and data analyzed using SPSS v.18.0 statistical software. The results showed that, the Fabaceae had significant trees (17 species), with a relative diversity index of 20% followed by the Mimosaceae with 14 species and a relative diversity index of 16%. *Millettia laurentii*, *Pentaclethra eetveldeana*, *Eriocoelum macrocarpum*, *Millettia pinnata* and *Sorindeia juglandifolia* were listed as most important species in the area based on their relative frequency. Further study has been done to identify and determine the trees taxon for Lesio-louna tropical forest and their phytogeographical distribution. Flora of Lesio-louna protected area has a remarkable diversity and the floristic richness is very considerable in its specific composition.
Keywords: *species diversity, Iboubikro, Blue Lake Forest, Lesio-Louna Forest, tree species*

Introduction

Central African forests comprise the second largest continuous block of tropical forests in the world after the Amazonian forests. These Central African forests harbour up to 20,000 different plant species including 8,000 tree species and store some 200 tons of carbon per hectare in live trees (Fayolle et al., 2014a). Tropical forest ecosystems apart from being carbon sinks are known to harbour over 50% of terrestrial

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biodiversity (Yoka et al., 2013; FAO, 2016). This is facilitated by biogeophysical microclimatic processes (e.g. arbor coverage) that influence surface albedo, rate of evapotranspiration, temperature and precipitation (Mukete and Sun, 2014). In Central Africa, most timber species require high light levels at the seedling stage for survival and growth (Ouedraogo et al., 2014). Congo basin forests house over of Earth's biodiversity have an important influence on the climate system (Ekoungoulou et al., 2015). Congo basin tropical forests ecosystems in Central Africa play an important role in the global carbon cycle, especially in carbon sequestration (Ekoungoulou et al., 2014a). This carbon results from global warming and is partly linked to anthropogenic activities such as loss in forest cover (FAO, 2008; Ngomanda et al., 2013).

The Republic of Congo is located in Central Africa and with a surface area of 342,000 km^2, covered by 65% dry and moist forests and 35% grassland and shrub landscapes (Legendre, 2014). However, among the two types of vegetation, there are savannas included in forest zones and the forest features of low savanna areas (Legendre, 2014). Interest in forest biodiversity has recently increased in response to the damage caused to ecosystem by anthropogenic activity.

Regarding the flora of Lesio-louna sanctuary, Nkounkou (2003) mentioned that the grassland is dominated by *Hyparrhenia diplandra* (Poaceae) and *Panicum maximum* (Poaceae) and the bushland dominated by *Hymenocardia acida* (Hymenocardiaceae) and *Annona arenaria* (Annonaceae). Therefore, the Lesio-louna forests are semi-tropical evergreen forests and are zones of transition between evergreen and semi-deciduous forests (PLL, 2010). These forests are characterized by a complex structure of high plant diversity exhibiting a heterogeneous appearance (Ekoungoulou et al., 2015). The canopy is generally discontinuous with separated tree crowns, dense undergrowth made of both short and tall grasses and lianescentes of Marantaceae, Zingiberaceae and Commelinaceae. Large trees (often commercial species) if present, always exceed 50 m in height (PLL, 2010), hence their canopy structure and undergrowth are easily distinguishable. This is because woodlands and forests have open Marantaceae with dense undergrowth (Nkounkou, 2003; Ekoungoulou et al., 2014b).

The Lesio-louna natural forest is a contiguous humid closed canopy forest with large areas of savannah and forest-savannah mosaics. These are often composed of forest groves either along the river bank or resulting from ancient local human activities (Ekoungoulou, 2014). The forests are highly dynamic ecosystems already encroaching into the savannah area (Koubouana et al., 2015).

Biodiversity is the integration of the variety and variation of all living organisms as related to their habitats and ecological complexes (CBD, 2006; Mukete and Sun, 2014). With its three primary attributes composition, structure and process, biodiversity is organized into a nested hierarchy from genes to species, populations and ecosystems. But recently, these vital ecosystems are being threatened by population growth and agricultural expansion (Ernst et al., 2013). It has therefore become very imperative to assess the in situ biodiversity such as to better understand its evolution (Yoka et al., 2013). The floristic diversity of a forest is a parametric description of its in-situ plant communities (FAO, 2008). It expresses the richness of a given community relative to its number of families, genus and species. It also takes into account the distribution of the species within the community (consistency or fairness) (Yoka et al., 2013). This can be expressed by several diversity indices with the most frequently used being the Shannon-Weaver diversity index (Fedor and Spellerberg, 2013). This index is mostly used for a

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comparative study of populations because it is independent of the size of the studied population (Chen and Li, 2011; Folega et al., 2014).

Species richness is a measure of the variety of species in a sampled area (Ekoungoulou et al., 2017). It is often a representation based simply on a count of the number of species in a particular sample (Fedor and Spellerberg, 2013; Fayolle et al., 2014b). Species diversity can be calculated and expressed in different formats with many indices such as Berger-Parker index, Margalef index, Menhinick index while others are based on complex mathematics (e.g. Brillouin index). Selection of appropriate index depends on required sample size sensitivity (from low in Simpson or Berger-Parker index to high in Margalef index), discriminant ability or simply on how widely an index is applied in ecological (FAO, 2008). Estimating biomass is necessary (Fayolle et al., 2014a) especially for those who stand to benefit from mechanisms such as reducing emissions from deforestation and forest degradation, and forest conservation, sustainable management of forest, and enhancement of forest carbon stocks (REDD+). This is an initiative aimed at encouraging less developed countries to voluntarily reduce their national deforestation rates and greenhouse gas emissions (Ngomanda et al., 2013; Ekoungoulou et al., 2014a). Hence, assessing floristic diversity such as to enable Congo enhance its floristic tank and sustainably manage its forests has become obvious. This study mainly focus on aimed the floristic distribution and structure of a Republic of Congo's tropical equatorial rainforest. It also aimed at assessing the trees composition of the Lesio-louna natural forest in Republic of Congo.

Materials and Methods

Study area

The study area is located in Lesio-louna natural forest of the Republic of Congo. It is managed by the Lesio-louna Project supported by The Aspinall Foundation and Congolese Ministry of Forest Economy and Sustainable Development. This is a wildlife reserve that extends over 173,000 ha (PLL, 2010; Ekoungoulou et al., 2014a) right into the country's Teke-plateau. These are a series of plateaus arising from Gabon, crossing the Republic of Congo and ending up in the Democratic Republic of Congo. Studied sites are located between 15°28.353'E, 03°16.241'S and 15°28.425'E, 03°16.056'S for Ikoubikro and between 15°28.841'E, 03°18.843'S and 15°28.919'E, 03°19.032'S for Blue Lake Forest (*Fig. 1*). Average annual rainfall ranges between 1,500 to 2,000 mm (ANAC, 2013) while mean annual temperature ranges between 20°C to 35°C (ANAC, 2013; Ekoungoulou, 2014; Ekoungoulou et al., 2014b). This gives the Lesio-louna a characteristic tropical equatorial climate with varying long dry seasons and low temperatures (ANAC, 2013; Ekoungoulou et al., 2014a). Meanwhile, the relative humidity ranges from 52% in August to 92% in December (*Fig. 2*) with several rivers such as the Ngambali and Lesio arising from and running through it (PLL, 2010; Ekoungoulou et al., 2015). Humidity data was provided by the main meteorological station around the study area which is from Congo's National Agency of Civil Aviation, Republic of Congo (ANAC, 2013).

Sampling design

The data regarding this study have been collected from August to October 2012 in Lesio-louna protected area. The double decameter was used (model TAJIMA-20m)

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made by Forestry Suppliers Inc, USA to measure the diameter at breast height (DBH) for each tree at both the Iboubikro and Blue Lake Forests.

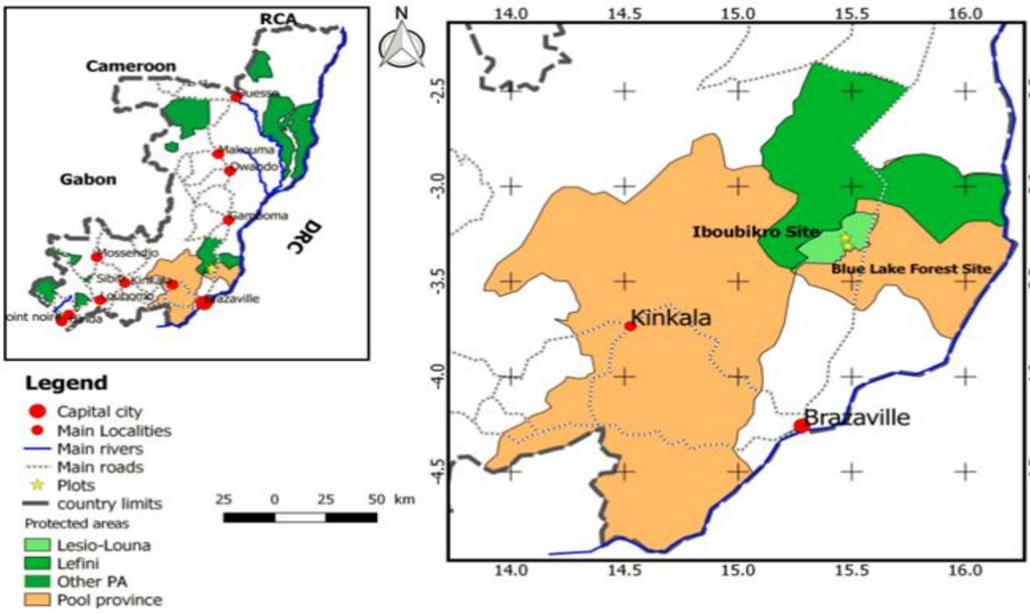

Figure 1. Study area location

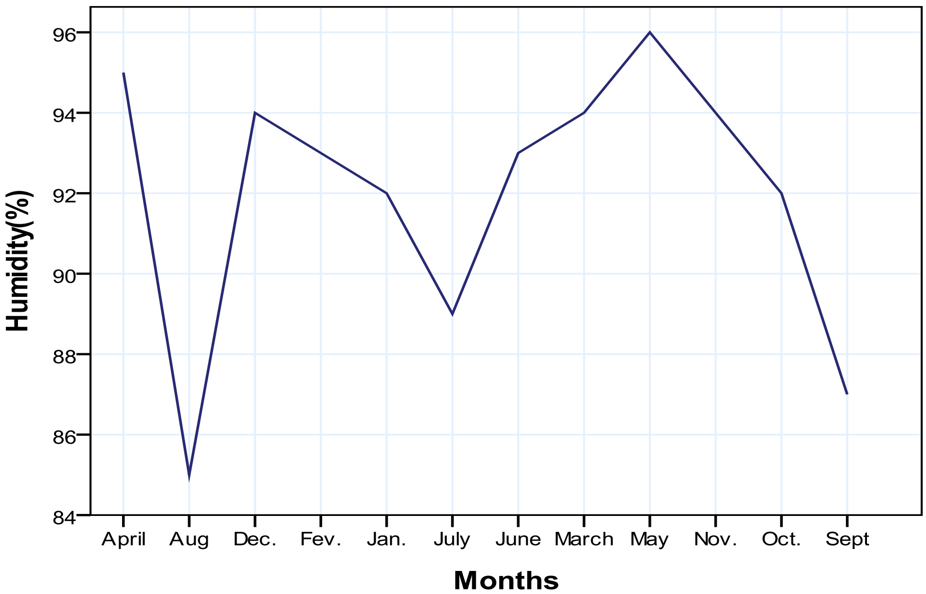

***Figure 2.** Average Monthly variation of relative humidity in Lesio-louna area from 1990 to 2012*

Each forest site was divided into three circular plots of size 1,256 m^2 (six plots in all) separated by a 100 m distance from each other (*Table 1*). Each circular plot was further divided into three circles (*Fig. 3*). From the circular plot center to the first circle (small circle), the radius was 0-6 m or 12 m of diameter and tree inventory 10-29.99 cm DBH (only trees with DBH ≥ 10 cm were measured). From the plot center to the second circle

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(medium circle), the radius was 0-14 m or 28 m of diameter, with tree inventory 30-60 cm DBH (*Fig. 3*). From plot center to the third circle (large circle), the radius was 0-20 m or 40 m of diameter, with tree inventory > 60 cm DBH. In each case, using the tape, the tree was measured at a chest height of 1.30 m above ground. Each measurement was done from the plot center to the north; south; east part and lastly from the center to the West according to Pearson and Brown (2005). Each recorded tree was then marked and labeled with a nail (model number 5 made by NASAFA Plc, Congo) carrying a 10 cm^2 plastic label obtained from PLASCO Plc, Congo. A compass was used (model SILVA-2S, Scale 1:24000) to determine cardinal points (N-S & E-W) or orientations of each plot. Finally, the GPS (model Garmin 60CSx) has been used to record the plot coordinates (location) in minutes, degrees and seconds. Data from each plot was then recorded (*Fig. 1*).

Table 1. *Study sites and their characteristics. IBK: Iboubikro (Plot1, Plo2, Plot3); BLF: Blue lake forest (Plot4, Plot5, Plot6); n: Number of sampled trees by site; E: Pielou evenness index; H': Shannon-Weaver index of species diversity (in bit); G: Basal area (in m^2 ha^{-1}); D': Diversity of Simpson index; K: Similarity of Sorensen index for both of two sites (in %); SR*: Species richness; DBH: Average of diameter at breast height (in cm); Pi: Relative abundance of the species; S: Number of species*

Site	***Flora richness***			***Diversity indices***				***Structure***		K
	S	*n*	SR*	H'	*Pi*	E	D'	G	DBH	
IBK	17	52	52	2.48	1	0.89	0.10	22.31	27	43.7
BLF	15	33	33	2.29	1	0.86	0.14	23.08	35	

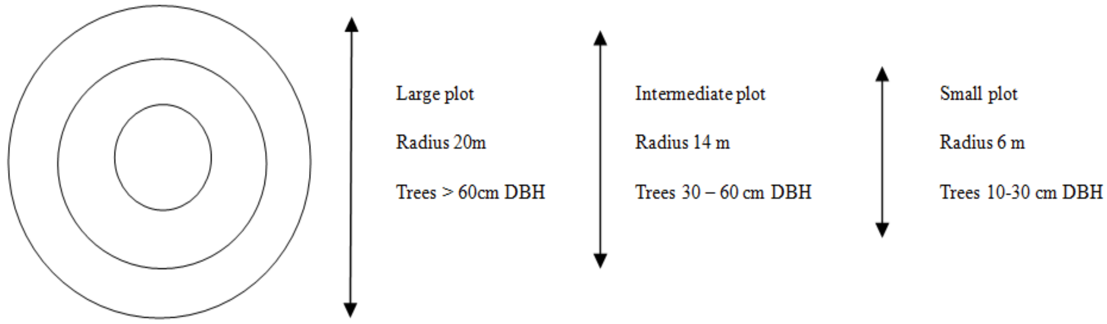

Figure 3. *Sample design: Circular plot of data census in study area. The schematic diagram represents a sample plot consisting of three concentric circles*

Data analysis

The floristic data was calculated in order to obtain various indices of diversity, phytogeographical determination of species and life forms as mentioned by Spellerberg and Fedor (2003). A diversity index was used since it provides detailed information on community composition and relative abundance. To know regarding the variation among plant communities in Blue lake forest and Iboubikro, ANOVA has been performed using SPSS 18.0 program. This data was analyzed using SPSS version18.0 software while the survey area map was performed with QGIS version 2 and ArcGIS

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version 9.3. We used the Shannon (*Eqs. 2* and *3*) and Pielou evenness (*Eq. 4*) and Sorensen & Simpson's index (*Eqs. 5-7*), to evaluate floristic diversity and accuracy indices (Spellerberg and Fedor, 2003; CBD, 2006; Fedor and Spellerberg, 2013). Using Sorensen's similarity index (Spellerberg and Fedor, 2003; Folega et al., 2011; Ekoungoulou et al., 2017), to perform the basal area and specific richness study for the Lesio-louna forest.

(a) Relative frequency:

The relative frequency and specific contribution of each tree species and family have been performed as follows (*Eq. 1*):

$$F_{(\%)} = \frac{n_i}{N} \times 100 \qquad \text{(Eq.1)}$$

where F is relative frequency of species and family (in %), n_i is the number of individual i and N is the total number of individuals found in the communities.

(b) Shannon-Weaver index, H':

$$H' = -\sum_{i=1}^{n} P_i \ln P_i \qquad \text{(Eq.2)}$$

According to Shannon-Weaver's diversity equation: H' is Shannon index, P_i is the proportion of individuals of one particular species found divided by the total number of individuals found, ln is the natural log, Σ is the sum of the calculations and n is the number of tree species.

P_i is calculated as follows:

$$P_i = \frac{N_i}{N} \qquad \text{(Eq.3)}$$

However, where H' is the Shannon-Weaver index of species diversity, P_i is the relative abundance of the species I, N_i is the number of the species i and N is the total number of species.

(c) Pielou evenness, E:

It represents the relationship between observed diversity and the possible maximum diversity for a given number of species of tree (N).

Small values of equitability represent a big importance of some dominant species.

$$E = H' / \ln N \qquad \text{(Eq.4)}$$

with:

E = Pielou evenness
H' = Shannon index
N = number of species
ln = natural log

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(d) Diversity of Simpson index, *D':*

It gives the probability that two individuals taken at random from a population studied belong to the same species (Ekoungoulou et al., 2015). This index measures how individuals are distributed among species of a community. It is a measure of the inverse diversity.

$$D' = \sum (N_i / N)^2 \qquad \text{(Eq.5)}$$

with:

D' = Simpson's index
Ni = number of species *i*
N = total number of species

(e) Species richness, SR:

It is the total number of floristic richness (families, genus and species) in the communities studied.

(f) Similarity of Sorensen index, *K:*

The degree of quantitative resemblance of two lists can be measured by the formula of the similarity coefficient of Sorensen (Spellerberg and Fedor, 2003; Folega et al., 2011; Ekoungoulou et al., 2017). From 2 floristic lists A and B, a designating the number of species of sample A and b designating the number of species of sample B and c designating the number of common species to both floristic lists, the coefficient of community Sorensen, K is calculated in the following manner:

$$K = \frac{2*c}{(a+b)} \times 100 \qquad \text{(Eq.6)}$$

with:

a = number of species in sample 1,
b = number of species in sample 2,
c = number of species common to both.

This index can used to evaluate the floristic affinities between 2 samples. If *K* > 50%, then the two samples belong to the same plant community in the area.

(g) Basal area, *G:*

This is a measurement taken at the DBH (1.3 m) of a tree above the ground and includes the complete diameter of every tree. To estimate a tree's basal area, we used the tree's DBH in inches with the following formula:

$$G = \sum (D_i)^2 \times \frac{\pi}{4} \qquad \text{(Eq.7)}$$

where *G* = basal area (m^2 ha^{-1}), D_i = diameter (cm) at 1.3 m above the ground and $\pi = 3.14$.

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Results and Discussion

A total of 85 trees divided into 25 species and 14 families have been recorded within the six circular plots studied (*Table 2*). Amongst the 85 trees, 52 trees were in the diameter class of 10-29.99 cm; 28 trees in the diameter class of 30-60 cm and 6 trees in the diameter class > 60 cm (*Fig. 5*). The Iboubikro site (Plot1, Plot2 and Plot3) had more measured trees (52 trees) while the Blue lake forest site (Plot4, Plot5 and Plot6) with 33 trees found. At the plot level, plot 3 had more recorded trees (20 trees) followed by plot 1 with 17 trees recorded (*Table 1*). The average diameter at breast height (DBH) for the 6 plots studied was 30.80 cm. Meanwhile, the average recorded diameter at breast height varied from 41.91 cm in plot 6 to 24.49 cm in plot4. The investigation revealed that in this ecosystem Fabaceae (20%), Mimosaceae (16%), Sapindaceae (12%) and Annonaceae (9%) where the most representative families (*Fig. 4a*). *Millettia laurentii* De Wild. (14%), *Pentaclethra eetveldeana* De Wild. & T. Durand (14%), *Eriocoelum macrocarpum* Gilg ex Radlk. (8%), *Millettia pinnata* Panigrahi (6%), *Sorindeia juglandifolia* (A.Rich.) Planch. Ex Oliv. (6%) and *Xylopia aethiopica* (Dunal) A.Rich. (6%) was the most dominant species (*Fig. 4c*). In Flacourtiaceae (1%), Euphorbiaceae (1%) and Rubiacea (1%) a very low proportion of relative frequency of families were found (*Fig. 4a*). *Treculia obovoidea* N.E.Br. (1%), *Oxyanthus speciosus* DC. (1%) and *Macaranga barteri* Mull. Arg. (1%) had a very low proportion of species relative frequency (*Fig. 4c*). The revision and homogenization were also applied on the taxonomy according to the working list of all plant species (accessed 8 January 2017 from http://www.theplantlist.org and from http://www.xycol.net/index.php?categorie=0&sess _langue=430).

Biodiversity analysis was made also by using Shannon-Weaver diversity index. For this research, the floristic biodiversity analysis between Iboubikro and Blue lake forest have been considered (*Table 1*). Shannon diversity index regarding six studied plots were between 2.29 and 2.48 bits (*Fig. 4d*). The highest Shannon index recorded was in Iboubikro site (2.48 bit), while the lowest was recorded in Blue lake forest site (2.29 bit). *Table 1* show that Pielou's evenness index was 0.86 for Blue lake forest (86.9%) and 0.89 for Iboubikro (89.4%). Iboubikro site had the highest index of Shannon-weaver and Pielou index compared with Blue lake forest site (*Fig. 4d*). An evenness index > 60% characterized an undisturbed ecosystem (Spellerberg and Fedor, 2003; Fedor and Spellerberg, 2013). One of non-disturbance reason about Lesio-louna forest is because this ecosystem is a protected area.

Similarity of Sorensen index regarding studied sites was 43.7% (*Table 1*). These results show that there is a very low similarity of species between Iboubikro and Blue lake forest, and between six study plots. All species of two communities studied are not similar. The results show that in total of 25 species only 7 similar species (species common to both communities) have been recorded in Iboubikro as well as in Blue lake forest. The two sites could be totally similar if Sorensen's index was 100% (Legendre, 2014; Koubouana et al., 2015). If this index is less than or greater than 100%, it is inferred that the two ecosystems are not completely similar (Legendre, 2014).

The Simpson diversity index was ranged from 0.10 regarding Iboubikro to 0.14 for Blue lake forest (*Table 1*). This index will have a value of 0 to indicate the maximum diversity, and a value of 1 to indicate the minimum diversity (Folega et al., 2011; Ekoungoulou et al., 2015). In this area, an important trees biodiversity have been measured. The value of Simpson diversity index recorded show a maximum trees biodiversity in Lesio-louna forest.

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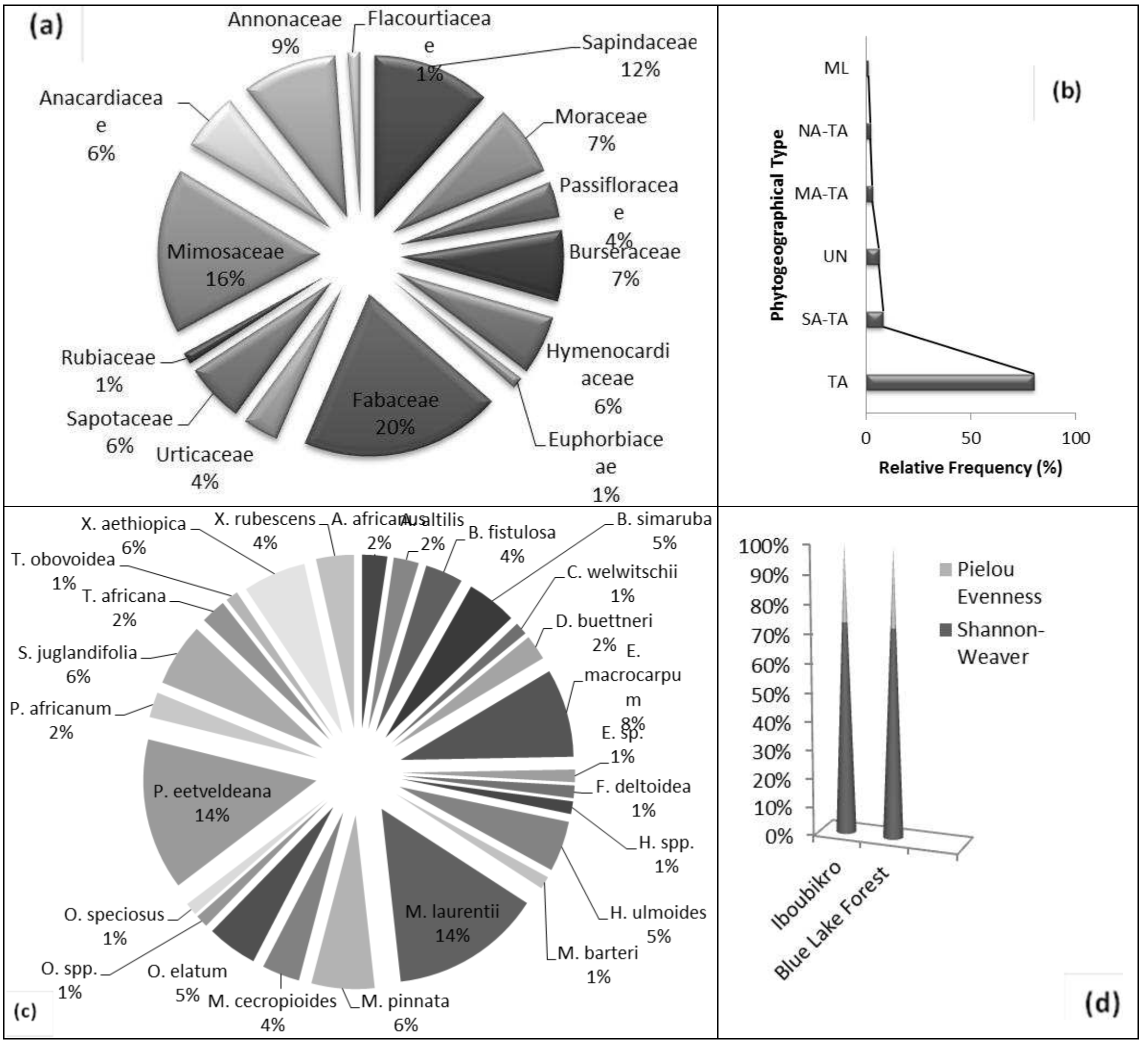

Figure 4. *Floristic diversity overview of forest ecosystem: (a) Relative frequency of specific spectra families recorded in study area; (b) phytogeographical type distribution— TA: Tropical Africa Area (EPFAT Area, country-based, south of Sahara, complementary to the following), SA: Southern Africa Area (South Africa, Namibia, Botswana, Lesotho, and Swaziland), NA: North Africa (Mauritania, Morocco, Canary IsI., Algeria, Tunisia, Libya, Egypt, and Madeira), ML: Malaysia (Tropical Asia), UN: Undetermined, and MA: Madagascar (Malagasy Republic); (c) Distribution of diversity among species taxa; (d) Variation of Shannon-Weaver diversity and Pielou Evenness indexes*

Therefore, Basal area of Blue lake forest (23.08 m^2 ha^{-1}) was higher compared with Iboubikro (22.31 m^2 ha^{-1}) as showed in *Table 1*. Basal area provided better measurement about the relative importance of tree species than simple stem count. It was obvious that dendrological parameters in Iboubikro were higher than those of Blue lake forest (*Fig. 5*). In order to know whether there is variation among the Iboubikro and Blue lake forest in species richness, evenness and species diversity, one way ANOVA was done using SPSS version 18.0 statistical software. The result of ANOVA performed showed that there was statistically significant difference among plant communities in species richness, evenness and species diversity at 92% confidence interval for Blue lake forest and Iboubikro (*Table 2* and *Fig. 4*). A two sample t-test was

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done in order to know the existence of variation within a community utilizing species richness, evenness and diversity of species. The result of 2 sample t-test shows that, there is a significant difference at 92% confidence interval within community types (*Table 2* and *Fig. 4*).

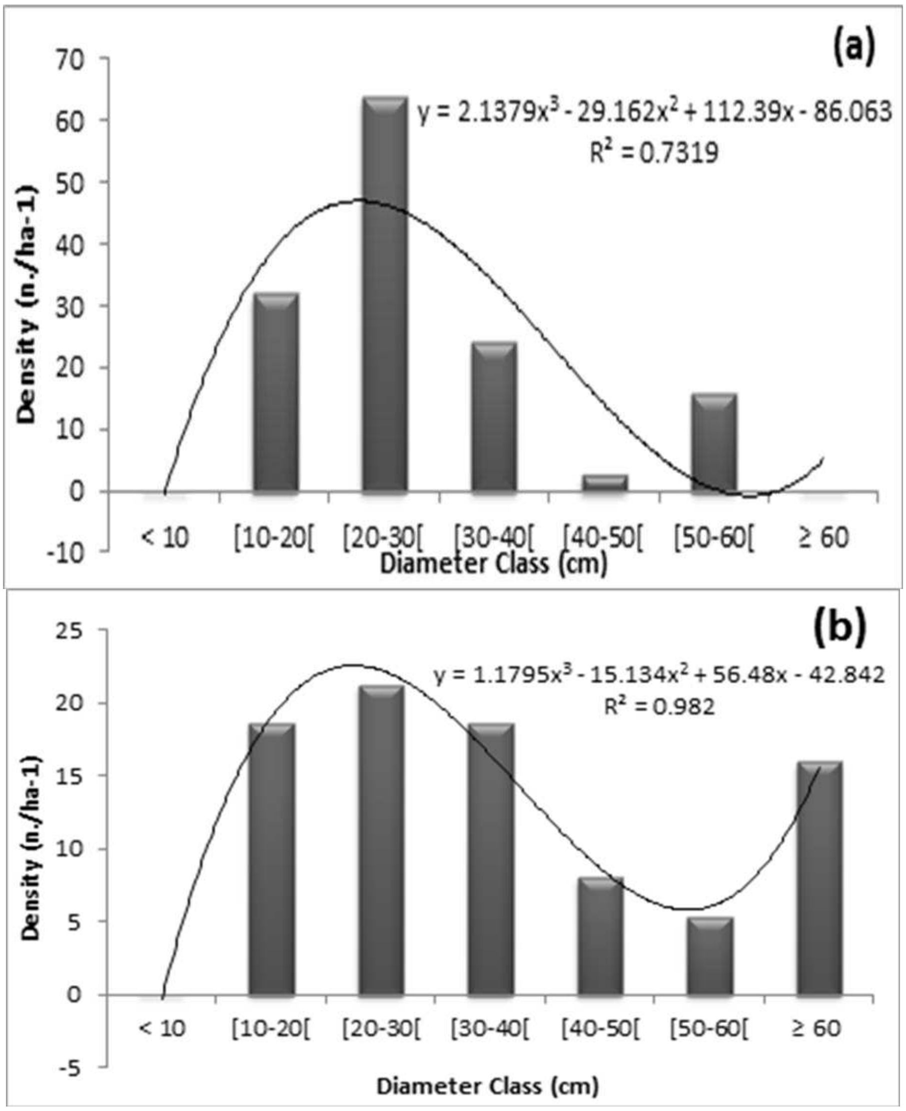

Figure 5. *Diameter class distribution of trees in Blue lake forest site (a) and Iboubikro site (b)*

However, on chorological level, Tropical African species (80%) were the most important about the Phytogeographical type (*Fig. 4b*) followed by Southern African-Tropical African species (8%). Malaysian species (1%) had a very low proportion of phytogeographical type followed by Northern African-Tropical African species (*Fig. 4b*). This study shows that, the Fabaceae family was dominant with 17 tree species, with relative diversity index of 20% (*Fig. 4a*). Among these 17 tree species, 12 tree species were *Millettia laurentii* De Wild., with a diameter at breast height (DBH) ranging from 11.1- 63.1 cm (*Table 2*). This was followed by the Mimosaceae with 14 tree species of which 12 species were *Pentaclethra eetveldeana* De Wild. & T. Durand, and the diameter at breast height (DBH) ranging from 10.9-59.2 cm. Other regular families

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included the Sapindaceae with 10 tree species; Annonaceae with 8 tree species; Euphorbiaceae, Flacourtiaceae and Rubiaceae with one tree species each (*Fig. 4a*). Fabaceae and Mimosoideae are dominant in this forest ecosystem because this environment is favourable for them. The trees from these families (Fabaceae and Mimosaceae) have a rapid growth than others, because they receive more light than other trees. These are trees that usually have an abundant canopy. Fabaceae and Mimosoideae are the Leguminosae (particularity absorbs the atmospheric nitrogen through nodules located in its roots). Leguminosae enrich the soil with nitrogen. The study show that the number of families varies from one site to another and from one plot to another. The trees of this forest are not producing since the inception of the Lesio-louna project, to sustainable forest management, also for the flora conservation. Any logging is strictly forbidden in Lesio-louna area (PLL, 2010).

Table 2. *Distribution of species recorded in the moist forest of Lesio-louna (study area) by family. Phytogeographical type has been provided by African Plants Database (version 3.4.0) of Conservatoire et Jardin botaniques de la ville de Geneve, Switzerland & South African National Biodiversity Institute, Pretoria (Retrieved 6 January 2017 at http://www.ville-ge.ch/musinfo/bd/cjb/africa/), and Global plants (Accessed 6 January 2017 at http://plants.jstor.org/). DBH: diameter at breast height (in cm), n: Number of tree species recorded, PT: Phytogeographical type, TA: Tropical Africa Area (EPFAT Area, country-based, south of Sahara, complementary to the following), SA: Southern Africa Area (South Africa, Namibia, Botswana, Lesotho, and Swaziland), NA: North Africa (Mauritania, Morocco, Canary IsI., Algeria, Tunisia, Libya, Egypt, and Madeira), ML: Malaysia (Tropical Asia), UN: Undetermined, and MA: Madagascar (Malagasy Republic)*

Species	Family	PT	*n*	DBH range
Allophylus africanus P.Beauv.	Sapindaceae	SA-TA	2	25.6-27.5
Artocarpus altilis (Parkinson ex F.A.Zorn) Fosberg	Moraceae	NA-TA	2	21.6-31.4
Barteria fistulosa Mast.	Passifloraceae	TA	3	14.6-21.2
Bursera simaruba (L.) Sarg.	Burseraceae	TA	4	29.8-79.0
Caloncoba welwitschii (Oliv.) Gilg	Flacourtiaceae	TA	1	18.3
Dacryodes buettneri (Engl.) H.J.Lam	Burseraceae	TA	2	22.2-24.3
Eriocoelum macrocarpum Gilg ex Radlk.	Sapindaceae	TA	7	13.5-25.9
Eriocoelum spp.	Sapindaceae	TA	1	41.1
Ficus deltoidea Jack	Moraceae	ML	1	17.7
Hymenocardia spp.	Hymenocardiaceae	TA	1	28.5
Hymenocardia ulmoides Oliv.	Hymenocardiaceae	SA-TA	4	16.9-50.3
Macaranga barteri Müll.Arg.	Euphorbiaceae	TA	1	10.3
Millettia laurentii De Wild.	Fabaceae	TA	12	11.1-63.1
Millettia pinnata (L.) Panigrahi	Fabaceae	UN	5	32.0-65.7

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Musanga cecropioides R.Br. ex Tedlie	Urticaceae	TA	3	12.2-35.2
Omphalocarpum elatum Miers	Sapotaceae	TA	4	15.4-34.2
Omphalocarpum spp.	Sapotaceae	TA	1	10.6
Oxyanthus speciosus DC.	Rubiaceae	SA-TA	1	84.3
Pentaclethra eetveldeana De Wild. & T.Durand	Mimosaceae	TA	12	10.9-59.2
Piptadeniastrum africanum (Hook.f.) Brenan	Mimosaceae	TA	2	22.8-26.0
Sorindeia juglandifolia (A.Rich.) Planch. ex Oliv.	Anacardiaceae	TA	5	12.9-26.5
Treculia africana Decne. ex Trécul	Moraceae	MA-TA	2	21.1-30.8
Treculia obovoidea N.E.Br.	Moraceae	TA	1	17.3
Xylopia aethiopica (Dunal) A.Rich.	Annonaceae	TA	5	11.6-60.5
Xylopia rubescens Oliv.	Annonaceae	TA	3	20.3-36.6

With regard to the phytogeographical type of species, this study shows that most of species recorded in Lesio-louna forest are from tropical Africa area, precisely the South of Sahara's countries (*Table 2*). The countries from South of Sahara (Africa) are dominated by the moist forests and some dry forests.

In the circular plots studied, 52 trees belonged to the diameter class 10-29.99 cm while 28 trees belonged to the diameter class 30-60 cm and 6 trees to the diameter class > 60 cm could be because the forest is to recover with a considerable regeneration as asserted by PLL (2010). *Figure 5* shows that, regarding the DBH classes of trees, an important number of trees have been recorded with the diameter class 20-30 cm for Iboubikro site as well as for Blue Lake Forest site compared to others diameter classes. In this study a measurement of more trees with low DBH and few trees with high DBH have been done (*Table 2* and *Fig. 5*). The horizontal growth of these trees depends on the vertical growth. Observation also showed the regeneration in Lesio-louna edge by the forest that moves towards Savannah.

More trees have been recorded in Iboubikro site (52 trees) as compared with the Blue lake site (33 trees), that can be by the fact that Blue lake forest is located in a cavity trough which is like a recess. Trees of this ecosystem (Blue lake forest) receive less light, because the forest is surrounded by hills. It is well known that floristic composition is determined by ecological factors. Other reason is the fact that Blue lake forest is often visited by tourists (disturbance of the ecosystem by anthropogenic activities). Therefore, it influences biodiversity patterns at both local and a regional scale further reflects both human and natural disturbances. Thus, in this ecosystem, floristic characteristics and biodiversity patterns coincide with the climate factors and anthropogenic actions. This study showed a variation in the DBH (diameter at breast height) and tree species amongst the plots because there is a forest atmosphere and individuals are heterogeneous (*Fig. 5*).

Similarly, studying floristic biodiversity and natural regeneration in three forest islands, Koubouana (2015) found 120 species and 47 families. These included the Rubiaceae (25.6 to 34.2%) and the Fabaceae (5.9 to 13.9%); characteristic species

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density varying from 0.7 to 60 trees ha^{-1} and biological diversity for Shannon index (H') averaged 1.9 ± 0.3 for Woody as against 3.5 ± 0.25 for the total flora (Koubouana, 2015). In the study by Koubouana (2015), the various indices showed their referral ecosystem had a low floristic diversity accompanied by heterogeneity and dominance in floristic composition.

In a related study Kimpouni (2008) asserted that, the species *Aucoumea klaineana* Pierre exhibited a highly heterogeneous and spatio-temporal floristic diversity in its specific composition. Their study concluded that, though *Aucoumea klaineana* Pierre is the characteristic and dominant element for this feature, its dynamics has a regressive evolution in relation to the step of development. According to their study, the Fabaceae has a relative diversity index of 18.31%. This does not tally with our study which found the relative diversity index for Fabaceae to be 20% (*Fig. 4a*).

In this study, floristic heterogeneity between Iboubikro and Blue lake forest was studied from structural analysis using the Sorensen similarity index. The result showed on the one hand that the sampled sites of measurement belong to the forest type and, on the other hand, that there is floristic variability due to the substrate variation as mentioned by PLL (2010) and Koubouana (2015). The different sites (Iboubikro and Blue lake forest) inventoried belong to the same plot type (with various area and climate condition) highlighted by the Sorensen similarity index. This cross-site heterogeneity is also observed in the site tailgates. The variability of edaphic factors such as soil composition, dewatered and hydromorphic soils and others could be an indicator concerning this kind of floristic richness about Lesio-louna protected area.

In Lesio-louna's Teke-plateau, along of streams and rivers, are the galleries forests (moist forest), but it varies considerably from one point to another of the hydrographic network (PLL, 2010). Near the heads of rivers, these galleries can be very narrow and reduced to a simple screen of trees, but they can also be continuity with shreds of dry forests hooks a slope shape. In this case, it is sometimes very difficult to draw a strict line between the riparian forest (gallery forest) and dry forest or secondary forest. In Lesio-louna, narrow galleries however is a rather special environment very different from the great forest. In fact, they are composed of two strips joined together back-to-back housing and a narrow strip of dense humid vegetation along a stream. When the stream that feeds the forest is enclosed between two steep banks, as it often happens in the upper part of the stream, the undergrowth may however be relatively dry. In general, even in very narrow tunnels, measuring no more than 20 or 30 m wide, however there are two areas. From both sides of the river, there is a central very wet and susceptible to some flooding during heavy flood area (Ekoungoulou, 2014).

A distance of rivers, on the foot of the hill lays a marginal area which is never flooded but the vegetation is more resistant to drought (Ekoungoulou et al., 2015). Further downstream, these galleries of Lesio-louna widen, and their edges depart, leaving more room for the central zone. Imperceptibly merges riparian gallery then in the great forest. Ecological difference between a gallery and a great forest is not always very clear, but in extreme cases it is well marked (Ekoungoulou et al., 2015). In a narrow gallery forest**,** vegetation undergrowth, at least in the wetland is generally very dense because of the proximity of the edge. Here, the microclimate is not as stable as in the great forest. The canopy is rarely continues (Nkounkou, 2003). It receives a very important side lighting and is much more exposed to the wind, so to drying, the canopy of the rainforest. Epiphytes are generally concentrated over the central stream and vines are extremely abundant, they constitute a very significant proportion of plant biomass (FAO, 2008). Another very important aspect of moist forests is related to the fact that

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they represent a quasi-linear medium. Animals that live in these galleries are forced to make important trips. Many species of forest, especially birds, avoid narrow galleries forests or moist forests (PLL, 2010).

Thus, understanding the factors that control species distributions and forest composition is a central issue in ecology and conservation because floristic diversity of Lesio-louna is influenced by ecological factor. This study has allowed to deduct that the advance of the forest to savannah in Lesio-louna and observed during our field work is may be caused by the wealth of organic matter soil. This wealth of organic matter soil can be by the fact that the forest is dominance of Fabaceae (20% of relative diversity index) and Mimosaceae (16% of relative diversity index). Many tree species from Fabaceae and Mimosaceae have been recorded during this study in natural forest of Lesio-louna. The species from Fabaceae and Mimosaceae families enrich the soil with nitrogen, and nitrogen is important for the nutrition of plants. Given the significant differences in floristic diversity, Lesio-louna forest ecosystem could play an important role in climate change mitigation and thus could provide a carbon sink in all Teke-plateau, and also in the whole basin forest of Central Africa (Congo Basin). However, knowing the Lesio-louna's floristic richness by this study, this forest ecosystem is important to contribute to the global environmental protection and support the reducing emissions from deforestation and forest degradation, and forest conservation, sustainable management of forest, and enhancement of forest carbon stocks ($REDD^+$) guidelines as set the Kyoto protocol. Also, in this ecosystem the growth of the forest to savanna is remarkable, because several species of bush land are found in the forest edge and even in the middle of forest as observed by PLL (2010).

Moreover, in this study area, the reconstitution of the wood stand can be characterized by diversity, basal area and the distribution of individuals in diameter classes. Diversity establishes links between the richness and the abundance of individuals; it reflects the degree of heterogeneity or stability of vegetation in Lesio-louna. The basal surface reflects the dynamism of vegetation and recovery speed. Plant diversity can therefore be integrated several parameters such as the structure and dynamics of vegetation. The relationship between diversity and structure in a community can be associated with various changes: diversity is a function of the structure dynamics and the structure diversity is a function of dynamic. This shows interest to appreciate the diversity of Congolese forest ecosystems for their sustainable management.

Conclusion

The vegetation of Central Africa is largely composed of Congo drainage basin and contiguous humid closed canopy forests less well known, large areas of savannah and forest-savannah mosaics often composed of forest groves either along the river bank or resulting from ancient local human activities. In Lesio-louna forest (Republic of Congo), the floristic diversity presented a dynamism and floristic richness very considerable in its specific composition, from Iboubikro to Blue Lake Forest sites. The number of trees recorded in Iboubikro site was important compared to the number of trees recorded in Blue Lake Forest site. In this Lesio-louna forest, DBH of trees vary from one plot to another and the number of trees species sampled varies from plot to plot. There is observation of a heterogeneous floristic diversity in this forest ecosystem. This study may enable us to contribute in the promotion of Lesio-louna forest ecosystem, which includes species with importance for humanity. Increasing

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knowledge of plants distributions in Congo demonstrated clear smaller-scale variation in forest composition and its relationship with environmental conditions, notably rainfall but also geological substrate and soil, or a combination of soil and rainfall. Also, in this forest there is abundance of trees with large diameter which was the main reason for these differences. Generally, the Lesio-louna forest is in the regeneration period, and can be a considerable carbon sink for the Republic of Congo and the world. Knowing the floristic richness of Lesio-louna by this study, this moist forest is an important ecosystem to contribute to the global climate change mitigation and support the REDD+ process.

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Conflict of interests. The authors declare that there is no conflict of interests regarding this paper.

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THE EFFECT OF CROPS CULTIVATION ON SOIL EROSION INDICES BASED ON IMPELERO MODEL IN NORTHEAST IRAN

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Abstract. In the present study an integrated neural network based model named ImpelERO was applied to estimate the impact of conventional cropping system on erosion indices, including soil vulnerability index, erosion risk class and soil loss rates by potato, alfalfa and maize plantations. Our results revealed that the soil vulnerability indices ranged from 0.21 to 0.52, 0.15 to 0.41 and 0.2 to 0.5 by potato, alfalfa and maize cultivations, respectively. The values of erosion risk classes by potato and maize cultivation ranged from V1 to V4 in which categorize the region as non-sensitive to very sensitive to erosion and by alfalfa cultivation varied between V1 to V3 which classify the study area as non-sensitive to sensitive to erosion The values of soil losses varied between 7.1 to 59 t $ha^{-1}yr^{-1}$ with an average of 15.11 t $ha^{-1}yr^{-1}$ by potato, 4.9 to 32.4 t ha^{-1} yr^{-1} with an average of 8.42 t ha^{-1} yr^{-1} by alfalfa and 6.8 to 52.7 t ha^{-1} yr^{-1} with an average of 13.65 t ha^{-1} yr^{-1} by maize cultivation. It was concluded that planting perennial alfalfa compared to row planted potato and maize has a great effect on controlling soil erosion and its indices at the study area.
Keywords: *neural network, erosion, potato, alfalfa, maize, GIS*

Introduction

Nowadays, soil erosion issues are considered one of the most important topics in the management of agriculture, natural resources, the environment, and water resources; which is the comprehensive management of watershed areas. The extent of soil erosion is determined by numerous factors such as relief, soil type, precipitation, farming practices, etc. (Evans, 2002). While soil and relief characteristics of fields have little change from year to year, precipitation and farming practices may vary strongly over time. The time-dependent combined effects of precipitation and cultivation practices and crop patterns are crucial for soil erosion in any given area (Fiener et al., 2011). The plant cover and cultivation practices are among the most important factors explaining the intensity of soil erosion, comparing rainfall intensity and slope gradient (Kosmas et al., 1997; Wainwright and Thornes, 2004). A number of studies demonstrate that the erosion caused by conventional tillage practices results in increased soil erosion, reduced surface soil quality on curving slope positions, loss of soil organic matter, revelation of subsoil with low quality and increased spatial variability of crop production (Kosmas et al., 2001; Papiernik et al., 2005). It has been shown that the main driving force for the soil redistribution is the erosion caused by conventional tillage practices which is strongly associated with the soil properties and grain yields (Quine and Zhang, 2002). Tillage erosion is the redistribution of soil within a farm caused directly by tillage. It has been shown that tillage erosion is a potential contributor to the

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total soil erosion on cultivated fields (Govers et al., 1999). The importance of tillage translocation on erosion in cultivated areas have been studied worldwide (Govers et al., 1999; Van Muysen et al., 2000, 2002; Lindstrom et al., 2001; de Alba et al., 2006; Van Oost et al., 2006). In recent decades much effort has been put into understanding the mechanisms of soil erosion by studying the factors affecting soil loss. In this regard several empirical or process-based models have been developed and practiced (Merritt et al., 2003; Russell and William, 2001). Modeling studies have mostly been limited to direct impact investigations without considering the potential for increased or even decreased erosion which may result from changes in land use and management (Mullan et al., 2012). Resource degradation is also an important problem for semi-arid areas and water erosion is common (Maji et al., 2010). One of the most important characteristics of precipitation in arid and semi-arid regions as the case of the study area is thunderstorm which causes huge runoff in a short time which resulted in massive amount of soil losses. In an investigation by Bagherzadeh (2014) for estimating soil losses in Mashhad-Chenaran Plain, the values of soil losses varied between 0 and 0.25 t ha^{-1} yr^{-1} alongside the kashaf-rud river in the middle of the plain and 2-10 t ha^{-1} yr^{-1} at the edges of the plain. Conventional down-slope cultivation on 1-2% slopes of silty loam soils accelerated the erosion process and adversely influenced soil structure (Kurothe et al., 2014). The Agricultural Soil Erosion Evaluation Model (ImpelERO) as an artificial neural network model developed for prediction of vulnerability to water erosion, productivity reduction and optimal management strategies for an agricultural parcel (de la Rosa et al., 1999). The aim of present study was to investigate the applicability of the proposed approach for predicting agricultural soil erosion vulnerability (ImpelERO model; De la Rosa et al., 1999) in14 selected benchmark sites from Chenaran Plain, northeast Iran was carried out in order to investigate the impact of conventional cultivation system of potato, alfalfa and maize as main strategic crops on soil erosion indices including vulnerability index, Risk class and Soil loss rate.

Materials and methods

Geographic position of the study area

The present study was conducted in Chenaran Plain with an area of 1305.3 km^2, Khorasan-e-Razavi province, northeast Iran. The study area is located between latitude 36.51° to 37.07° N and longitude 58.38° to 59.07° E including lands less than 1500 m above sea level (asl). The topographical elevation values of the study area vary between 1131 and 2907 m asl, while the main topographical elevation range over 2019 m asl. The general physiographic trend of the plain extends in a NW–SE direction surrounded between two mountainous zones of Kopetdagh at northward and Binaloud at southward based on visual interpretation of satellite image and field observations (*Fig. 1*).

The database in our study were the soil samples which derived from the Soil and Water Research Center of Khorasan-e-Razavi province, northeast Iran and the climate data were collected from local weather stations in study area. The main land use practice at the study area is irrigated farming around Kashaf-rud River. The study area has a semi-arid climate with mean annual precipitation of 208 mm and mean annual temperature of 13.5 °C. The rainiest month is March (46.7 mm) and the driest month is August (0.7 mm). The soil physical and chemical characteristics and the land terrain of the selected sites have been presented in *Tables 1* and *2*.

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Table 1. *Soil physical and chemical characteristics of the study area*

Site	Longitude	Latitude	Sand	Silt	Clay	Texture	ESP (%)	OM (%)	Bulk density
1	59.010	36.684	26	62	12	Silt loam	0.88	0.81	1.455
2	58.935	36.749	14	58	28	Silty clay loam	6.02	1.91	1.308
3	59.013	36.746	36	60	4	Silt loam	7.71	1.09	1.636
4	58.782	36.818	34	48	18	Loam	0.88	0.62	1.411
5	58.861	36.815	26	48	26	Loam	12.2	0.72	1.342
6	58.939	36.812	25	48	27	Clay loam/loam	8.59	1.03	1.334
7	58.707	36.884	34	56	10	Silt loam	0.58	0.93	1.497
8	58.786	36.881	29	49	22	Loam	13.3	0.59	1.372
9	58.554	36.953	32	52	16	Silt loam	0.43	0.86	1.425
10	58.633	36.950	48	40	12	Loam	0.58	0.88	1.498
11	58.711	36.947	25	52	23	Silt loam	6.42	1.84	1.358
12	58.558	37.016	32	54	14	Silt loam	0.43	1	1.444
13	58.636	37.013	30	52	18	Silt loam	0.58	0.59	1.403
14	58.697	37.012	24	58	18	Silt loam	0.29	0.76	1.392

Table 2. *Land terrain values of the study area*

Site	Slope		Aspect degree	Sub soil stoniness class[2]	Internal drainage[3]
	%	class[1]			
1	2.00	A	3.00	C	M
2	1.00	F	3.00	C	V
3	2.00	A	3.00	C	M
4	1.00	F	5.00	C	M
5	1.00	F	3.00	F	M
6	2.00	A	7.00	F	V
7	1.00	F	3.00	F	M
8	1.00	F	5.00	F	M
9	2.00	A	3.00	F	M
10	2.00	A	3.00	F	M
11	2.00	A	7.00	F	M
12	2.00	A	3.00	F	M
13	2.00	A	7.00	F	M
14	7.00	U	7.00	F	M

[1]Slope class: F: flat, A: almost flat, U: undul
[2]Sub soil stoniness class: F: few, C: common
[3]Internal drainage: M: moderate, V: very slow

The ImpelERO model

The data of the soil samples have been extracted from the studies of soil monitoring of Chenaran Plain. Geologically, main alluvial nature of the plain has been developed into a thick sediment dominated environment that belongs to quaternary period. Crop management systems includes farming systems, interactions with crop residue after harvesting, plowing systems during the growing season, row spacing, basic

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functionality and yield prediction as well as the frequency of application and type of equipment used for plowing from planting to harvesting (*Table 3*).

Table 3. *Agricultural management practices for potato, alfalfa and maize cultivation, and assumed to be used in the selected sites*

Crop type	Management	Residue treatment	Tillage system	Row spacing (m)	Baseline yield[1] ($t\ ha^{-1}$)	Estimated yield[2] ($t\ ha^{-1}$)	Tillage operation		
							Implement	Time[3]	Workability[4]
Potato	Convention	Grazing	Traditional	0.6	31	35	Plow moldboard	2	Yes
							Drill deep furrow	1	Yes
							Disc cultivator	2	Yes
							Harrow-roller	3	Yes
							Planter row	1	Yes
							Spray implement	4	Yes
							Fertilizer applicator	3	Yes
Alfalfa	Convention	Grazing	Traditional	0.5	10	12	Plow moldboard	1	Yes
							Disc cultivator	2	Yes
							Harrow-roller	1	Yes
							Planter row	1	Yes
							Spray implement	2	Yes
							Fertilizer applicator	2	Yes
Maize	Convention	Grazing	Traditional	0.75	40	45	Plow moldboard	1	Yes
							Drill deep furrow	1	Yes
							Disc cultivator	2	Yes
							Harrow-roller	3	Yes
							Planter row	1	Yes
							Spray implement	2	Yes
							Fertilizer applicator	4	Yes

[1]Baseline yield is the actual value of crop production from statistical sources
[2]Estimated yield is the predicted value of crop production by using simulation models
[3]Number of times that an implement is used
[4]Workability status makes reference if (yes or no) the optimum soil water content for each tillage operation is considered by the farmer

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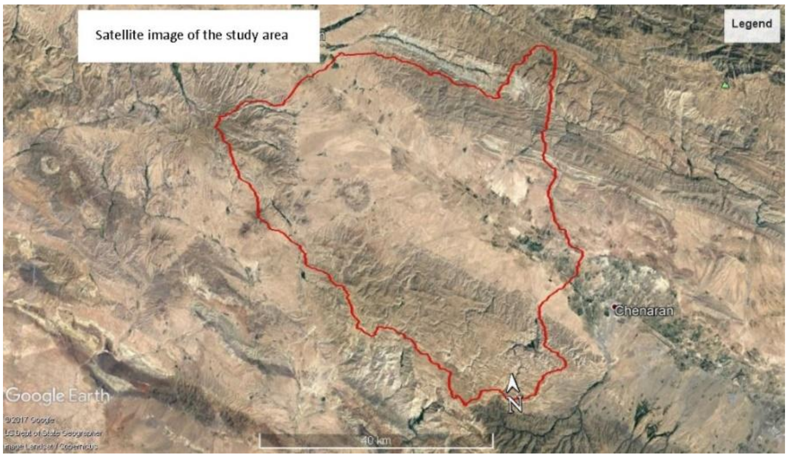

Figure 1. *Satellite image of the study area*

Soil depth loss was calculated using the ImpelERO model (De la Rosa et al., 1999). This model was developed as a Universal Soil Loss Equation-type model following traditional land evaluation analysis and advanced empirical modelling techniques. Using expert-decision trees, soil survey information and expert knowledge of the soil erosion process were combined with land and management qualities (de la Rosa et al., 2000).

An artificial neural-network approach was applied to capture the interactions between the land and management qualities and one output, vulnerability index to soil erosion. This computerized approach of agricultural management strategies on soil erosion reduction is summarized in *Figure 2*. As a first step and for one particular field-unit (fixed land qualities, LQs), the user can establish a percentage of vulnerability reduction (R) of the actual vulnerability index (V_a) in order to calculate the target vulnerability index (V_t). As a second step, 64 applications of the neural-network (four possible values of the three management qualities, MQs, = 4^3) were made in order to calculate the vulnerability index (V_j) which is closer to the target index. Then, the combination of MQs which corresponds to the V_j was selected. As a third step, the decision trees were backtracked by using the selected combination of MQs to finally formulate the optimum management strategies (de la Rosa et al., 2000).

Spatial analysis

An IDW interpolation function was applied in GIS to produce interpreted maps and visualize the zonation of the erosion indices including vulnerability index, erosion risk class and soil loss rate in the study area.

Results

The values of soil vulnerability indices by potato, alfalfa and maize cultivations varied between 0.21 to 0.52, 0.15 to 0.41 and 0.2 to 0.5, respectively (*Table 4*). The most vulnerable regions to erosion by potato and maize cultivations were some parts in north and east of the study area, while by alfalfa production the prone areas to high erosion vulnerability restricted to scattered parts in north of the plain (*Fig. 3*). The value of soil vulnerability index for maize and potato by conventional management system were greater than the similar values by alfalfa cultivation.

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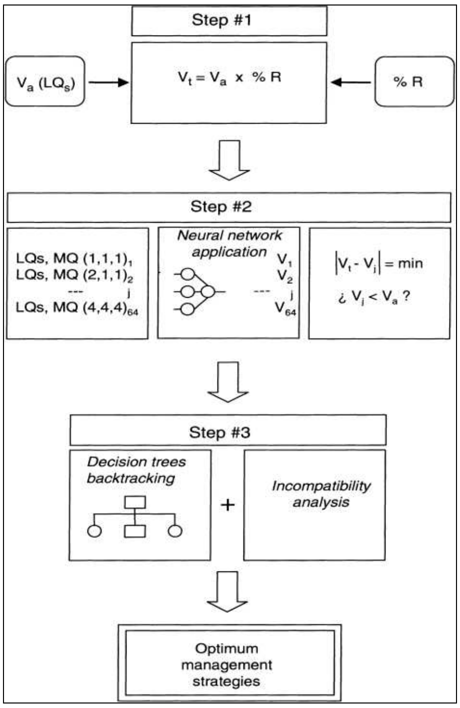

Figure 2. *General scheme of the automated neural-network-based search and the decision trees backtracking to accommodate the management practices (MQs= management qualities) to a percent of soil erosion reduction, where V_a= actual vulnerability index, V_t= target vulnerability index, R= desired vulnerability reduction and V_j= possible vulnerabilities for fixed land qualities (LQs) (de la Rosa et al., 2000)*

Table 4. *Soil erosion parameters by conventional practice for potato, alfalfa and maize cultivation*

	Potato			Alfalfa			Maize		
Site	**Vulnerability index[1]**	**Risk class[2]**	**Soil loss rate ($t\ ha^{-1}\ yr^{-1}$)**	**Vulnerability index**	**Risk class**	**Soil loss rate ($t\ ha^{-1}\ yr^{-1}$)**	**Vulnerability index**	**Risk class**	**Soil loss rate ($t\ ha^{-1}\ yr^{-1}$)**
1	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
2	0.36	V3	24.5	0.27	V2	8.9	0.35	V3	20.4
3	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
4	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
5	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
6	0.41	V3	36	0.31	V3	12.7	0.4	V3	31.2
7	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
8	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
9	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
10	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
11	0.21	V2	7.1	0.15	V1	4.9	0.2	V2	6.8
12	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
13	0.25	V2	8.5	0.18	V2	5.9	0.24	V2	8
14	0.52	V4	59	0.41	V3	32.4	0.5	V4	52.7

[1]Vulnerability Index ranged from 0-1
[2]Erosion risk class ranged from V1-V6

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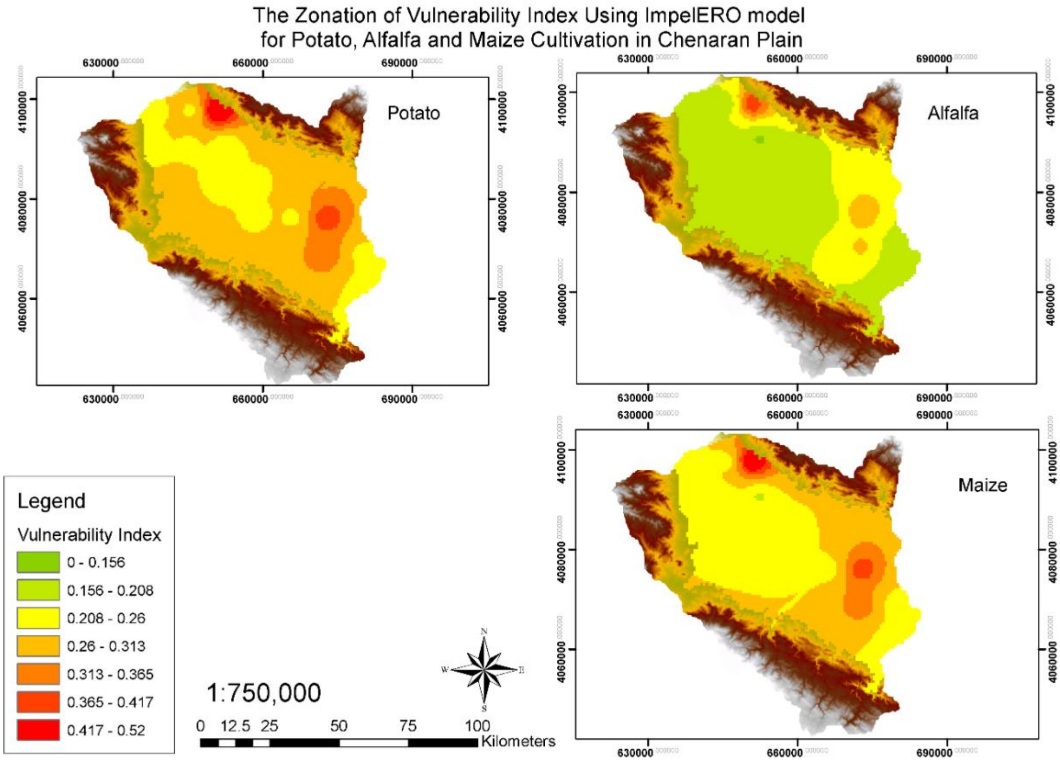

Figure 3. *The zonation of vulnerability index for potato, alfalfa and maize cultivation in Chenaran Plain*

The zonation map of the erosion risk classes by both potato and maize cultivations revealed that 27.84% (363.39 km^2) of the plain were categorized into V2 class, 70.63% (921.98 km^2) into V3 class and 1.53% (19.93 km^2) were classified into V4 risk class. The zonation map of erosion risk class by alfalfa cultivation exhibited that 0.17% (2.22 km^2) of the plain were categorized into V1 class, 79.58% (1038.82 km^2) into V2 class and 20.25% (264.26 km^2) of the study area were classified into V3 risk class (*Tables 4* and *5*).

Table 5. *Measurement areas of zonation map of vulnerability index, erosion risk class and soil loss rate*

		Potato		Alfalfa		Maize	
		Area (km^2)	**%**	**Area (km^2)**	**%**	**Area (km^2)**	**%**
Vulnerability index	0.0-0.156	0	0	2.07	0.16	0	0
	0.156-0.208	0	0	900.55	68.99	2.07	0.16
	0.208-0.260	384.72	29.47	331.09	25.37	718.71	55.06
	0.260-0.313	749.64	57.43	54.14	4.15	450.58	34.52
	0.313-0.365	126.06	9.66	10.31	0.79	99.55	7.63
	0.365-0.417	29.06	2.23	7.14	0.55	21.52	1.65
	0.417-0.52	15.82	1.21	0	0	12.86	0.99
Risk class	V1	0	0	2.22	0.17	0	0
	V2	363.39	27.84	1038.82	79.58	363.39	27.84
	V3	921.98	70.63	264.26	20.25	921.98	70.63
	V4	19.93	1.53	0	0	19.93	1.53

Soil loss rate (t ha^{-1} yr^{-1})	0.0-5.0	0	0	0.16	0.01	0	0
	5.0-10.0	217.54	16.67	1208.19	92.56	386.66	29.62
	10.0-15.0	706.97	54.16	63.19	4.84	639.99	49.03
	15.0-20.0	222.63	17.06	13.20	1.01	184.98	14.17
	20.0-25.0	86.38	6.62	9.16	0.70	45.97	3.52
	25.0-30.0	31.91	2.44	7.79	0.60	22.98	1.76
	30.0-35.0	17.77	1.36	3.61	0.28	8.25	0.63
	35.0-40.0	6.15	0.47	0	0	4.21	0.32
	40.0-60.0	15.95	1.22	0	0	12.26	0.94
Total		1305.304	100	1305.304	100	1305.304	100

The geographical distribution of the erosion risk classes revealed the same pattern for potato and maize cultivations, where most parts of the study area are sensitive to erosion with high vulnerability. In contrast to other crops the risk classes by alfalfa production exhibit relatively low sensitivity in most parts of the plain to erosion (*Fig. 4*).

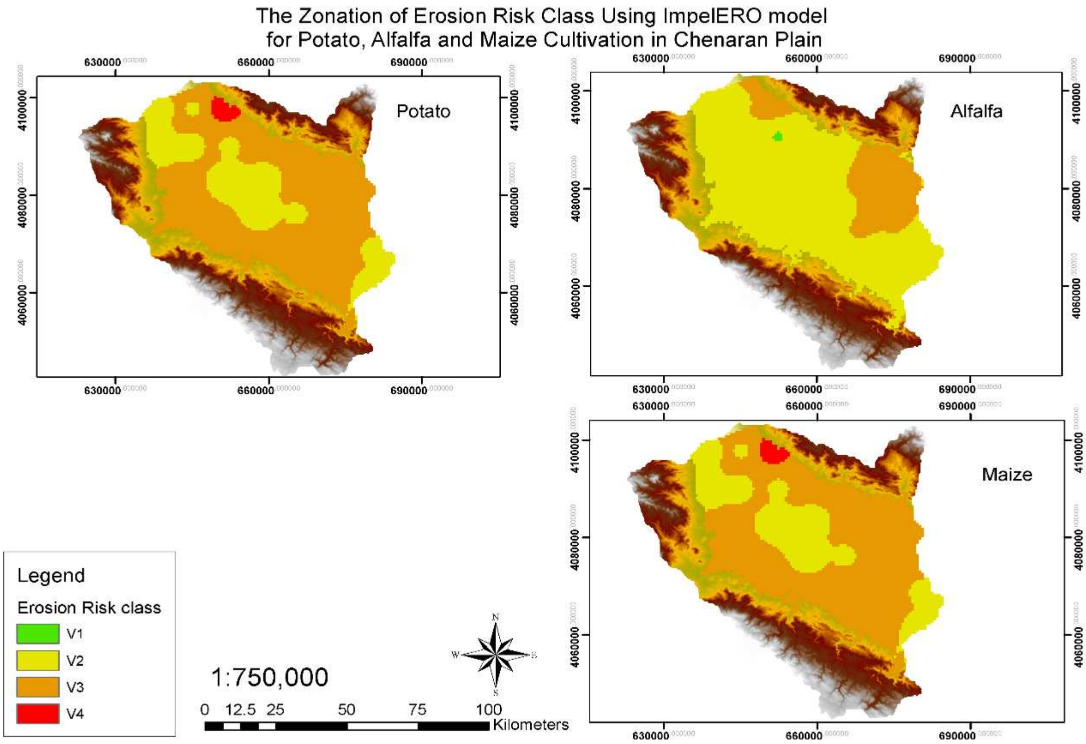

Figure 4. *The zonation of erosion risk class for potato, alfalfa and maize cultivation in Chenaran Plain*

The values of soil losses varied between 7.1 to 59 t ha^{-1} yr^{-1} with an average of 15.11 t ha^{-1} yr^{-1} by potato, 4.9 to 32.4 t ha^{-1} yr^{-1} with an average of 8.42 t ha^{-1} yr^{-1} by alfalfa and 6.8 to 52.7 t ha^{-1} yr^{-1} with an average of 13.65 t ha^{-1} yr^{-1} by maize cultivation (*Table 4* and *Fig. 5*).

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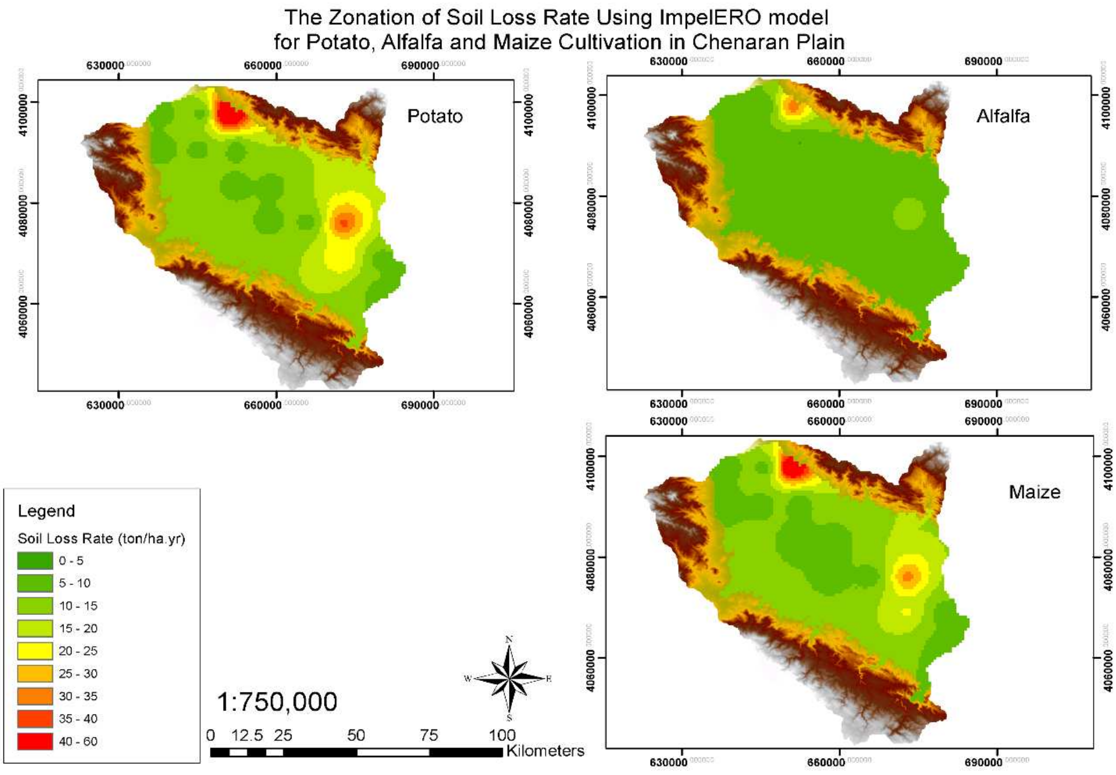

Figure 5. *The zonation of soil loss rate for potato, alfalfa and maize cultivation in Chenaran Plain*

As shown above, the values of soil losses by potato and maize cultivations were higher than the corresponding values by alfalfa cultivation. The reduction percent of soil losses by maize production compared to potato cultivation was about 9.66%, while by alfalfa cultivation the soil losses reduction reached to 44.28% compared to potato production. Shifting cultivation from maize to alfalfa resulted in 38.32% reduction in soil losses.

Our results revealed that the effect of row crops cultivation including potato and maize on undulated slopes in mainly southward aspects, medium to fine silty soil textures, the drainage is very slow and the soil organic matter is commonly low in some parts at the north and east of the plain were considered as the most important factors increasing soil vulnerability to erosion, soil loss rates and erosion risk classes.

Discussion

Results showed that all three erosional classes are moderate in the region, and alfalfa cultivation displays a lower erosional class compared to potato and maize cultivations. Soils with silt and fine sandy texture were the most erodible soils due to their lack of both the cohesiveness of clay minerals and the weight of large particles (Morgan, 2005).

The row crops such as potato and maize plants are highly susceptible to erosion because the vegetation does not cover the entire soil surface (Southgate and Whitaker, 1992; Stone and Moore, 1997).

Our results showed that alfalfa cultivation provides better protection against soil erosion compared with our previous study on sugar beet cultivation (Afshar et al., 2016). Cultivation of potatoes has been reported to be accompanied by enhanced tillage

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erosion and soil loss (Ruysschaert et al., 2006; Auerswald et al., 2006; Evans, 2002). Evans (2002) observed a soil loss rate of 2.53 t ha^{-1} yr^{-1} by potato cultivation. Jung et al. (2003) found an average erosion rate of 47.5 t ha^{-1} yr^{-1}, and Choi et al. (2005) reported erosion rates between 4.2 t ha^{-1} yr^{-1} and 29.6 t ha^{-1} yr^{-1} by potato cultivation. Potato fields also have a high erosion risk after harvesting because the crop leaves little residue or the residues are ploughed into the soil. Ruysschaert et al. (2006) reported that extensive soil loss may occur if the harvested potato field would not immediately planted with a subsequent crop. Maize cultivation has also a high erosion risk because of its slow early growth and large row spacing (Boardman and Poesen, 2006). Evans (2002) found the contributions of maize on soil loss to 4.48 t ha^{-1} yr^{-1}.

The time-dependent combined effects of precipitation and state of the crop fields with regard to the crop planted, soil cover, soil tillage, and soil looseness are crucial for soil erosion in any given area (Fiener et al., 2011). Boardman et al. (2009) reported on serious erosion on fields with post-harvest potato and maize crops in the United Kingdom.

Maize was most frequently affected by erosion. Erosion was greatest by far when the land was planted with potatoes. Thus, crops may show differences in their impact on erosion, but crop sequences and crop rotations should also be taken into account, because positive and negative carry-over effects of previous crops may have an influence on the extent of erosion. As well, the areas which covered by grass-clover as main and intermediate crop markedly decreased the extent of erosion relative to the other crops and winter fallow (Prasuhn, 2012).

Conclusion

Agricultural activities, especially those concerning soil tillage, can be accommodated to reduce soil erosion by using expert system/neural-network technologies adapted to erosion prediction risks. An example of these models has been satisfactorily used as an optimization tool for selecting the land use and management practices for the reduction of soil erosion. Applying ImpelERO model in estimating soil erosion indices is considered as practical approach to optimize soil use and preventing soil loss. With respect to obtained results we can identify vulnerable areas to erosion and manage the cultivation techniques in order to reduce the values of soil vulnerability and controlling the risk of erosion. According to the ImpelERO model application in benchmark sites of Chenaran Plain, there is a significant vulnerability to soil erosion in some parts in north and east of the study area. It was exhibited that shifting crop pattern to alfalfa cultivation is considered as an alternative in controlling erosion in more vulnerable areas of Chenaran Plain. The cultivation of alfalfa as a perennial plant improves the soil structure, increases organic matter content of the soil and enhance the soil's biological activities. The decreasing effect of alfalfa on soil erosion is primarily based on its long-lasting growing period and extensive canopy which cause the subsequent crops may benefit from improved fertility conditions even up to many years later.

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PERCEPTIONS AND EXPECTATIONS ON FOREST MANAGEMENT CERTIFICATION OF FORESTERS IN STATE FOREST ENTERPRISES: A CASE STUDY IN TURKEY

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Abstract. Determination of the perception of employees in the forestry organization involved in forest management certification (FMC) in state forests is considered important in increasing the success level of this process. In this study, some questions were asked to evaluate the opinions of the forest management directorate employees [forest engineers (FE), rangers (R), and forest workers (FW)] about the forest management certificate and to know the expectations about certification. Data has been collected by conducting face-to-face interviews with 51 FEs, 56 Rs, and 80 FWs. This data collection was done at the certified forest enterprise of the Kastamonu Regional Directorate of Forestry, which has been selected as the work area. The research methods included descriptive statistics, one-way analysis of variance, and correspondence analysis for hypothesis testing. When a statistical significance (p = 0.05) was found, the intergroup differences were analyzed using a post hoc test. Our results showed differences in the perceptions of foresters, with 90% of FEs, Rs, and FWs stating that the certification process contributes positively to forest management. Moreover, 35.8% of the foresters stated that FMC encouraged selection of the most suitable forest management, 34.5% stated that FMC ensured forest management according to sustainable forest management (SFM) criteria and in compliance with international agreements, and 30.1% stated that FMC contributed to the conservation and survival of forest areas.
Keywords: *sustainability, sustainable forest management, forest management certification, Forest Stewardship Council certification, Turkey*

Introduction

Although forests have been considered as firewood and timber supply areas until very recently, forest and related ecosystems have important roles as sources of food, round wood, firewood, water, fresh air, fossil fuels, shelter and are important in climate regulation, flood protection, disease control, water conservation, all of which support human life (Tolunay and Başsüllü, 2015; Krieger, 2001). However, despite all these unique qualities, human beings continue to destroy forests due to negligent consumptive behaviour. Specifically, as a result of excessive use, 60% of the functions of the forests have been impaired or jeopardized in terms of sustainability worldwide (Brockhouse and Botoni, 2009). Therefore, activities to manage existing forests in an orderly manner and establish new forest areas have gained importance (Eler, 2010).

Although degradation and destructions in the world's forests continue, programs to halt this trend, such as the tropical Forestry Action Plans, the International Tropical Timber Agreement, the Convention on the International Trade in Endangered Species of Flora and Fauna (CITES), and the Global Environment Facility, have not been fully effective in addressing forest degradation and destruction. This shortcoming was especially pronounced at the Earth Summit in Rio de Janeiro in 1992. In addition to

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many governments, many non-governmental organizations have also pressed for overcoming these deficiencies. As a result, although no legally binding commitments were made, the Agenda 21 Forestry Principles set out an action plan to delve into sustainable forestry issues (Asan, 2010; Durusoy, 2002; Pfeifer, 2003).

Although regional processes around the world have their distinct characteristics, seven important points are common in all processes, and these are listed as SFM criteria: (1) size of forest resources; (2) biodiversity in forests; (3) health and vitality of forests; (4) protection functions of forests; (5) production functions of forests; (6) socioeconomic functions of forests, (7) legal, political and institutional frameworks (CICI, 2003).

While processes of developing criteria for SFM continue, forest certification has begun to take shape through non-governmental organizations (Perera and Vlosky, 2006). The forest certification system is the most important concept that non-governmental organizations have developed to achieve SFM. This concept has been developed for certifying and labelling forests and forest products, and forest certification emerged in the 1990s as an instrument to facilitate sustainable use of natural resources (Cubbage et al., 2007). As a result, certification efforts were initiated by a voluntary non-profit organization called the Forest Stewardship Council (FSC), 1993 World Wide Fund for Nature (WWF) coalition, and other leading environmental organizations (Perera and Vlosky, 2006; Rametsteiner, 2002). Today, there are many organizations that perform certification, including Canadian Standards Association SFM System (CSA), Malaysian Timber Certification Council, Programme for the Endorsement of Forest Certification (PEFC), CERTFOR, Australian Forestry Standard (AFS) etc. (Klingberg, 2003; Ozinga, 2008; Eryılmaz and Tolunay, 2015). Although certification processes initially focused on tropical forests, they were expanded to cover continental forests and temperate forests (Perera and Vlosky, 2006; Nussbaum and Simula, 2005).

Approximately 8% of the world's forests are certified. The highest share in this certification processes belongs to PEFC and FSC (Yıldırım, 2010). FSC Principles and Criteria for Forest Stewardship are used as the basis for independent, third-party certification of forest management operations around the world. FSC principles and criteria are used as the basis for independent, third-party certification of forest management operations around the world. FSC has certified 194,478,017 hectares of forests across the world with a total of 1519 certificates in 84 countries (FSC, 2017).

Especially in recent years, many studies have been conducted to determine perceptions of stakeholders about forest certification (Bass et al., 2001; Cashore et al., 2002; Carrera et al., 2004; Pinto and McDermott, 2013; Trishkina et al., 2014; Pratiwi et al., 2015). Some studies in this field have been conducted in Turkey (Durusoy, 2002; Akyol and Tolunay, 2006; Tolunay and Türkoğlu, 2014; Ayan et al., 2009; Şenöz, 2014); however, a study that evaluates every aspect of forest management certification (FMC), especially one questioning the views and opinions of the stakeholders, has not been reported yet.

Decisions taken in the process of certification of forests in Turkey have been made within the framework of a top-down hierarchy. Forest certification studies in Turkey have started with the studies that the General Directorate of Forestry has initiated in line with the requests of Turkish companies wanting to export wood-based products to Europe. In this context, no preliminary studies or assessments have been made that involved participation of all stakeholders. The perceptions of employees of the forestry

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organization on certification itself are important for obtaining certification and maintained it. To what extent will it be possible to achieve success and to maintain these activities with staff are not convinced about the certification process and its positive effects on forestry? For this reason, it is very important to determine the opinions of the employees in the forestry organization who will directly implement certification process. The results obtained will help decision makers on what needs to be done before certification processes take place in the future.

Forest assets in Turkey are 22.3 million hectares, accounting for 28.6% of the total area of the country. Approximately 57% of this area consists of productive forest areas (GDF, 2016), and 99% of the forests in Turkey are owned by the state. Turkey's total industrial wood production is 7,895,000 m^3, and total firewood production is 8,408,400 m^3. In the last decade, an annual average of 13 million m^3 of industrial wood was supplied, as well as 1.5 million m^3/year of wood raw material was imported (KRDF, 2016).

The first management plan for forests in Turkey was prepared in 1963 (Eler, 2010). The General Directorate of Forestry (GDF), which is the organization that operates and manages forest assets in Turkey, has the following aims: (i) to protect, develop, and expand forests; (ii) to provide society with sustainable multiple benefits from forest resources, (iii) to improve capacity of the institution to realize these services faster and better; and (iv) operation and management of forest areas with these aims within a sustainability framework (GDF, 2012). In this context, Turkey has been in sync with the developments around the world, and has participated in pan-Europe and FAO-UNEP Near East processes.

The certification studies, which have made significant improvements in the last twenty years in the world, were initiated in 2010 in Turkey. Although Turkey has been involved in this process only for a short duration, approximately 10% of Turkey's forests have been included in the scope of certification program. All FMC studies that were conducted in Turkey were executed by the FSC. By year of 2015, FMC has been performed for 3,249,999 hectares forest area in Turkey and this figure is expected to be 5 million hectares in 2019 (Şen et al., 2013; Şenöz, 2014).

In this study, the perceptions and expectations of forestry organization employees in state forest regarding FMC were evaluated. In addition, expectations of the forestry groups about possible developments that may take place after FMC and differences between the groups in terms of their expectations were also revealed. In a country like Turkey, where forests are almost entirely state-owned, revealing the points of view and expectations of forestry organization about forest certification is expected to contribute both to the success of certification efforts as well as its sustainability and help decision makers in the other similar countries on the pre-certification procedures. Determination of the differences in opinion of foresters groups on FMC will contribute to and assist decision makers and relevant stakeholders in the new regulations to be made regarding the tasks of these groups with different job descriptions as well as in the plans to be made. In addition to these, the results of the study will be helpful in formulating national forest certification criteria and in the identifying forestry policy objectives. Additionally, comparisons between the FMC applications adopted in private forests and state forests can be performed and relevant evaluations can be conducted.

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Materials and methods

Description of the research site

This study was carried out conducted in Kastamonu Regional Directorate of Forestry (KRDF). KRDF is ranked first for total wood wealth and total wood sales volume in 27 Regional Directorates of Forestry in Turkey. The certified forest areas in KRDF constitute 20.8% of the total certified forest areas in Turkey (Şen et al., 2013; Güneş Şen, 2015). KRDF is located approximately between the 41^{st}–42^{nd} northern parallels and the 33^{rd}–35^{th} eastern meridians. KRDF is located in the West Black Sea Region of Turkey and includes Sinop and Kastamonu provinces within its borders (*Fig. 1*). There are a total of 1542 villages in the area, most of which are forest villages (1488). The total population in the study area is 576,766 (TSI, 2016). Approximately 245,586 of these individuals are forest villagers (living in villages located in the forest or at the edges of the forest) (KRDF, 2016). Two national parks are included in the study area—Küre Mountains National Park and Ilgaz Mountain National Park with Protected Areas Network (PAN) park certification (Anonymous, 2014; Öztürk and Ayan, 2015). Moreover, Küre Mountains are home to five distinct habitat types categorized as endangered habitats as per the Convention on the Conservation of European Wildlife and Habitats (Bern Convention) (LTDP, 2008). KRDF operates approximately 1.2 million hectares of forest area on a 2 million hectares area via 21 Forestry Enterprise Directorates (FED) within its body (Şen et al., 2013). Among these forest enterprises, Daday, Araç, Ayancık, Taşköprü, and Tosya FEDs have received FMC according to the FSC standards (*Fig. 1*).

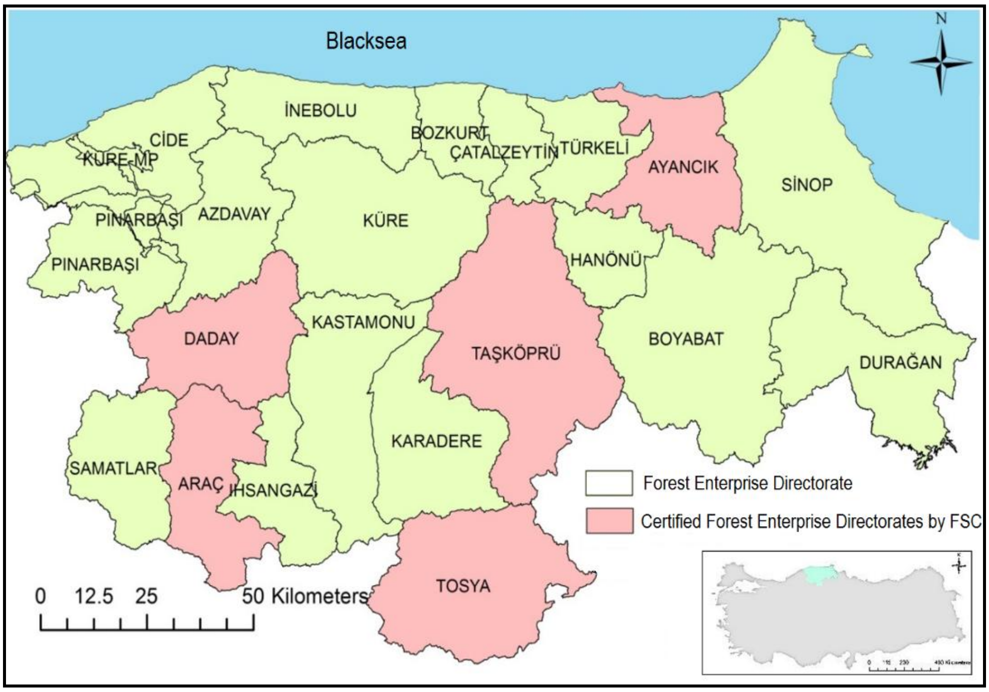

Figure 1. *Locations of KRDF and certified FEDs*

Distribution of forest lands of KRDF and certified FEDs are shown in *Table 1* (KRDF, 2014).

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Table 1. *Distribution of forest land (ha)*

FED	High forest (ha)		Coppice (ha)		Forested land (ha)	General land (ha)
	Productive	**Degraded**	**Productive**	**Degraded**		
Daday	52422.9	11444.9	-		63867.8	85465.6
Vehicle	35264.0	11258.0	-	4102.0	50624.0	76062.0
Ayancık	54189.0	4869.0	-		59058.0	80195.3
Taşköprü	82469.5	31049.4	-		113518.9	176647.9
Tosya	55499.3	24807.1	-		80306.4	122636.1
Certified FED	279844.7	83428.4	-	4102.0	367375.1	541006.9
KRDF	930357.1	285885.9	8570.5	25778.1	1250591.6	2011116.2

As shown in *Table 1,* the total area of five FEDs included in KRDF is 541,006.9 ha, which constitutes 26.9% of total KRDF area and 29.38% of forest lands. Approximately 30% of productive high forests and 29% of degraded high forests of KRDF were certified. 29% of total forest lands have FMC.

Identification of target groups of the study

Because 99.9% of the forests in Turkey are state-owned, a successful certification process is directly proportional to the selfless work of the employees. Most of the current employees are permanent staff. Their salary does not depend on their work performance. In this context, a new system to be brought to forestry can mean disruption of conventional order and added workload for most of the employees. Therefore, evaluating the level of awareness of the foresters engaged in land and office work during the certification process, their thoughts on certification, and their expectations from certification is important for the establishment of forestry policies. In this context, a survey was conducted with forestry groups directly working in FMC tasks. Therefore, in this study, we collected original data from the surveys conducted for forest engineers (FE), rangers (R) and forest workers (FW) staff of forestry organizations. Employees working in Daday, Araç, Ayancık, Taşköprü, and Tosya FEDs, which have received FSC from KRDF, participated in the surveys. Moreover, the results of previous studies and the KRDF documents were used.

In this study, participants are divided into three groups. FEs are employees with a 4-year undergraduate degree in forest management and working as engineers or managers. Rs are employees with a 2-year associate degree in forest management and are employed in forest protection duties of enterprises and other related tasks. FW's are high-school graduates or represent/have lower educational level and are generally employed in heavy jobs such as production and fire extinguishing. Most FWs are villagers living near or in forests. Identification of differences between the groups will help to improve foresters' trust in and internalization of the work as well as awareness about the work, which are important factors in the success of certification process.

According to KRDF data, there are 55 FE, 102 R, and 313 FW working in FED's which were certified. The following formula (*Eq.* 1) was used for determining the sample size (Serper, 2000; Orhunbilge, 2000):

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$$n = \frac{N * t^2 * P * Q}{(d^2 (N-1)) + (t^2 * P * Q)}. \quad \text{(Eq.1)}$$

The various parameters in this equation are as follows—n: sample size; t^2: confidence level 90% (1.64); N: Population (55 FE; 102 R; 313 FW); P: probability of the presence of the aspect aimed to be measured within the main group (taken 50% because of the multipurpose nature of this study); Q: 1 − P; d: Sampling error considered 10% (0.1). Thus it was calculated that: n = 35 FE; 50 R; 74 FW

To increase the survey reliability level, 50 FE; 57 R; 80 FW foresters instead of 35 FE; 50 R; 74 FW were interviewed. Participants for surveying were selected by random sampling method. The main mass and sample sizes according to the enterprise directorates are shown in *Table 2*.

Table 2. *Sample sizes and number of survey participants*

FDD	Number of engineers	Number of officers	Number of workers
Daday	9	19	68
Vehicle	6	14	42
Ayancık	17	20	52
Taşköprü	14	28	97
Tosya	9	21	54
Total	55	102	313
Sample sizes	35	50	74
Survey participants	50	57	80

Table 3 shows the general demographics of the foresters according to their age, level of education, and gender.

Table 3. *Education, work position, and age of the foresters*

Age groups (years)		18-30		31-40		41-50		51-60		60<		Total	%
Gender		F	M	F	M	F	M	F	M	F	M		
Graduation level / Work position													
Primary education	FW	-	3	-	5	-	36	-	16	-	-	60	32.1
	R	-	-	-	-	-	7	-	4	-	1	12	6.4
High school	FW	-	3	-	4	-	7	-		-	-	14	7.5
	R	-	-	-	3	-	18	-	5	-	-	26	13.9
Undergraduate degree	FW	2	2	-	-	-	1	-	-	-	-	5	2.7
	R	1	5	-	1	2	5	-	-	-	1	15	8.0
Bachelor's degree	FW	-	-	-	1	-	-	-	-	-	-	1	0.5
	R	-	3	1	1	-	-	-	-	-	-	5	2.7
	FE	2	7	4	19	-	7	-	-	-	-	39	20.9
Master's degree	FE	-	4		6	-	-	-	-	-	-	10	5.3
Total		5	27	5	40	2	81	-	25	-	2	187	100
%		2.7	14.4	2.7	21.4	1.1	43.3	-	13.3	-	1.1	100	

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26.2% of the participants in the questionnaire were FEs, 31% were Rs and 42.8% were FWs. Sixty percent of these people are elementary school graduates, 10% have associate degrees, 24% have bachelor's degrees, and 6% have graduate degrees. The forester groups that participate in certification operations consist of 6.5% women and 94% men. Sixty percent of these people are elementary school graduates, 10% have associate degrees, 24% have bachelor's degrees, and 6% have graduate degrees. While workers and public servants are of 41–50 age group, engineers are typically aged 30–40 years.

Survey design and statistical analyses

The results of the study were obtained from questionnaire forms designed to determine foresters' thoughts about and expectations from FMC. Following the first part of the questionnaire about the general characteristics of the forestry personnel, opinions and evaluations of the participants about SFM, forest certification, objectives of certification, contribution of certification to forestry, and the changes that certification will bring about in forestry activities were examined. In the last part, expectations of the participants about possible developments after certification were queried. The questionnaire questions were prepared as closed and open-ended questions. Some questions are for obtaining descriptive information. In general, when asked about perceptions and expectations, questions were prepared on a 5-point Likert scale. The response options were as follows: agree = 5, partly agree = 4, neither agree, nor disagree = 3, partly disagree = 2, and disagree = 1. Surveys were conducted through face-to-face interviews. The questionnaires were first tested with a preliminary application, after which the necessary revisions were made.

The H_0 hypothesis was formulated as "there is no difference between the perceptions and expectations among staff groups' about FMC," and the H_1 hypothesis was formulated as "there is a difference among the staff groups' perceptions and expectations of staff groups about FMC." The statistical analyses were performed using the SPSS 17 Statistics Package Program. The Cronbach's alpha coefficient was calculated at 0.788. Descriptive statistics were used to analyze the data and one-way analyses of variance (ANOVA) ($p = 0.05$) were used to measure significant differences between means (Özdamar, 2002; Vidal et al., 2005; Büyüköztürk, 2010), whereas correspondence analysis (Toksoy et al., 2008) was used for testing the hypothesis. When there was a statistical significance ($p = 0.05$), the differences between the groups were analyzed using a post hoc test. Tukey test was used variance was equal ($p > 0.05$), and Tamhane's T test was used when variances were different (Özdamar, 2002).

SCA is a useful method for evaluating two-dimension spaces so that values are geometrically shown by rows and columns forming a contingency table. When the contingency table identifies clustered dots, this may signal problems with the data (Toksoy et al., 2008). To asses whether or not there is a problem, researchers conduct a three-stage analytic process of compatibility analysis (Clausen, 1998). In order to determine both inter-relations and intra-relations between two different categorical variables a suitable multivariate technique, Simple Correspondence Analysis (SCA) is built by reviewing the closeness and remoteness between them (Başpınar and Mendes, 2000; Bendixen, 2003; Aktürk, 2004; Abdi and Bera, 2014). SCA reduces the dimensionality of contingency tables arranged from categorical data (Özdamar, 2002). Compared to other statistical techniques such as Chi-square analysis, G-tests, Z-tests, Fisher Exact tests, or Log-linear models, SCA presents data more visually so that

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reserachers are more easily able to see data relationships (Devillers ve Karcher, 1991; Greenacre, 1998; Başpınar and Mendes, 2002; Özdamar, 2002). Variable levels studied through SCA are represented with a dot in a two-dimensional space. Dots shown to be closer to one another are more closely related and correlated, whereas dots represented as further from each other are not as strongly related (Dunteman, 1989).

SCA is a useful method for evaluating two-dimension spaces so that values are geometrically shown by rows and columns forming a contingency table. When the contingency table identifies clustered dots, this may signal problems with the data (Lee, 1996). To asses whether or not there is a problem, researchers conduct a three-stage analytic process of compatibility analysis (Clausen, 1998).

In running SCA for this study, X and Y variables acquired N monad and are illustrated as k1*k2 in a dimensional cross section table (*Table 4*). Row and column profiles were first calculated; second, row and column profiles were drawn the same a two-dimensional space; and finally, these profiles were shown on a two-dimensional map. In order to understand SCA, it is necessary to describe the profile, mass, and chi-square distance and total inertia.

Table 4. *k1*k2 dimensional cross selection table*

		y(ci)				
		y_1	y_2		y_i	Total (f_{+i})
	x_1	f_{11}	f_{12}	…	f_{1j}	f_{1+}
$x(r_i)$	x_2	f_{21}	f_{22}	…	f_{2j}	f_{2+}
	:	:	:		:	:
	i	f_{31}	f_{32}	…	f_{ij}	f_{i+}
	Total (f_{i+})	f_{-1}	f_{-2}	…	f_{-j}	f

f_{ij}: Frequency value of column i and row j
f: Total number of observation

Profiles: When creating a contingency table, frequencies should not be sown in each cell because each row and column contains different responses. Relative frequency was instead calculated as 1 value for each row and column (Greenacre, 1998; *Eqs. 2* and *3*).

$$\text{Row profiles: } r_{ij} = f_{ij} / f_{i+} \qquad \text{(Eq.2)}$$

$$\text{Column profiles: } c_{ij} = f_{ij} / f_{+j} \qquad \text{(Eq.3)}$$

Mass is defined by dividing marginal frequencies by the sum of rows and columns. This system of calculating mass provides an equal contribution for every answer and every profile point. In this analysis, masses are a way to measure the importance of a given profile (Uzgoren, 2007; *Eqs. 4* and *5*).

$$\text{Row Mass: RM} = f_{i+} / f \qquad \text{(Eq.4)}$$

$$\text{Column Mass: CM} = f_{+j} / f \qquad \text{(Eq.5)}$$

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Chi-square distances: In SCA, the distances of every categories to other one is defined as chi-square distances. Firstly, expected (theoretic) values (tij) of frequencies (fij) in cells are calculated (*Eqs. 6* and *7*).

$$t_{ij}\text{: mass}_i \text{ x mass}_j \text{ x N} = \left(f_{i+} + f_{+j}\right) / \text{ N} \quad \text{(Eq.6)}$$

Then, chi-square values of every cells are calculated with this formula:

$$x^2_{ij} = \left(f_{ij} - t_{ij}\right)^2 / \ t_{ij} \quad \text{(Eq.7)}$$

Total inertia (Λ^2): Variance notion, in SCA, is associated with chi-square distances. For this, generally inertia notion is accepted and inertia is used as synonym with variance notion. Total inertia is the measured distance about the distribution of profile points around the centre, which can be calculated by the below-mentioned formula (Ozdamar, 2002; *Eqs. 8* and *9*):

$$\text{Intertia}_i\text{: } x^2_i / \ x^2_{i+} \quad \text{(Eq.8)}$$

$$\text{Intertia}_j\text{: } x^2 / \ x^2_{+j} \quad \text{(Eq.9)}$$

SCA also can be understood as a technique for representing the chi-square (or Phi-square $\phi^2 = (x^2 / f) = \Lambda^2$ = total inertia) value of a frequencies table (Clausen, 1998). Total inertia decomposes by an eigenvalue cluster. In a bidirectional table, the number of eigenvalues and also number of dimensions are equal to minimum of (i-1) and (j-1) (Anonymus, 2017). These eigenvalues convey the comparative importance of dimensions and calculate the percentage of total inertia for every dimension. When eigenvalue of data matrix is computed, total inertia is maximum at the first dimension, then begins to decrease in subsequent dimensions (Clausen, 1998).

Results

The survey was conducted on 187 forestry workers working in certified FDDs in study area. Firstly, SCA results where views of the forester groups on SFM and FMC were assessed were presented (see *Tables 5–13*). Then, Likert scale average scores (see *Tables 14* and *15*), ANOVA and post hoc test results (see *Table 16*), where the forester groups were tested for their expectations about possible developments that may take place after FMC and whether or not there was a difference between the forester groups in terms of their expectations, were included.

Participants' views on SFM and FMC

To determine the level of awareness of the forester groups about SFM, they had to answer the following question: "In your opinion, which component of SFM is the most important?" (Question 1). Before asking the question, the participants were explained that SFM is composed of economic (a type of management which yields more returns regarding wood and non-wood forestry products), ecologic (a type of management that

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respects the environment and focuses on protecting the environment), and social (a type of management that protects the rights of the local societies and forest workers) (Rahman, 2015). The participant's views were tested by SCA. Frequency values of the SCA analysis results are shown in *Table 5*. Similarly, *Table 6* shows the row and column profiles and *Table 7* indicates the inertia, chi-square, significance level, standard error, and correlation values. *Figure* 2 shows the tendencies of forester groups regarding the components of SFM.

Table 5. *Correspondence table: frequencies of people's responses about the questionnaire*

Forester groups	The most important SFM component							
	Ecologic	%	Social	%	Economic	%	Active Margin	%
FE	31	62.0	19	38.0	0	0.0	50	100
R	29	50.9	24	42.1	4	7.0	57	100
FW	40	50.6	32	40.5	7	8.9	79	100
Active Margin	100	53.8	75	40.3	11	5.9	186	100

Table 6. *Row and column profiles*

Row profiles					Column profiles				
Forester groups	The most important SFM component				Forester groups	The most important SFM component			
	1	2	3	Active Margin		1	2	3	Mass
FE	0,620	0,380	0,000	1,000	FE	0,310	0,253	0,000	0,269
R	0,509	0,421	0,070	1,000	R	0,290	0,320	0,364	0,306
FW	0,506	0,405	0,089	1,000	FW	0,400	0,427	0,636	0,425
Mass	0,538	0,403	0,059		Active margin	1,000	1,000	1,000	

*These values show comparative frequencies. The values in the last row are row mass values

Table 7. *Summary table of SCA results on the views of foresters regarding the SFM component they considered most important*

Dimension	Singular value	Inertia	Chi-square	Sig.	Proportion of inertia		Confidence singular value	
					Accounted for	Cumulative	Standard deviation	Correlation 2
1	0,166	0,028			0,988	0,988	0,038	0,144
2	0,019	0,000			0,012	1,000	0,075	
Total		0,028	5,210	0,266	1,000	1,000		

In terms of the three main objectives of SFM is based on, 53.8% of the participants found ecological dimension the most important, 40.3% of the participants found social dimension and 5.9% of the participants found economic dimension the most important. According to the SCA analysis, all groups seem to agree on the importance of the

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ecological basis of SFM. However, the social basis of SFM is not seen very important by FE. The group that considers this component of SFM as the most important component is the R group. The group that sees the economic dimension of SFM as the most important component is the FWs.

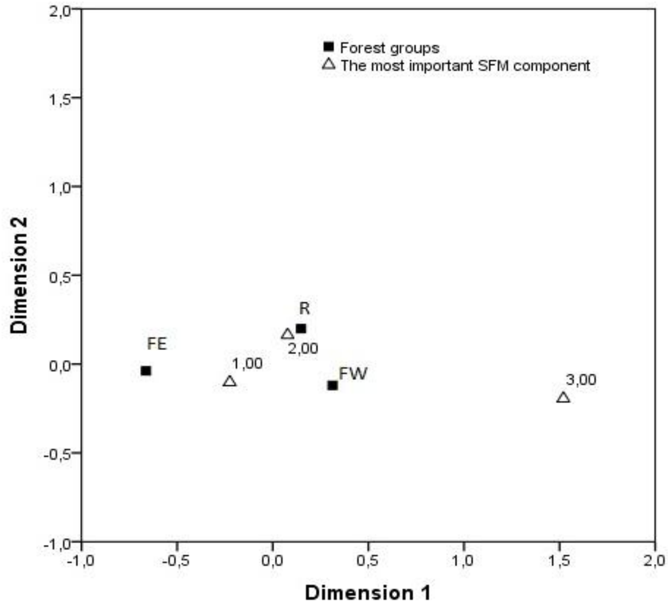

Figure 2. *Row and column points symmetrical normalization (for perceptions of forest groups about SFM component). 1: Ecological (forest management with respect for environment and focused on environmental protection). 2: Social (forest management protecting rights of local people and FWs). 3: Economic (it is a management that yields more return in terms of production of wood and non-wood forest products)*

We asked the participants a close-ended question, "What do you think the purpose of FMC is?" to determine the views of forester groups on the purposes of FMC (Question 2). The following three propositions were provided to the participants: a) to encourage the most appropriate way of management for all the forests around the world, b) to help manage forestry activities in accordance with the principles of SFM and to spread it internationally, and c) to assure protection and lasting presence of forested areas. The views of the participants regarding the purposes of FMC/Frequency values of the SCA analysis results were tested by SCA and the results are shown in *Table 8*. Similarly, *Table 9* shows the row and column profiles, and *Table 10* indicates the inertia, chi-square, significance level, standard error, and correlation values. *Figure 3* shows the tendencies of forester groups regarding the purposes of FMC.

Table 8*. Correspondence table: frequencies of people's responses about the questionnaire*

Forester groups	Objectives of forest certification							
	1	%	2	%	3	%	Active margin	%
FE	11	23.9	23	50.0	12	26.1	46	100
R	18	32.7	22	40.0	15	27.3	55	100
FW	35	44.9	16	20.5	27	34.6	78	100
Active margin	64	35.8	61	34.1	54	30.1	179	100

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Table 9. *Row and column profiles*

Row profiles					Column profiles				
Forester groups	Objectives of forest certification				Forester groups	Objectives of forest certification			
	1	2	3	Active margin		1	2	3	Mass
FE	0,239	0,500	0,261	1,000	FE	0,172	0,377	0,222	0,257
R	0,327	0,400	0,273	1,000	R	0,281	0,361	0,278	0,307
FW	0,449	0,205	0,346	1,000	FW	0,547	0,262	0,500	0,436
Mass	0,358	0,341	0,302		Active margin	1,000	1,000	1,000	

These values show comparative frequencies. The values in the last row and column are row mass and column values

Table 10. *Summary table of SCA results on the views of the foresters regarding the objectives of certification*

Dimension	Singular value	Inertia	Chi-square	Sig.	Proportion of inertia		Confidence singular value	
					Accounted for	Cumulative	Standard deviation	Correlation
								2
1	0,268	0,072			0,995	0,995	0,070	-0,068
2	0,018	0,000			0,005	1,000	0,071	
Total		0,072	120,876	0.012	1,000	1,000		

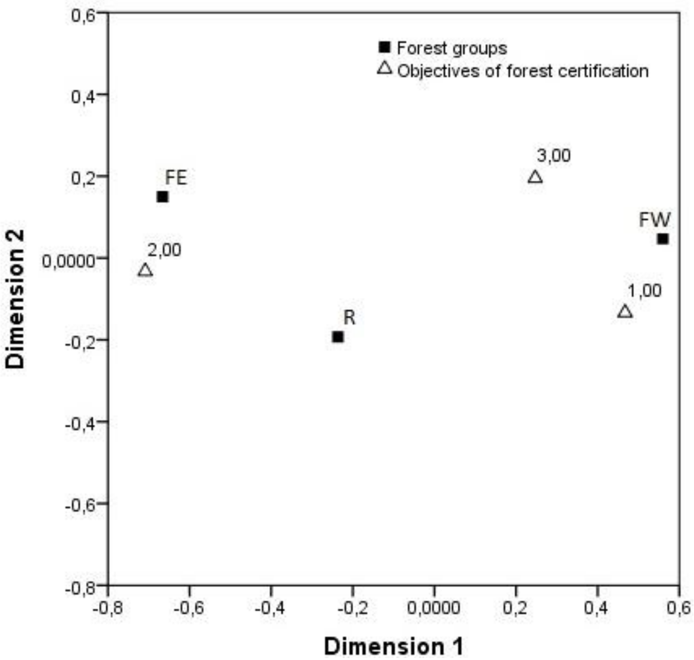

Figure 3. *Row and column points symmetrical normalization (for perceptions of forest groups about objectives of FMC). 1: Encouraging the management of all forests in the world most properly. 2: Helping manage forestry according to SFM principles and promoting it internationally. 3: Ensuring preservation and sustainability of forest areas*

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Approximately 35.8% participants thought that the objective of FMC encourages forest management policy selection in the most appropriate way. This was followed by 34.1% participants considering that forest management according to SFM criteria was effective and as well as followed international. Approximately 30.1% participants agreed with the view regarding ensuring preservation and sustainability of forest areas. While examining the SCA analysis, it is important for the success of applications to determine the objectives of FMC and which objective is prioritized. These perceptions vary among the workers in the KRDF. The FEs explicitly state that the certification will help manage forestry according to the SFM criteria and will contribute to making the Turkey's forestry products more visible in the international arena. In general, the FWs who live in forest villages and meet their livelihoods with forestry work and agricultural activities hold the view that the objective of certification is to provide preservation and sustainability of forest areas. The Rs, on the other hand, accepted all three viewpoints equally important.

We asked the participants, "has there been a change in your workload during or after the certification?" to determine the effects of FMC on the current workload (Question 3). *Table 11* shows the frequency values of the SCA analysis results that measured the views regarding the change in the workload of foresters during certification. Similarly, *Table 12* shows the row and column profiles, and *Table 13* indicates the inertia, chi-square, significance level, standard error, and correlation values. *Figure 4* indicates the direction of the views of the forester groups on the effects of SFM on their workload.

In the analyses regarding the impact of certification on workload were examined, 76.6% of the participants thought that there was an increase in workload, and 16.3% think that there was no change in workload. With regard to the individual groups, the general tendency of the FEs among forester groups is that there is an increase in workload.

Table 11. *Correspondence table: frequencies of people's responses about the questionnaire*

Forester groups	**Impact of certification on workload**							
	1	%	2	%	3	%	Active margin	%
FE	46	92.0	0	0.0	4	8.0	50	100
R	44	78.6	1	1.8	11	19.6	56	100
FW	51	65.4	12	15.4	15	19.2	78	100
Active margin	141	76.6	13	7.1	30	16.3	184	100

Table 12. *Row and column profiles*

Row profiles					**Column profiles**				
Forester groups	**Impact of certification on workload**				**Forester groups**	**Impact of certification on workload**			
	1	**2**	**3**	**Active margin**		**1**	**2**	**3**	**Mass**
FE	0,920	0,000	0,080	1,000	FE	0,326	0,000	0,133	0,272
R	0,786	0,018	0,196	1,000	R	0,312	0,077	0,367	0,304
FW	0,654	0,154	0,192	1,000	FW	0,362	0,923	0,500	0,424
Mass	0,766	0,071	0,163		Active margin	1,000	1,000	1,000	

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Table 13. Summary table of SCA results regarding the impact of certification on workload

Dimension	Singular value	Inertia	Chi-square	Sig.	Proportion of inertia		Confidence singular value	
					Accounted for	Cumulative	Standard deviation	Correlation 2
1	0,305	0,093			0,896	0,896	0,054	0,026
2	0,104	0,011			0,104	1,000	0,066	
Total		0,104	19,146	0,001	1,000	1,000		

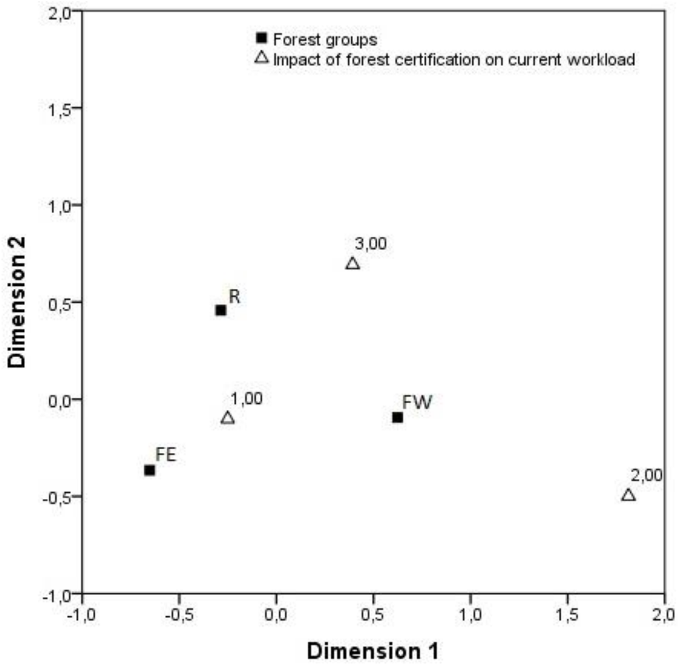

***Figure 4.** Row and column points symmetrical normalization (for perceptions of forest groups about impact of FMC on workload). 1: There is an increase in workload. 2: There is a decrease in workload. 3: There is no change in workload*

The percentage of the Rs who think that there is an increase in workload and the percentage of Rs who think that there is no change in workload were close to each other. Among the FWs, there are participants showing tendency toward all the options and their percentages are close to each other.

When the reasons for this change in workload were examined, the FEs and Rs stated that business security activities, training activities, and paperwork increased after certification and the number of staff is inadequate. The FWs, on the other hand, stated that basic trainings given during the certification process decreased workload.

Expectations of forester groups regarding post-certification conditions

We also examined the expectations of the participants regarding possible developments that might occur during the certification process. For this purpose, we provided 25 propositions to the participants (*Table 14*) and asked them whether they agree with these propositions or not (Question 4). *Table 15* shows the average Likert

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scores based on the obtained survey results, and *Table 16* shows the results of the ANOVA analysis conducted to test the variation among the groups.

Table 14. *Propositions about possible developments after certification*

1	Provisions of international agreements will be reflected to practice in a more effective way.
2	During the certification process, foresters will be aware of their rights defined by international conventions and be able to use these rights.
3	Illegal benefits gained from forests will be decreased as a result of trainings in the certification process.
4	Professionalization of local people to work in production will be ensured.
5	Access to and use of non-wood forest products will be recorded.
6	Laws will be strictly complied with regarding employment of children, women and young workers in the forest and they will be given all their rights.
7	Housing and nutrition conditions of the workers performing forestry activities will be improved.
8	Mobbing on exercising the right of unionization and collective bargaining will be eliminated.
9	Potential conflicts between stakeholders will be identified and solutions will be developed.
10	Sustainable production plans will be made for non-wood services and products.
11	Forestry production techniques will be improved and thus production losses will be minimized.
12	The quality of forest products and services will be improved after certification.
13	Employees of forestry organization, especially managers will increase their skills to evaluate forests in many aspects by improving themselves with the help of trainings.
14	Before all forestry activities, environmental impact assessment will be done and measures will be taken wherever required.
15	Expert opinion will be taken in all forestry activities beforehand.
16	Preservation of wetlands will be provided.
17	Creation of natural corridors will be provided wherever required by preventing deterioration of forests.
18	Monitoring, mapping, and registration of forestry activities and social, economic, and ecological effects of these activities will be provided.
19	Environmental and social impact assessment, identification, and protection of endangered species and descriptions of production techniques and equipment will be added to management plans.
20	Results of monitoring plans will be shared with the public.
21	Forests with high preservation value will be identified with the support of stakeholders and experts and will be managed with a participative management mentality.
22	It will be paid more attention to occupational safety and health issues in forestry activities (use of personal protective equipment, first aid, etc.).
23	Awareness of stakeholders about endangered species will be increased.
24	Efficiency of stakeholders in forestry management will be increased.
25	The sales prices of products and services obtained from certificated forests will increase according to the existing values.

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Table 15. Likert results on the propositions about possible developments after certification

Expectations after FMC		1	2	3	4	5	6	7	8	9	10	11	12	13
Avg. Score*	FE	4.2	4.1	3.5	3.9	3.8	3.9	4.1	3.8	4	4	4.2	4.2	4.3
	R	3.8	3.7	3.7	3.7	3.7	3.8	3.6	3.6	3.6	3.8	3.8	3.7	3.6
	FW	3.6	3.7	3.8	3.8	3.8	3.8	3.6	3.7	3.5	3.9	3.8	3.8	3.7
Expectations after FMC		**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	
Avg. Score*	FE	4.2	3.9	4.1	3.9	4.4	4.2	4.2	4.1	4.4	4.2	4	4	
	R	3.7	3.7	3.7	3.7	3.8	3.7	3.7	3.8	3.8	3.8	3.8	3.8	
	FW	3.8	3.7	3.9	3.7	3.8	3.8	3.8	3.8	3.8	3.9	3.8	3.8	

*Agree (5.5–4.6), partly agree (4.5–3.6), neither agree nor disagree (3.5–2.6), partly disagree (2.5–1.6), disagree (1.5–0.5)

Table 16. ANOVA results on the views of the foresters regarding possible developments after certification

ANOVA results								Post hoc test results
Dependent variables	**Source of variance**	**Sum of squares**	**df**	**Mean square**	**F**	**p**	**Significant difference**	**Differences between the groups**
1	Between groups Within groups Total	9,074 132,113 141,187	2 184 186	4,537 0,718	6,319	0,002	*Yes*	FE - FW
2	Between groups within groups Total	6,275 174,003 180,278	2 184 186	3,137 .946	3,318	0,038	*Yes*	FE - FW
3	Between groups Within groups Total	2,352 169,274 171,626	2 184 186	1,176 0,920	1,278	0,281	No:	-
4	Between groups Within groups Total	1,366 141,703 143,070	2 184 186	0,683 0,770	0,887	0,414	No:	-
5	Between groups within groups Total	0,216 146,896 147,112	2 184 186	0,108 0,798	0,135	0,874	No:	-
6	Between groups Within groups Total	0,438 161,241 161,679	2 184 186	0,219 0,876	0,250	0,779	No:	-
7	Between groups within groups Total	8,362 154,750 163,112	2 184 186	4,181 0,841	4,971	0,008	*Yes*	FE - R FE - FW
8	Between groups Within groups Total	2,080 152,305 154,385	2 184 186	1,040 0,828	1,256	0,287	No:	-
9	Between groups Within groups Total	8,745 154,699 163,444	2 184 186	4,372 0,841	5,200	0,006	*Yes*	FE - R FE - FW

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10	Between groups Within groups Total	1,818 143,594 145,412	2 184 186	0,909 0,780	1,165	0,314	No:	-
11	Between groups within groups Total	6,336 138,733 145,070	2 184 186	3,168 0,754	4,202	0,016	*Yes*	FE - FW
12	Between groups Within groups Total	6,952 153,433 160,385	2 184 186	3,476 0,834	4,169	0,017	*Yes*	FE - R FE - FW
13	Between groups Within groups Total	13,415 138,970 152,385	2 184 186	6,708 0,755	8,881	0,000	*Yes*	FE - R FE - FW
14	Between groups Within groups Total	6,334 140,586 146,920	2 184 186	3,167 0,764	4,145	0,017	*Yes*	FE - R
15	Between groups Within groups Total	0,971 151,040 152,011	2 184 186	0,485 0,821	0,591	0,555	No:	-
16	Between groups Within groups Total	3,159 145,483 148,642	2 184 186	1,580 0,791	1,998	0,139	No:	-
17	Between groups Within groups Total	2,290 167,389 169,679	2 184 186	1,145 0,910	1,259	0,286	No:	-
18	Between groups Within groups Total	10,705 136,246 146,952	2 184 186	5,353 0,740	7,229	0,001	*Yes*	FE - R FE - FW
19	Between groups Within groups Total	7,294 122,117 129,412	2 184 186	3,647 0,664	5,495	0,005	*Yes*	FE - R FE - FW
20	Between groups Within groups Total	7,009 143,376 150,385	2 184 186	3,504 0,779	4,497	0,012	*Yes*	FE - R FE - FW
21	Between groups Within groups Total	3,041 138,413 141,455	2 184 186	1,521 0,752	2,022	0,135	No:	-
22	Between groups Within groups Total	13,966 115,841 129,807	2 184 186	6,983 0,630	11,092	0,000	*Yes*	FE - R FE - FW
23	Between groups Within groups Total	6,369 128,369 134,738	2 184 186	3,185 0,698	4,565	0,012	*Yes*	FE - R
24	Between groups Within groups Total	1,155 137,765 138,920	2 184 186	0,578 0,749	0,771	0,464	No:	-
25	Between groups Within groups Total	1,426 152,745 154,171	2 184 186	0,713 0,830	0,859	0,425	No:	-

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According to the ANOVA test results, which was conducted to determine whether there were differences between the forester groups' expectations regarding possible developments after FMC, there was a statistically significant difference between the FEs and the FWs regarding the views that the provisions of international agreements would be reflected to practice in a more effective way ($p = 0.002 < 0.05$), that during the certification process, foresters would be aware of their rights defined by international conventions and be able to use these rights ($p = 0.038 < 0.05$) and that forestry production techniques would be improved and thus production losses would be minimized ($p = 0.016 < 0.05$).

There was a statistically significant difference between the FEs and the Rs regarding the views that before all forestry activities, environmental impact assessment would be done and measures would be taken wherever required ($p = 0.017 < 0.05$) and that the awareness of stakeholders about endangered species would be increased ($p = 0.012 < 0.05$).

There was statistically significant difference sbetween the FEs and the Rs and between the FEs and the FWs regarding the following changes after FMC ($p = 0.008 < 0.05$): that potential conflicts between stakeholders would be identified and solutions would be developed ($p = 0.006 < 0.05$); that the quality of forest products and services would be improved ($p = 0.017 < 0.05$); that the employees of the forestry organization, especially managers would increase their skills to evaluate forests in many aspects by improving themselves with the help of trainings ($p = 0.000 < 0.05$); that monitoring, mapping and registering all kinds of forestry activities and social, economic and ecological effects of these activities would be provided ($p = 0.001 < 0.05$); that environmental and social impact assessment, identification and protection of endangered species and descriptions of production techniques and equipment would be added to management plans ($p = 0.005 < 0.05$) that results of monitoring plans would be shared with the public ($p = 0.012 < 0.05$) and that it would be paid more attention to occupational safety and health issues in forestry activities (use of personal protective equipment, first aid, etc.) ($p = 0.000 < 0.05$).

Discussion

SFM and FMC

In certification applications in the regions where private forestry is prevalent, the forest owners is informed in advance regarding the responsibilities they will assume. However, FMC processes in Turkey have started in line with the directives of senior executives of the forest management according to the requests from private companies engaged in export of forest products, because forests are owned by the state in Turkey. Before the certification process, the fact that the employees in the institution except a small number of the FEs had no knowledge about FMC has caused many problems in the certification application.

In many studies worldwide, the certification process conducted particularly in the areas where forests are under human pressure were considered as an important tool for SFM (Rametsteiner and Simula, 2003; Gambetta et al., 2006; Zhao et al., 2011). Therefore, foresters' views on SFM are important for implementation and maintenance of such techniques. However, in addition to considering certification important, knowledge regarding which aspects the forester stakeholders consider important will also be effective in solving the problems encountered in the certification process.

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In the study, when the views of the forester groups on FMC were examined, it was seen that the FEs see certification as a tool for SFM, the FWs approach it as a tool to encourage management of all forests in the world in the most appropriate way and to provide preservation of forest lands. The Rs, on the other hand, evaluate certification from a more general perspective, advocating these three views. The FSC certification organization has more effective criteria particularly in terms of nature conservation and this is reported to be seen as the reason why FSC is preferred in the USA, Canada, and Germany rather than other certification bodies that adopt economics-based approaches (Cashore et al., 2005). In this context, it can be said that the perspective on the certification process in Turkey show alignment with the rest of the world. Although forest certification initially dealt with environmental issues and then addressed social issues, the current economic aspects of certification have been also being discussed (Butterfield et al., 2005). A forest management understanding focused on respect for environment and protection of the environment, which constitutes the first phase of certification, is still on the agenda in Turkey considering that the certification efforts in Turkey begun in 2010. The economic aspect of certification is expected to come into prominence in the coming years with the demands of forest industry organizations for certified assets. Indeed, in a study conducted in 2015, 58.2% of the companies in the forest industry in Turkey indicate that certification is required for forest products (Tolunay et al., 2014).

The concern that the certification process will increase workload is one of the most important causes of negative outlook of foresters for certification. As forester groups are under heavy workload because of excessive bureaucracy, paperwork, and fieldworks presently, it is not actually an unexpected situation that they adopt a negative stance against certification. The fact that all forester groups, and particularly the FEs and the Rs, think that the workload will increase after the certification supports this prediction. One of the important points here is to find the line of work that will have an increase in workload and the causes for it. It was found at the end of the study that according to the FEs and the Rs, particularly because of inadequate staff numbers, there is an increase in workload in the fields such as safety activities, training programs, and paperwork. However, some of the FWs indicate that basic training given in the certification process causes a decrease in workload by allowing a more orderly and planned work. Similarly, in some scientific studies, it is stated that there is a requirement for proper paperwork regulations and an archiving system during adaptation to the FSC standards (Çavdar, 2012), however, it was discovered that paperwork density and redundancy of administrative requirements are seen as a worrying situation particularly in small scale businesses (Bass et al., 2001). In a study in the USA is reported that certification will increase workload in particular (Auld et al., 2003).

Post-certification expectations

Although a significant amount of material and nonmaterial costs are paid for the certification process, people's expectations for the benefits to be gained from the results of these studies constitute the most important driving force for the success of these processes. In this context, the results obtained in the study show that all forester groups have many positive expectations for the conditions after FMC. However, the FEs think negatively about the proposition that illegal benefits would be decreased after trainings given especially to forest villagers and the FWs think negatively about the proposition

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that conflicts between forester stakeholders would be ended. In general, the FWs and the Rs share the same thoughts with regard to expectations after certification.

Several other conclusions were noted in other studies with regard to such propositions. For example, in a study conducted in Brazil, it was proposed that the FSC certification would increase the value of certified products in economic sense (Azevedo and Freitas, 2003). In a study conducted in Guatemala, the stakeholders adopted the view that "in forest products market, the demand for certified forest products would gain positive momentum" (Rametsteiner and Simula, 2003). Finally, in a study in the American market, the proposition was that certification would provide market security for local products (Auld et al., 2003).

A study on feasibility of certification in Turkey stated that certification is offered as an opportunity for the solution of problems faced by the forest industry in Turkey in terms of economical, institutional, legal, and social problems as well as issues regarding planned tasks (Durusoy, 2002). Moreover, in a study conducted for preservation of the Amazon rainforest, it is indicated that there is improvement in FW rights and an increase in the standards of production activities in the forest after introduction of legislation and in the FSC harmonization process (Azevedo and Freitas, 2003). This is in agreement with the view that certification improve standards.

Inadequacies in the implementation of international agreements in the certification process have been reported. In fact, these inadequacies have been already indicated by the FSC inspectors that international conventions (ILO, CITES, BERN, etc.), which Turkey has signed, cannot be completely applied in Turkey because of "customary local mentalities." This reveals the necessity to make new policies as well as new arrangements in laws and regulations in coming years. In a study conducted in England, it is stated that certification affects forestry policies of the society (Bass et al., 2001).

Conclusion

In this study, we discovered that the success of FMC applications adopted by forests and managed by state forest enterprises is affected by the personnel structures. Especially if the workplace of the employees, who are in managerial positions, changes frequently and the replacing employees have a lower level of knowledge about certification, work gets delayed. In these cases, if attention is not paid toward the required observation and evaluation efforts for the continuation of certification, this makes the efforts superficial and prevents the certification from reaching the expected purposes.

Especially personnel policies need to be planned well to make sure that the certification practices, which are to be adopted in state-owned forested areas, are successful and provide the expected contributions. Filling the vacancies would increase the chances of success and sustainability of the efforts. In addition, it is necessary to conduct risk assessments and social impact evaluations before ensuring improvements in the use of legal rights owned by the forester groups. These generally include occupational safety and efficient labor issues, implementation of participatory management model, and creation and implementation of a management model.

It is wiser for countries to create their own national standards without deviating from the main purposes of certification rather than applying the standards determined by a certain certification institution due to their different legal, social, and physical structures. Certification efforts conducted in accordance with the standards that would

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be determined according to the domestic dynamics of countries would make it possible to solve many of the current problems at the very beginning. However, the most important two points that need to be applied to this type of a system are to keep the protection of forests and participatory approach in the foreground and having the audits always conducted by independent institutions.

With increasing the awareness of the groups forming the forestry association regarding sustainability and certification, trust in the certifications efforts that the expected benefits of certification would be realized in time and accordingly, the health, and sustainability of the forests would be guaranteed, is one of the most positive factors in the certification efforts.

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POLISH SOCIETY IN THE LIGHT OF THE USE OF RENEWABLE ENERGY SOURCES

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Abstract. The aim of the study was to determine the attitude of Polish society towards renewable energy sources and identify the benefits posed by actions that use renewable energy sources. A method of diagnostic survey was applied, conducting the study on the representative sample of 1067 respondents. The Statistica 10.1 GB program and the analysis of discriminant function were used in statistical analysis. It was shown that paying conscious attention to saving energy was the most important declaration of Polish society as the best way to save electricity. Polish citizens declared the use of energy-efficient sources of electricity, and in their opinion the greatest chance of use within the renewable energy sources had solar energy. In the opinion of Polish society, the greatest barrier to use the renewable energy sources is the lack of mechanisms supporting the reduction of energy intensity of the economy and the lack of policy coherence for sustainable development in terms of air protection. Additionally, the most important type of support that would encourage investing in the renewable energy sources is to raise funds from the ecological funds and European Union funds. The acquisition of renewable energy sources is highly influenced by the energy policy pursued by individual European Union countries, and the energy future depends on the political will and the capacity of societies to implement appropriate technologies.
Keywords: *renewable energy sources, Polish society, barriers and motives, production support*

Introduction

The demand for energy resources is continuously growing, and the reason for this tendency lies in the dynamic economic development and population growth (Warner and Jones, 2017). These factors caused that the energy resources in the form of non-renewable resources started to decrease, making it necessary to search for alternative sources (Hodana et al., 2012). Another important problem related to the exploitation of natural resources is the progressing degradation of the natural environment, affecting the proper functioning of the population (Adewuyi and Awodumi, 2017).

A model of functioning global energy, created by Jones and Warner (2016), has shown that the energy sector in the coming years will not deal with meeting the needs of the industrial economy and individual consumers in providing the energy without a support of renewable energy sources (Ahmed et al., 2014). A high impact on the growing demand for energy have rapidly developing countries of Asia in particular, but also countries of Europe, America and Australia as well as the Pacific Islands (Betzold, 2016).

There is observed an increase in the intensity of promotional campaigns aimed at the reduction of greenhouse gas emission, which is also of a great importance while promoting investments on the production of renewable energy (Al-mulali et al., 2013).

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An example is Europe, where since 2008 the majority of new energy installations have been based on renewable energy sources (Hvelplund et al., 2013).

In 2013 the produced renewable energy accounted for only 19.1% of the world consumption (Gasparatos et al., 2017). however, by 2014 the number of countries that have adopted policies aimed at the production of renewable energy has increased 3,5 times compared to 2004. Today, this number has increased to 164 countries from all regions of the world (REN21, 2015). European Union directive on the use of energy from renewable sources assumes that this energy by 2020 will be used in 20% by the EU Member States (EC. Directive, 2009).

The studies carried out so far have shown the greatest demand for the energy from wind, biomass and the sun (Banshwar et al., 2017b), which is most widely used by USA, China, and in Europe by Germany and the United Kingdom (Hammons, 2008). An important issue undertaken by researchers is the protection of environmental resources as well as motives related to generating income as a result of using energy from renewable sources (Bergek and Mignon, 2017).

In the studies Poland, like Ireland, Portugal, Finland, Greece, Romania and other countries that joined the European Union, is classified in Europe as a developing country (Amri, 2017). For this group of countries there was demonstrated a strong correlation between the introduction of solutions related to renewable energy sources and the GDP growth as well as the possibility to employ the local population in the energy sector (Arellano and Bond, 1991). However, fossil fuels are still the main source of the energy in Poland. This leads to high damages in natural environment and health of the population, although Poland has the potential for the use of renewable energy sources, which currently is used only to a very small extent (Igliński et al., 2016b).

The aim of the study was to determine the attitude of Polish society towards renewable energy sources. The efforts were made to determine the benefits posed by activities that use renewable energy sources and to identify the motives of potential recipients to use different techniques of obtaining the energy from renewable sources.

Methodology

A method of diagnostic survey was used in the study. The research tool was a questionnaire, which included 5 closed-ended questions. While measuring the attitudes, a five-point Likert scale was applied, after the use of activities related to the construction and validation. There was calculated the index of scale reliability, where Cronbach's alpha was set at 0.84. The methodological procedure allowed calculating the size of research sample, where the confidence level was set at 0.95, the estimated size of fraction at 0.50 and the maximum error at 0.03.

The sample size was selected from the whole adult population of Poland, which amounted to 31532048 persons (GUS, 2016). The sample size was set at 1067 respondents, who were chosen taking into account sex, place of residence, age and individual six Polish regions, and the number of respondents was representative for these regions: central - 220, southern - 222, eastern - 188, southwestern - 109, northwestern - 164 and northern - 164 persons. The study was conducted in September and October 2016.

The Statistica 10.1 GB program and the analysis of discriminant function were used in statistical analysis. The classification function was used in the form of calculating coefficients that were determined for each of created groups. Prior to the analysis there

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was examined a multivariate normality, verifying each variable for normality of distribution. It was assumed that the matrices of variances were homogeneous in groups. A standard deviation was not included due to a large number of respondents in each group. Statistically significant were those differences in means, of which the probability of randomness was less than $p < 0.05$.

Research results

Saving energy in Polish households

Paying conscious attention to the energy saving was the most common action pointed by Polish society as the best way to save electricity. Such declarations, to the most significant extent, occurred within the oldest age group at $p < 0.001$, as compared to the other two.

Regularly appeared, that the younger the group of responders, the less its interest in this way of saving energy. The opposite situation occurred in a declaration that the light is turned off in a situation of leaving the room. Significant differences occurred at $p < 0.001$, at the highest values of classification function in the case of the youngest group of respondents. The society, to a considerable extent, declared the use of energy-efficient sources of electricity. Such declarations, to the highest degree, appeared at the age from 40 to 54 years. The model also includes activities related to indications concerning energy consumption when buying RTV/AGD devices and avoiding the use of standby mode in RTV/AGD devices (*Table 1*).

Table1. *The level of saving electricity in Polish households*

Means of saving the electricity	Wilks' lambda: 0.632 F = 9.143 p<0.001*			Classification function (the age of respondents)			
	Wilks' lambda	F value	P level	From 18 to 24 years	From 25 to 39 years	From 40 to54 years	55 and more years
I follow the labeling on the consumption of electricity when buying RTV/AGD	0.678	30.381	0.001*	0.212	0.482	0.587	0.286
I consciously pay attention to energy saving	0.649	17.363	0.001*	1.908	2.041	2.775	3.053
I always turn off the light when leaving the room	0.652	18.458	0.001*	3.123	2.856	2.719	1.484
I use energy-efficient sources of electricity	0.721	4.999	0.019*	1.684	1.869	2.105	1.770
I avoid the use of standby mode in RTV/AGD devices	0.616	2.865	0.036*	0.722	0.640	0.712	1.226
Constans				13.748	17.219	22.002	19.195

*Level of significant difference at $p < 0.050$
Source: Author's own analysis based on study material

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The future of renewable energy sources

In the opinion of the society, renewable energy sources using solar energy are most likely to be used. The oldest respondents also perceived in biogas opportunity to produce renewable energy sources. This declaration at $p < 0.001$ was significantly the highest in relation to other groups. Respondents aged from 18 to 24 declared in substantially the highest degree, at $p < 0.001$, obtaining energy from sources related to water in the future, whereas respondents aged from 25 to 39 and from 40 to 54 years in the biomass. Apart from the oldest group surveyed, other groups also highly assessed the energy obtaining with the use of wind power installations (*Table 2*).

Table 2. *Preferences regarding the future of various renewable energy sources*

Type of renewable energy sources	Wilks' lambda: 0.713 F = 11.157 p<0.001*			Classification function (the age of respondents)			
	Wilks' lambda	F value	P level	From 18 to 24 years	From 25 to 39 years	From 40 to54 years	55 and more years
Water	0.771	22.381	0.001*	1.113	0.915	0.296	0.391
Biogas	0.758	17.162	0.001*	1.500	1.779	1.330	3.246
Biomass	0.730	6.407	0.001*	0.879	1.194	1.264	0.546
Wind	0.726	4.991	0.001*	1.280	1.389	1.466	0.677
Geothermal	0.724	4.046	0.007*	0.038	0.146	0.060	0.847
Solar	0.721	2.736	0.042*	4.422	4.414	4.694	4.916
Constans				16.654	19.275	19.246	21.474

*Level of significant difference at $p < 0.050$
Source: Author's own analysis based on study material

Investments related to renewable energy sources

The respondents sought the most important benefit from the use of renewable energy in the protection of natural environment. It was a declaration significantly more often expressed by the groups aged 25-39 and 40-54, at $p = 0.006$. The opposite situation occurred in the declaration of benefits from savings in energy use. Significantly higher values of classification function, at $p < 0.001$, occurred in the youngest and the oldest groups of respondents, comparing to respondents aged 25-39 and 40-54. An important task for Polish society is to become independent from fossil fuels and energy security as well as the gradual independence from external sources. The reduction of greenhouse gas emissions was significantly more important, at $p < 0.001$, at the age group from 25 to 39 (*Table 3*).

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Table 3. *The benefits of investing in renewable energy sources*

Type the benefits of the use of renewable energy sources	Wilks' lambda: 0.632 F = 9.877 p<0.001*			Classification function (the age of respondents)			
	Wilks' lambda	F value	P level	From 18 to 24 years	From 25 to 39 years	From 40 to54 years	55 and more years
Energy securityand the gradual dependence on external sources	0.661	10.949	0.001*	1.732	2.313	1.637	2.410
Local/regional development	0.677	16.876	0.001*	0.535	0.715	0.898	1.098
Reduction of greenhouse gas emissions	0.662	11.167	0.001*	0.864	1.236	0.618	0.078
Savings in energy use	0.653	7.880	0.001*	3.504	3.046	3.008	3.747
Independence from fossil fuels	0.645	4.767	0.002*	1.950	2.188	2.356	1.646
Protection of natural environment	0.643	4.115	0.006*	3.840	4.282	4.165	3.845
Constans				21.563	25.335	26.497	26.183

*Level of significant difference at p < 0.050
Source: Author's own analysis based on study material

Barriers in the use of renewable energy sources

In the opinion of Polish society, the greatest barrier in the use of renewable energy sources is the lack of mechanisms supporting the reduction of energy intensity of the economy. Significantly higher values of declarations, at p = 0.002, appeared among respondents from the two oldest groups, whereas the youngest groups, to a significantly higher degree, at p < 0.001, drew attention to the lack of policy coherence for sustainable development in the issue of air protection. Especially for residents aged 40-54, a significant obstacle was the lack of stability of the policy to promote renewable energy sources, associated with occurring monopoly on the energy market. The model also includes: barriers associated with exploitation of energy networks infrastructure, the increase of energy prices resulting from the lack of competition on the wholesale market and too complicated procedures of obtaining permits (*Table 4*).

Types of support for the production of renewable energy sources

It has been declared that the most important support which would encourage investments in renewable energy sources is to raise funds from the ecological funds and European Union funds. Especially respondents of the two oldest age groups expect such kind of support. Expectations are also directed towards relief from excise tax for the energy produced. Such solutions, to the most significant extend, at p < 0.001, are expected by residents aged 40-54. The model also includes expectations of legal nature that are associated with the volume commitments (*Table 5*).

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Table 4. *Barriers affecting the use of renewable energy sources*

Type of barrier	**Wilks' lambda: 0.628 F = 10.129 p<0.001***			**Classification function (the age of respondents)**			
	Wilks' lambda	**F value**	**P level**	**From 18 to 24 years**	**From 25 to 39 years**	**From 40 to54 years**	**55 and more years**
The lack of stable policy to promote renewable energy sources - monopoly of the energy market	0.699	26.847	0.001*	0.998	1.377	2.308	1.137
Lack of coherent policy on sustainable development in air protection	0.651	8.544	0.001*	2.214	2.427	1.818	1.440
The lack of mechanisms to support the reduction of energy intensity of the economy	0.641	4.721	0.002*	2.532	2.712	2.905	3.163
Exploited infrastructure of power grids	0.638	3.829	0.009*	1.289	1.536	1.042	1.635
The increase in energy prices resulting from the lack of competition in the wholesale market	0.636	2.847	0.036*	1.993	2.014	1.613	2.000
Too complicated procedures for obtaining permits and energy accounting	0.631	1.037	0.375	1.136	1.190	1.341	1.335
Constans				17.850	23.193	23.440	23.433

*Level of significant difference at p < 0.050
Source: Author's own analysis based on study material

Table 5. *Type of support that will encourage investments in renewable energy sources*

Type of support	**Wilks' lambda: 0.727 F = 8.011 p<0.001***			**Classification function (the age of respondents)**			
	Wilks' lambda	**F value**	**P level**	**From 18 to 24 years**	**From 25 to 39 years**	**From 40 to54 years**	**55 and more years**
Tax (relief in excise tax)	0.761	11.547	0.001*	2.469	2.608	3.107	2.257
Legal (quantity commitments)	0.756	9.789	0.001*	1.788	2.146	1.502	2.193
Financial (subsidies from environmental funds and EU funds)	0.740	4.436	0.004*	4.723	4.383	4.881	4.959
Constans				18.193	19.970	22.652	23.177

*Level of significant difference at p < 0.050
Source: Author's own analysis based on study material

Discussion

The aim of the study was to determine the attitude of Polish society towards renewable energy sources. Currently, Poland is a country where almost 90% of the energy is derived from coal and lignite that is from natural energy sources.

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Recommendations of the European Union as well as the willingness and readiness of Poland to environmental protection tend to obtaining energy from renewable sources (Paska and Surma, 2014; Igliński et. al., 2016a).

The conducted study showed that in Polish society there is awareness of the need to save the energy, especially this identified with the electricity. This approach is especially supported by older part of the population, although the level of declarations that the light is turned off in a situation of leaving the room indicates that also younger generations have a sense of concern for the energy.

The most popular way is to obtain the energy from solar collectors. It is a natural and targeted action, concerning the large promotion and additional investment of these projects from the EU funds. Confirmation of the validity of such actions is the West Pomeranian region, in which among holders of solar collectors there have been reported savings even up to 90% in summer and 40% in winter (Igliński et al., 2013). However, without financial support from the EU funds, as this type of installation is rather expensive, economic savings on such scale would not take place (Lambert and Silva, 2012).

It should be noted, however, that countries experienced in the production of energy from renewable sources prefer obtaining the energy from wind and biomass (Branshwar et al., 2017b).

The results of conducted studies are synonymous with European Union directives, which like the Polish society perceive the future of energy also inheat pumps and biomass stoves. In addition, such actions are aimed at reducing emission of greenhouse gases, of which excessive production has increasingly negative impact on healthy functioning of European societies. Similar expectations appeared also in Ireland, which like Poland has a problem with fossil fuels (Murphy et al., 2016).

The main benefits of the use of renewable energy sources are nature protection and savings associated with this kind of energy. These two factors are very important for developing countries, also Poland (Banshwar et. al., 2017a). Such declarations were also expressed by respondents in the conducted study.

This is a confirmation of the studies conducted in Sweden, focused on environmental issues and environmental protection (Bergek and Mignon, 2017).

An important issue for the Polish population is to become less reliant on fossil fuels, and energy security as well as gradual independence from obtaining energy from external sources (Lund et al., 2015). The local development is also very important. Through the introduction on the large scale devices for obtaining alternative energy, there is the possibility of creating new jobs for people operating these devices (Igliński et. al., 2012). Moving away from traditional fossil fuels to renewable energy sources, Europe is also able to eliminate nuclear power plants, which significantly damage the environment (Connolly et. al., 2016).

There were pointed out the most important barriers in the use of renewable energy sources which are the lack of support mechanisms to reduce the energy intensity of the economy and the lack of policy coherence for sustainable development of air protection. Such arguments have already been confirmed in the studies conducted by Igliński et al. (2012). Additionally, such barriers as the lack of stability of the policy to promote renewable energy sources, exploited infrastructure of energy network, higher energy prices resulting from the lack of competition in the wholesale market and overly complicated procedures for obtaining were also very important (Igliński et. al., 2012; Huijts et. al., 2012) for respondents taking part in the study.

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Respondents declared that the most important support which would encourage investments in renewable energy sources is to raise funds from subsidies of environmental funds and European Union funds. Expectations are also directed towards reliefs from excise tax for the energy produced and quantity commitments. It is also a focus on generating economic income, which is becoming a motivation to invest in renewable energy sources (Bergek and Mingnon, 2017). The conducted study and analysis of their results indicate a great importance of energy policy implemented by individual European Union countries, and the future of energy depends on political will and the capacity of societies to implement appropriate technologies (Lund and Hvelplund, 2012).

Conclusions

1. In Polish society there is awareness of the need to save the energy, especially this identified with the electricity. This approach is especially supported by older part of the population.
2. The most popular way to obtain the energy is the use of solar collectors.
3. The main benefits of the use of renewable energy sources are nature protection and savings associated with this kind of energy. An important issue for the Polish population is to become less reliant on fossil fuels, and energy security as well as gradual independence from obtaining energy from external sources.
4. That the most important support which would encourage investments in renewable energy sources is to raise funds from subsidies of environmental funds and European Union funds.

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ASSESSMENT OF THE POTENTIALLY ALGICIDAL EFFECTS OF *THYMUS SATUREIOIDES COSS.* AND *ARTEMISIA HERBA ALBA* L. AGAINST *MICROCYSTIS AERUGINOSA*

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Abstract. In search of an ecofriendly algaecide, aqueous extracts of two medicinal plants, *Thymus satureioides* Coss. and *Artemisia herba alba* L., were assessed for antialgal activity against *Microcystis aeruginosa.* An experiment was designed using five treatments (1%, 0.75%, 0.5%,0.25% and 0.1%). The growth of *M. aeruginosa*, morphological modifications, and photosynthetic pigments (chlorophyll a and carotenoids) on exposure to the extracts were explored. Also, phytochemical parameters in the extracts were analyzed to reveal the potential allelochemical compounds.
The results showed that both *T. satureioides* and *A. herba alba* extracts inhibited the growth of *M. aeruginosa* in a concentration-dependent way. After 8 days of treatment, the highest inhibition rates reached were 95%, 93% and 88.58%, and for *T. satureioides* and *A. herba alba* aqueous extracts, respectively. Chlorophyll a and carotenoid concentrations in cultures decreased especially in the 1% treatment group. Several morphological changes were observed in the treatment group compared to the controls. It was concluded that *M. aeruginosa* growth was suppressed by the potentially allelochemical compounds and probably by other allelochemical substances in aqueous extracts. Our results illustrated that both *T. satureioides and A. herba alba* extracts are able to control *Microcystis* blooms, and these may be recommended as a remedy for contamination of water bodies by harmful blooms.
Keywords: *Microcystis aeruginosa, Thymus satureioides Coss., Artemisia herba alba L., algicides, morphological and physiological changes, inhibitory effect*

Introduction

Cyanobacterial blooms are a major environmental problem in water bodies worldwide (Xiao et al., 2010). Their frequent appearance has been considered a consequence of eutrophication (Codd and Bell, 1985). *Microcystis spp.* are the most common bloom-forming cyanobacteria in eutrophic freshwaters (Douma et al., 2016; 2017). A break-out of *Microcystis* blooms releases cyanotoxins which can impair water quality, reduce productivity, decrease biodiversity, as well as causes severe illnesses to animals and humans (Rondel et al., 2008).

To control the adverse effects of cyanobacterial blooms, several strategies have been developed, often including the application of physical and chemical agents (Jeong et al., 2000). None of these methods can specifically control harmful algal blooms without causing secondary pollution or affecting aquatic organisms (Zhang et al., 2013).

Therefore, there is an urgent need to develop safe algaecides for controlling these harmful algal blooms.

Recent researches have exploited plants as an alternative to chemical agents (Chen et al., 2012; Zhou et al., 2014; Meng et al., 2015; Li et al., 2016). Numerous studies have reported the inhibition of cyanobacterial biomasses by several aquatic plants such as *Potamogeton maackianus* (Wu et al., 2007), *Myriophyllum spicatum* (Zhu et al., 2010), *Sagittariatrifolia* (Li et al., 2016), and *Eichhornia crassipes* (Zhou et al., 2014). To explore the potential of terrestrial plant extracts for controlling cyanobacteria provide a new guide for developing algicide (Shao et al., 2013).

Terrestrial medicinal plants have shown positive bioactivity and have demonstrated antibacterial, antifungal, antiviral, and insecticidal properties. The most important bioactive constituents of plants are phenolic compounds (Chen et al., 2012; Zhang et al., 2013). However, there are few reports on the use of medicinal plants to control *M. aeruginosa*, notably *Achillea ageratum L* and *Origanum compactum Benth* (Tebaa et al., 2017*); Portulaca oleracea* (Wang et al., 2016); *Ailanthus altissima* (Meng et al., 2015); *Shaddock Peel, Pomegranate Peel* (Wang et al., 2015).

In the Mediterranean area, medicinal plants are endemic (Bellakhdar, 1997). *T. satureioides* and *A. herba alba* are two well-known Moroccan medicinal plants (Ismaili et al., 2004; Bezza et al., 2010). *Thymus* is a Lamiaceae herbaceous species. It is a well-known aromatic perennial herb used extensively throughout the Mediterranean basin (Ismaili et al., 2004). A wide range of biological and pharmacological properties, including antiseptic, anthelmintic, and anti-inflammatory activities, have been reported for these species (Elhabazi et al., 2008; Ichrak et al., 2011). Several reports have demonstrated their strong antimicrobial, insecticidal, antifungal, antiviral, and antioxidant properties due to the rich content of phenolic compounds such as thymol and carvacrol (Jamali et al., 2013).

A. herba alba, belonging to the family Asteraseae, has been used by the local population as a medicinal plant as well as a flavoring additive in tea and coffee (Bezza et al., 2010). It has been extensively used to treat stomach disorders, hepatitis, and certain poisoning cases, as well as an antitumor, antispasmodic, antiseptic, antigenotoxic, antidiabetic, and antibacterial agent (Bezza et al., 2010; Mighri et al., 2010).

Phytochemical components and secondary metabolites such as flavonoids and others phenolic compounds present in these make them attractive for medicinal uses (Mohamed et al., 2010). However, little information is available on the use of medicinal plants for the treatment of bloom-forming cyanobacterium *M. aeruginosa* (Ni et al., 2011; 2012, Tebaa et al., 2017).

The aim of this study was to assess the potentially algicidal effects of *T. satureioides* and *A. herba alba* plants against *M. aeruginosa* with special attention to their photosynthetic pigments and allelochemicals compounds. To the best of our knowledge, this is the first report to study the impact of these two Moroccan specimens on *Microcystis spp.*

Materials and methods

Algal and floral materials

M. aeruginosa was isolated from the Moroccan eutrophic reservoir Lalla Takerkoust (31°21′36″ N; 8°7′48″ W) in October 2015. The isolated strain was grown in Z8 medium at 26±2°C under light intensity of 4000 $lx.m^{-2} s^{-1}$, with a light/dark cycle of

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15 h/9 h. The cyanobacteria were cultivated to an exponential growth phase (1–2 × 10^6 cells/ml). The two medicinal plants *T. satureioides* and *A. herba alba* were collected in May 2016 from two locations, Oukaimden and Tahanaout (High Atlas, Marrakesh area). Specimens were botanically identified, confirmed, and deposited in the Herbarium Mark (Faculty of Sciences Semlalia, Cadi Ayyad University of Marrakesh). The aerial plant parts were rinsed several times with distilled water to remove debris and epiphytic microbes. Leaves were separated and conserved for further use.

Preparation of aqueous extracts

The aqueous extraction of plants was carried out according to the method described by Ball et al. (2001), slightly modified by Li et al. (2016). Briefly, 10g of leaves were cut into tiny pieces and placed in 100 ml distilled water under agitation (37°C; 48 h). The macerate was filtered through a filter paper (Whatman GF/C, 0.22 µm). Then the filtrate was adjusted with distilled water to 100 ml and kept at 4°C as aqueous extract.

Algal bioassay

Experimental cultures were carried out in Erlenmeyer flask containing Z8 medium to a final volume (300 mL). Each flask was inoculated by a volume of *M. aeruginosa* in exponential growth phase to make an initial density (1–2 × 106 cells/ml). Microcystis cultures were exposed to different concentrations (0% [control], 0.1%, 0.25%, 0.5%, 0.75%, 1%) of aqueous extract. The cultures were incubated in a culture room at 26±2°C, illuminated in 15 h/9 h light-dark cycle with fluorescent tubes (4000 $lx.m^{-2}\ s^{-1}$). All experiments were carried out in triplicates.

Biomass estimation

M. aeruginosa growth under treatment was quantified through a daily sampling (every 24 h) of a constant volume of culture (2 ml each replicate) using Malassez hemocytometer. Growth inhibitory rate (IR) of *M. aeruginosa* under different concentrations was determined according to *Equation 1:*

$$\mathrm{IR}\left(\%\right) = \ (\mathrm{N0} - \mathrm{N} / \mathrm{N0} \times 100, \qquad \text{(Eq.1)}$$

where N0 and N (cells/ml) are cell density in treatment and control cultures, respectively.

Pigment quantification

Chlorophyll-a and carotenoid concentrations were extracted in the dark with 95% ethanol at 4°C for 48 h, and measured using a UV spectrophotometer (Carré 50 Scan) at 470, 649 and 665 nm. Pigment quantification was calculated according to Lichthentaler and Wellburn (1983). The following formulas (*Eq. 2* and *3*) were used to calculate the concentrations:

$$\text{Chlorophyll-a} \ = \ 13.95 \times \mathrm{OD665} \ - \ 6.88 \times \mathrm{OD649} \qquad \text{(Eq.2)}$$

$$\text{Carotenoids} = \ \left(\left[\left(1000 \times \mathrm{OD470}\right) \ - \ \left(2.05 \ \text{chlorophyll-a}\right)\right]\right) / \ 2 \qquad \text{(Eq.3)}$$

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Determination of Total phenolic (TPs) , Total flavonoids (TFs), Total tannins (TTs)

TP concentration was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). The total phenolic compounds were expressed as µg gallic acid equivalents per milliliter of aqueous extract.

TF concentration was determined by the method described by Kim et al. (2003). Measurements were calibrated to a standard curve of prepared catechin (Fluka) and the results were expressed as µg catechin equivalents per milliliter of aqueous extract. TT content was determined using the Folin-Denis test described by Salunkh et al. (1990), with slight modification. This method quantifies both condensed and hydrolysable tannins. A calibration curve was obtained using a tannic acid solution and the results were expressed as µg tannic acid equivalent per milliliter of aqueous extract.

Statistical analysis

The statistical significance between control and treatment groups was confirmed by analysis of variance (ANOVA) using SPSS V20.0 Windows 2010. Two-way ANOVA and Tukey's test were used to test differences between exposure concentrations and control at $p = 0.05$.

Results

Inhibitory effects on M. aeruginosa

As shown in *Figure 1 A-B* and *Table 1*, both *T. satureioides* and *A. herba alba* extracts exhibited a marked inhibitory effect on *M. aeruginosa.* In contrast to the control group, the cell densities of *M. aeruginosa* at all three concentrations (0.5%, 0.75% and 1%) for *T. satureioides* and *A. herba alba* extracts, respectively, were significantly reduced during the 8-day test period ($p < 0.05$). Moreover, the inhibition rates were dose-dependent within 8 days. For both *T. satureioides coss* and *A. herba alba* extracts, the maximum inhibition of cell growth (94.46%, 94.60%, and 95.93%, and 88.58%, respectively) was achieved at 0.5%, 0.75% and 1%, and 1% concentrations, respectively. The results suggest that *T. satureioides* showed a strong inhibitory effect on algal cells, and the effective doses ranged from 0.5% to 1% (*Fig. 1 C-D*).

Table 1. *Total phenolic (TPs) , Total flavonoids (TFs), Total tannins (TTs) amounts in A. herba alba and T. satureioides LA extracts; and correlations between all amounts and IRs of the three high concentration (0.5, 0.75, 1%) after 8 days of exposure*

	PT[1]	**FT[2]**	**TT[3]**
Plant	***T. satureioides***		
Concentrations	285 ± 34.82	25.83 ±4	0.032± 0.002
Coefficient of correlation	-0.044	-0.458	**0.941**
Plant	***A. herba alba***		
Concentrations	290±19.8	37.61±0.66	0.024±0.005
Coefficient of correlation	-0.999	**0.536**	-0.215

[1]µg Gallic acid equivalent ml-1 Aqeous extract
[2]µg catechin equivalent/ml Aqueous extract
[3]µg tannic acid equivalents ml-1 Aqeous extract

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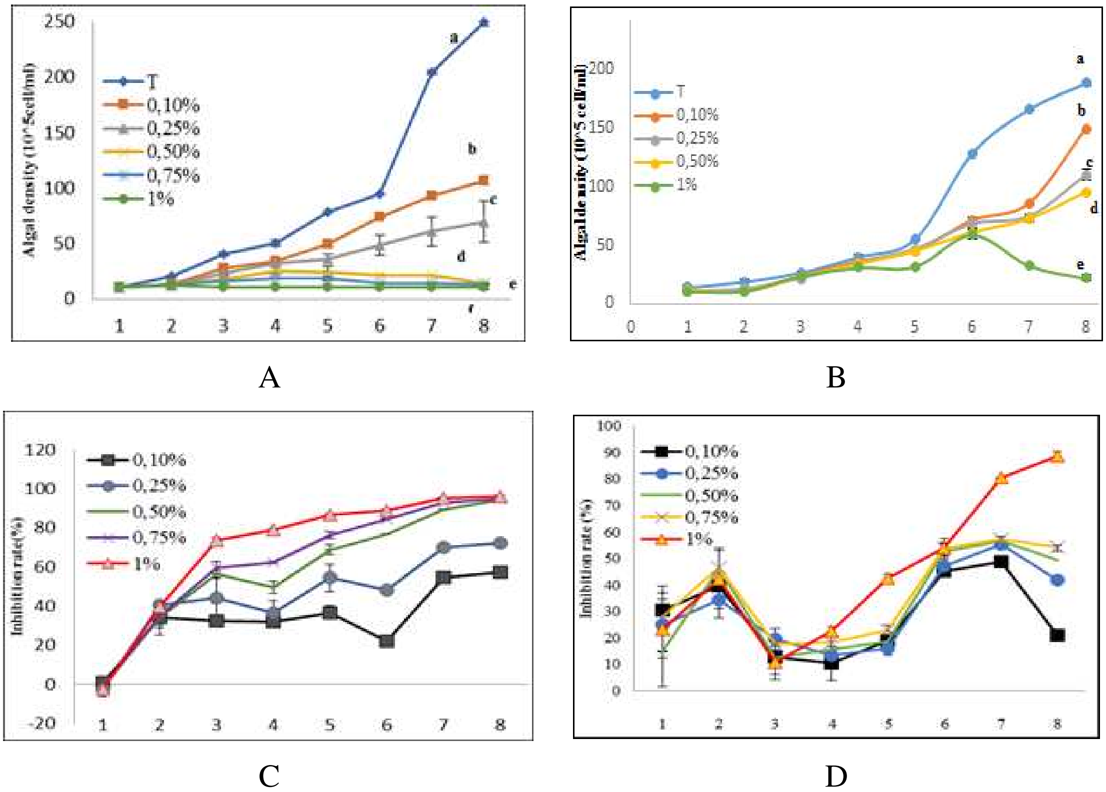

Figure 1. *Inhibitory effect of aqueous extracts of T. satureioides and A. herba alba on M. aeruginosa growth. A and B: density curves of T. satureioides, A. herba alba, respectively. C and D: IR inhibition rate curves of T. satureioides, A. herba alba, respectively. The values are means ± standard deviations calculated from different repetitions (n = 3). The value of p < 0.05 was considered significant. Letters a to f: groups not sharing the same letter are significantly different means*

Effects on photosynthetic pigments

The levels of two photosynthetic pigments (chlorophyll-a and carotenoids) were used as physiological indicators of the inhibition of *Microcystis.* In the control, the levels of both pigments increased as a function of tested concentrations (*Fig. 2*). Whereas in treatments, these globally decreased with the increase of concentrations. This demonstrated the significant difference between the treatment and control groups ($p < 0.05$) (*Fig. 2*). The levels of pigments appeared to be strongly inhibited at 95.93% and 88.58% concentrations of *T. satureioides* and *A. herba alba,* respectively.

Inhibitory mechanism of plant extracts on M. aeruginosa

According to the values of TPs, TFs, and TTs shown in *Table 1*, the concentrations in *T. satureioides* are higher than those in *A. herba alba*. As well, a significant correlation in IR was obtained between three significant concentrations (0.5–1%) with regard to TT concentrations (coef > 0.8) for *T. satureioides* sand with regard to TF concentrations (coef > 0.5) for *A. herba alba*.

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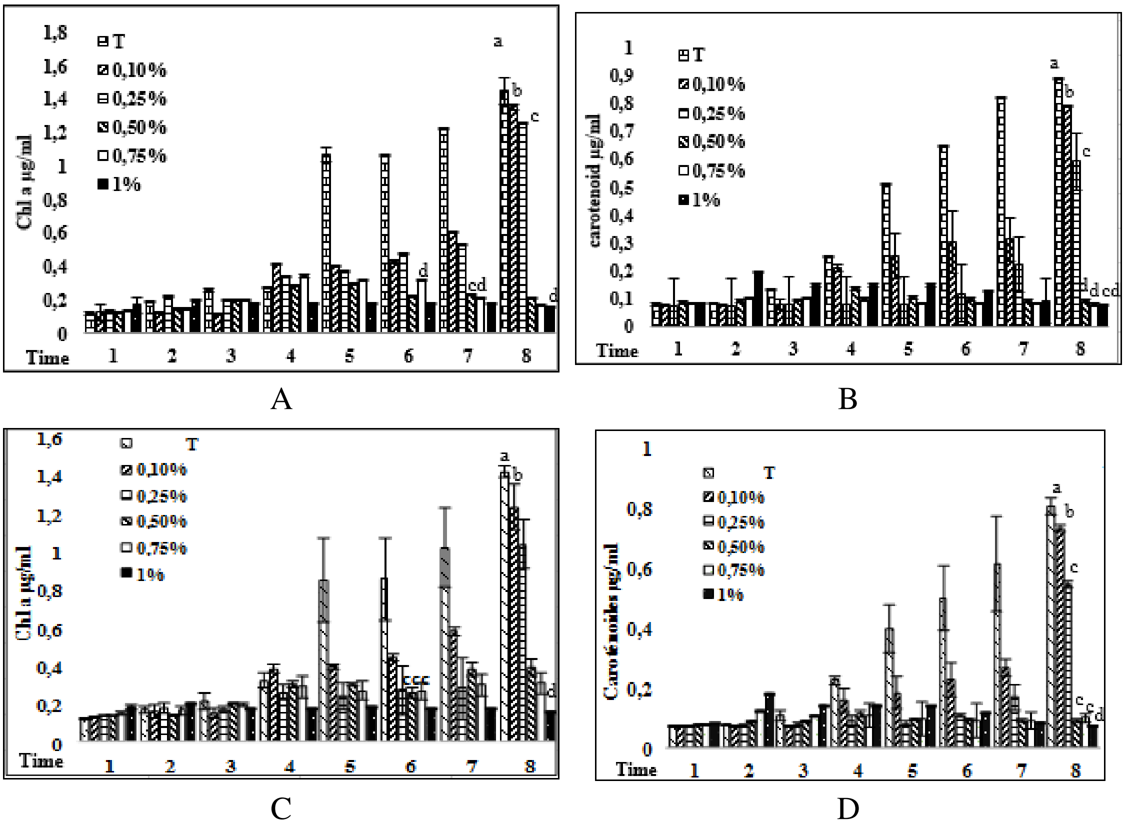

***Figure 2.** Effect of aqueous extracts on photosynthetic pigments of M. aeruginosa. A and C: Contents of chlorophyll a of T. satureioide and A. herba alba, respectively. B and D: Carotenoid contents of T. satureioides and A. herba alba, respectively*

Discussion

According to this study, both *A. herba alba* and *T. saturoides* aqueous extracts had potential algicidal effects on *Microcystis* growth. They significantly reduced the biomass and photosynthetic pigments of *M. aeruginosa.*

After 7 days of treatment at 1% concentration, the maximum IRs of 95%, 93%, and 88.58% were reached using *T. satureioides* and *A. herba alba* aqueous extracts, respectively. Meng et al.(2015) demonstrated that *A. altissima* extracts showed an IR of 90% against *M. aeruginosa* while completely destroying the organelle responsible for photosynthesis in algal cells.

In a study by Li et al. (2016), *S. trifolia* tuber aqueous extract demonstrated the highest inhibition rate of 70% on *M. aeruginosa* after 6 days of treatment. Ye et al. (2014) studied several Chinese herb aqueous extracts on *M. aeruginosa* and obtained the maximum IRs in the range of 51–98% after 10 days. Besides, the lowest concentrations of aqueous extracts had only negligible inhibition effect on *M. aeruginosa* (Ye et al., 2014; Li et al., 2016). Similar trends were reported by Xiao et al. (2010).

On the other hand, *T. saturoides* at a concentration of 0.5% was sufficient to elicit a strong inhibition on the growth of *M. aeruginosa*. The difference between the present results and that of Nakai et al. (1999) might be explained by the importance of aerobic decomposition to generate more inhibitors for algal suppression (Li et al., 2016).

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For *A. herba alba*, only the concentration of 1% was significantly inhibitory compared to the controls, which suggests that the potentially bioactive substances inhibited the growth of *M. aeruginosa*. At all other concentrations (0.1%, 0.25%, 0.5%, 0.75%), the growth retardation of *M. aeruginosa* was dose-dependent.

Furthermore, by microscopic observations, the presence of green color signified the abundant growth of *M. aeruginosa* in control cultures, whereas under treatments, the cultures became transparent with yellow sediment at the bottom after 6 days of treatment with *A. herba alba* (1%) and *T. saturoides* (0.5%,0.75% and 1%) aqueous extracts (*Fig. 3*).

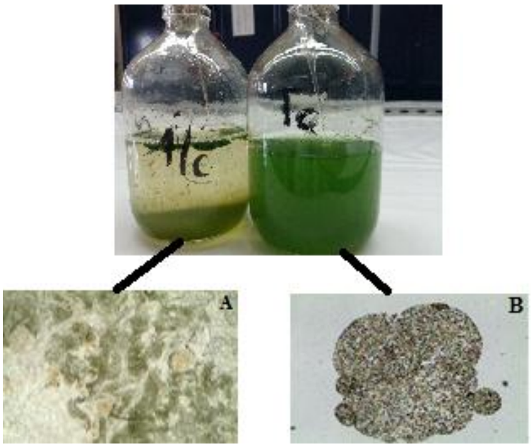

Figure 3. *Microscopic observation of the behavior of M. aeruginosa in the concentration groups 1% of T. satureioides aqueous extracts. A: (Gr. x 40) with sedimented cells, completely devacuolated and decomposed. B control group: (Gr. x 40); with M. aeruginosa in normal growth*

The results indicate that the extracts had a potential to accelerate *M. aeruginosa* cell lysis. These observations are in agreement with previous studies that investigated the cell morphology variations under treatments (Meng et al., 2015; Li et al., 2016). In our study, the photosynthetic pigments of M. *aeruginosa* were destroyed by aqueous extracts.

The antialgal allelochemicals reported in literature include fatty acids, polyphenols, terpenoids and polyethers. Previous studies have found that *Myriophyllum spicatum* releases four polyphenols and fatty acids that have an inhibitory effect on algae (Nakai et al., 2000).

In this study, tannins and flavonoids as potential allelochemicals made significant contribution to algal inhibition. These describe a group of phenolic compounds with a wide variety of allelochemical actions. Potential synergy between tannins and flavonoids may account for the maximum inhibition 95.93% ± 0.49 noted in *T. satureioides.*

Tannins have a toxic activity against filamentous fungi, yeasts, and bacteria, the antimicrobial activity of tannins could be due to their ability to form complexes with transport proteins (Scalbert, 1991). The inhibition of algal growth can be attributed to allelochemicals, including tannic acids. But because the correlation coefficient was less than 0.5 for *T. saturoides*, it is assumed that the phenolic compounds are one type among other secondary metabolites that cause algal inhibition (Chen et al., 2012).

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When investigating the antialgal activity of leaves of aquatic species *Iris wilsoni* on *Microcystis* sp., Whittaker and Feeny (1971) have shown that flavonoids, terpenoids, steroids, alkaloids, and organic cyanides are among allelochemicals to side polyphenolic compounds.

Chlorophyll-a and carotenoids are major pigments in microalgal photosynthetic systems (Yang et al., 2012). Their decrease shows the disturbance of photosynthesis affecting the growth and reproduction of *M. aeruginosa* (Li et al., 2016).

Allelopathic compounds behave like natural algicides; they often have multiple sites of action and various effects on the target organism (Ni et al., 2012). Some allelochemicals act by inhibiting photosynthesis, slowing down the growth of *M. aeruginosa.* They also attack the reactive oxygen species (ROS) on cell membranes by degrading unsaturated phospholipids and consequently increasing their permeability, leading to a disorder in cell organization (Meng et al., 2015).

The results of this study show that the use of these two plant extracts could bring great results in the biological control of harmful algae in aquatic ecosystems. However, further works will be needed to verify the application of these extracts in lakes for HAB inhibition. Also, it should be noted that the specific compounds responsible for such effects should be isolated and identified to explore the mechanism of inhibition in future studies and for field applications.

Conclusion

The present work demonstrated the inhibitory effect of aqueous extracts of *T. satureioides and A. herba alba* on *M. aeruginosa* growth. In addition to their antimicrobial, anti-inflammatory, analgesic, and antipyretic potentialities, these two medicinal plants can be used for ecofriendly restoration of aquatic environments contaminated by *Microcystis* blooms. Other future approaches will be necessary to optimize the allelochemical agents, as well as the conditions of interactions with other potential components, especially other pathogens, in the aquatic ecosystem.

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