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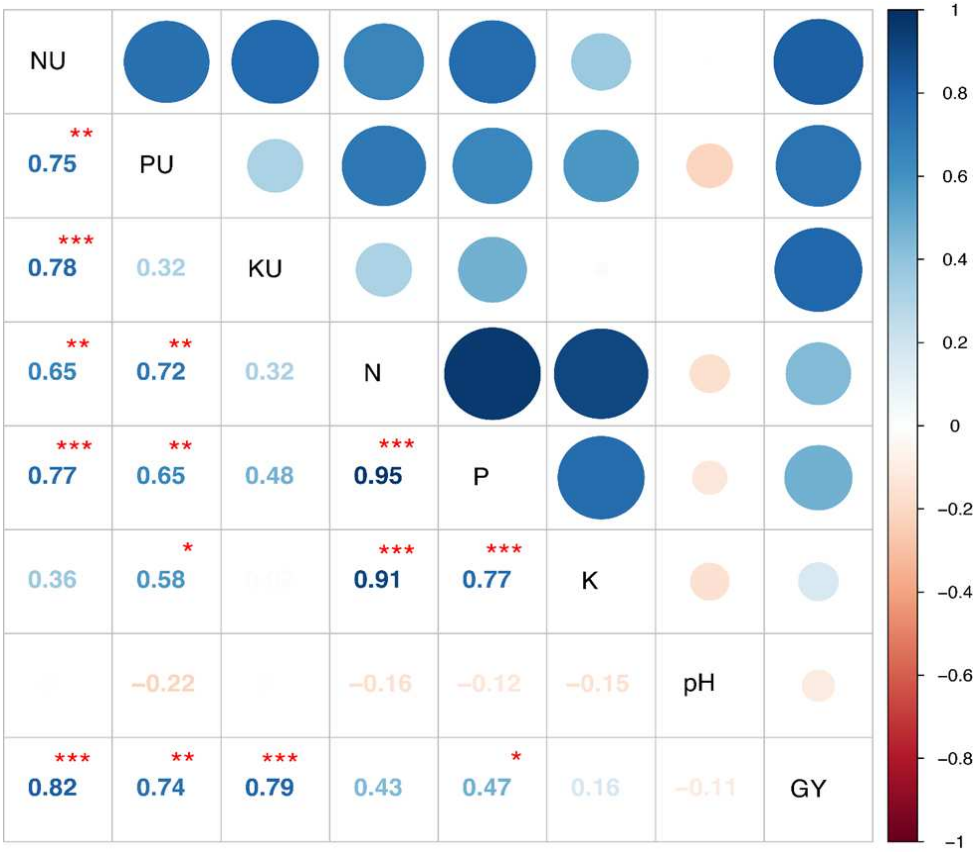

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PHYTOPLANKTON FUNCTIONAL GROUPS AND THEIR RELATIONSHIP WITH ENVIRONMENTAL FACTORS IN A EUTROPHIC AND HIGH-ALKALINITY LAKE IN THE COLD REGIONS OF NORTHERN CHINA

LI, X. C. – SUN, X. – MA, C. X.* – YU, H. X.*

Department of Ecology, College of Wildlife and Protected Area, Northeast Forestry University, Harbin 150040, China
(e-mail: Lixingchun0612@126.com – Li, X. C.; phone: +86-186-1248-9321)

**Corresponding authors*
e-mail: mch007@vip.163.com; phone: +86-180-0366-8291 (Ma, C. X.)
e-mail: china.yhx@163.com; phone: +86-131-0096-0911 (Yu, H. X.)

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Abstract. The investigation of phytoplankton was carried out from May to October month by month in 2018 in Hulun Lake, north China. Our purpose is to explore the community structure characteristics of phytoplankton by using the method of functional groups, combined with the monitoring results of water environmental factors, discussed the correlation between them in this special lake with high alkalinity, turbidity and eutrophication, so as to further explore the mechanisms of cyanobacteria bloom. Altogether 21 functional groups were identified, including 7 predominant functional groups. The predominant functional groups demonstrated strong spatio-temporal variations. It should also be noted that group H1 and M were the main culprits for the cyanobacteria bloom in the lake. Redundancy and Pearson analysis revealed that WT, DO, TP and TN were the most significant environmental factors controlling the distribution of phytoplankton functional groups. Furthermore, this study also evaluated the water quality in Hulun Lake by using Q index which shows it in a "medium" state. In a word, our results evidenced that a timely water environment dynamic can be reflected by monitoring the phytoplankton in Hulun Lake, which is also a superb way for the real-time monitoring of cyanobacteria bloom in this area.
Keywords: *cyanobacteria bloom, spatio-temporal succession, nutrient, ecological status, multivariate analysis, growth strategy*

Introduction

Water is the source of life. Freshwater resources which is available to human beings accounting for 0.26% of the global water resources. Among them, lake has provided more than half of drinking water resources for urban in inland China. It also plays a pivotal role in regulating river runoff, developing irrigation, multiplying aquatic organisms and improving regional ecological environment. Therefore, the protection of lake has always been one of the most significant themes in the process of protecting the global water environment. However, under the joint promotion of climate change and human activities, lakes have undergone various changes in recent years. Among them, the issue of eutrophication has been highly valued.

As an international environmental problem, eutrophication of the water body is threatening the ecological security of water areas all over the world. Eutrophication refers to a phenomenon where the water body is enriched in a large amount of nutrients like nitrogen and phosphorus, resulting in abnormal reproduction of algae and other aquatic organisms, ultimately leading to the deterioration of lake water quality, inhibition and destruction of both water ecosystem and self-purification ability. Eutrophication will increase the content of organic matter in water to a certain extent, and even lead to harmful

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algal blooms (Paerl, 1988). However, a large-scale of cyanobacteria bloom will foul waterways and water intakes, disrupt food webs, fuel hypoxia and produce secondary metabolites which are toxic to water consumers and users, including zooplankton, fish, shellfish, cattle, domestic pets, and human (Shang, 2017). Cyanobacterial bloom dynamics have been frequently reported in lake (Solis et al., 2018; Rose et al., 2019) which associated with many environmental factors, including low turbulence, low light, low ratio of euphotic zone to mixing zone and high temperature. Among them, temperature plays a considerable role. Generally speaking, the area of cyanobacteria bloom is mainly concentrated in tropical and subtropical areas. Therefore, the research on cyanobacteria bloom is also mainly concentrated in these areas. For domestic in China, the research is mainly concentrated in the area with high temperature, such as Taihu Lake and Chaohu Lake (Chuai et al., 2011; Zhang et al., 2016) which in the south of China. The research on cyanobacteria bloom region which in the north cold area is almost none.

Phytoplankton is an important primary producer of aquatic ecosystems and a major component of the aquatic food chain. It has become a pivotal indicator species which can reflect the water quality because of its rapid regeneration and sensitivity to environmental changes (Camp et al., 2015; Zwart et al., 2015). Therefore, the study of phytoplankton is helpful for us to evaluate the water quality and the nutritional status of water body. Many methods have been developed to evaluate water quality by using different idexes of phytoplankton, such as abundance, biomass and community composition (Birk et al., 2012). However, these traditional methods ignore the environmental and ecological characteristics of phytoplankton population, so it exposes many deficiencies in specific application. Reynolds et al. (2002) proposed the theory of phytoplankton functional group. Padisák et al. (2009) supplemented and improved the theory on the basis of Reynolds. Using this method to classify phytoplankton from the perspective of ecological function, a new concept of functional group was proposed. This method is conducive to us to describe the habitat characteristics of phytoplankton more accurately, so as to predict specific life. In this paper, the functional groups of phytoplankton are studied to explore the community distribution of phytoplankton in the environment.

The growth of phytoplankton will be affected by various environmental factors, such as water temperature, light availability, acid and alkali. Therefore, the species and biomass of phytoplankton vary greatly under different environmental conditions. Hulun Lake is a typical alkaline eutrophication water body in the cold area of north China. In recent years, a series of ecological problems caused by serious eutrophication of water body have been emerging, such as seriously cyanobacteria bloom occurs in summer with the highest temperature every year, miniaturization of zooplankton and fish, etc., which have attracted great attention of the state and government. As phytoplankton is increasingly common used in bioassessments, studying the lake organisms in relation to abiotic factors and identifying spatial patterns of biodiversity as well as their driving mechanisms have become a major trend of ecology as basis for prioritizing global and regional conservation efforts (Myers et al., 2000; Wang et al., 2016c). Therefore, this paper takes Hulun Lake as the research object, further studies phytoplankton and the relationship between phytoplankton and environmental factors by means of functional groups, reveals which environmental factors have influence on the distribution of phytoplankton functional groups by means of RDA and Pearson and comprehensively evaluates the water environment state of Hulun Lake basin with Q index, so as to explore the temporal and spatial distribution mechanism of cyanobacteria bloom. In response to the call of the state, it also provides a new monitoring method and important theoretical

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basis for the study of phytoplankton functional groups in the high alkalinity water body which in cold region.

Materials and methods

Investigation area and sampling sites description

Hulun Lake (48°31'-49°20'N, 116°58'-117°48'E), which is also known as Dalai Lake, lies in the hinterland of Hulunbuir prairie, Inner Mongolia, China. It is the fifth largest freshwater lake in China with the size of 2339 km^2 with the deepest water level of 9 m. Mean annual precipitation is 247-319 mm, and 80-86% of the annual precipitation falls in June to September. The lake is covered with 1m of ice from early November to late April. Except for rainfall and groundwater, the water supply of Hulun Lake mainly depends on direct surface runoff. The main rivers flowing into Hulun Lake include Crulen River and Orshen River which between Baikal Lake and Hulun Lake. Moreover, Hailar River is one of the most important water sources of Hulun Lake through the project of "Diversion of rivers into lake". And the Xinkai River which locates in the northeast of Hulun Lake is a throughput river regulating the water level of Hulun Lake. Hulun Lake Basin is a crucial ecological barrier in the north and plays an important ecological role in regulating the climate, conserving water resources, preventing desertification, and maintaining the balance of the grassland ecosystem.

According to the ecological environment characteristics of the Hulun Lake, following the principle of uniform distribution of sampling points, a total of 10 sampling points (*Table 1, Fig. 1*) are arranged in the lake body of Hulun Lake, including four sampling points specially set at the entrance of the lake due to the special relationship with the surrounding four rivers (1#-Xinkai River Estuary; 4#-Crulen River Estuary; 6#-Orshenn River Estuary; 7#-Hailar River Estuary). In addition, there are also 2#-3# located in the west of Hulun Lake, 5#-6# from the eastern region and 8#-10# in the central region.

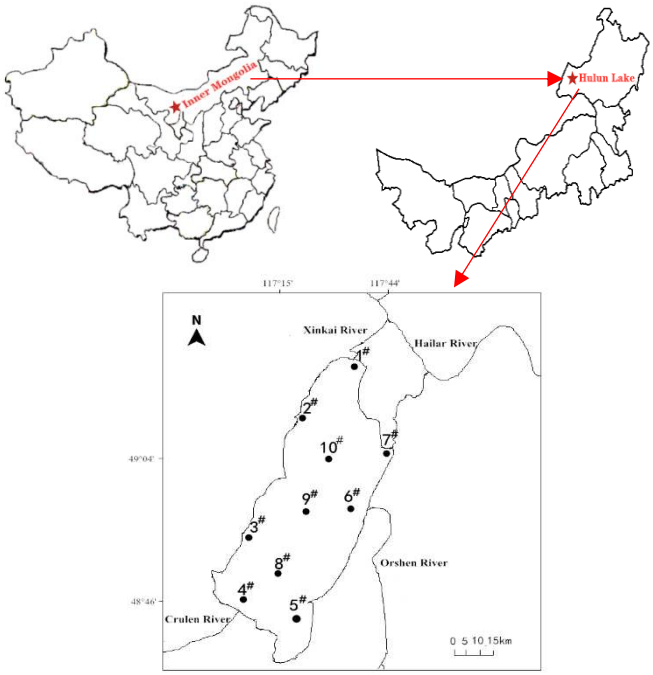

Figure 1. *Location of the study area and the sampling points in Hulun Lake*

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Table 1. *Ten sampling sites coordinates in Hulun Lake*

Sampling sites	Latitude	Longitude
1#	N49°18′10.61″	E117°38′40.75″
2#	N49°09′36.88″	E117°25′24.04″
3#	N48°55′06.01″	E117°08′36.90″
4#	N48°46′44.12″	E117°04′52.88″
5#	N48°43′15.38″	E117°17′45.55″
6#	N48°58′55.21″	E117°37′26.59″
7#	N49°06′44.45″	E117°44′53.27″
8#	N48°49′51.94″	E117°15′51.25″
9#	N48°57′32.13″	E117°25′49.68″
10#	N49°04′07.70″	E117°33′07.93″

Sampling collection and analysis

Many studies focus on how different environmental and spatial parameters, such as hydrological, physical and chemical ones (Isabwe et al., 2018; Wu et al., 2018) shape phytoplankton community. This approach may result in inaccurate prioritizations (Wu et al., 2018). So, there is a need for a parallel monitoring of the biota and the number of environmental drivers synchronously in space and time (Belletti et al., 2017; Latinopoulos et al., 2020). Different types of phytoplankton have different requirements for the environment. The environmental conditions of a certain season are more suitable for some types, while others are not. For phytoplankton, not only will the species composition change in different seasons, but also the biomass of phytoplankton will have very significant seasonal changes. In recent years, scholars have been committed to exploring the temporal and spatial succession of phytoplankton functional groups and their influencing factors. Therefore, they took samples at monthly intervals to explore their succession characteristics and further confirm which environmental factors influence the phytoplankton community structure (Yang et al., 2020; Liao et al., 2020). The monthly monitoring of physicochemical indices, especially WT and nutrients, is more effective in analyzing the environmental thresholds required for the cyanobacteria bloom which could be used to forecast the rapid formation of a cyanobacteria bloom. Therefore, we use the method of synchronous monitoring of phytoplankton and physico-chemical index, took water samples at monthly intervals in ten sampling sites from May to October in 2018. Physical and chemical indicators such as water temperature (WT), pH and conductivity (Cond) are measured directly by a portable multi-probe (YSI 6600, YSI Inc., USA) in sampling site. Secchi disk is used for the determination of water transparency (SD). Analysis of nutrients like total nitrogen (TN) and total phosphorus (TP) requires that we take water samples back to laboratory and then measured by oxidation with alkaline persulfate and persulfate acid as described by Costa et al. (2009). In addition, we used the Chinese standard methods proposed by Ministry of Environmental Protection of People's Republic of China (MEP, 2002) to determine the ammonium nitrogen (NH_4^+-N), chemical oxygen demand (COD_{Cr}) and dissolved oxygen (DO).

Phytoplankton analysis was sampled with calibrated 1 L van Dorn sampler at the surface and the bottom of the water column and integrated over depth for every site, then transfer to 1 L phytoplankton bottle and add 10-15 mL Lugol's solution immediately.

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After being taken back to the laboratory, sedimented for 48 h then concentrated to a final volume of 30 mL. Species were identified and counted using an inverted microscope at 400 magnification. The estimation of phytoplankton biomass is based on a method proposed by Hillebrand et al. (1999) and Chen et al. (2003) then transforming biovolume to biomass by using a formula of 1 mm^3/L=1 mg/L. The classification and determination of phytoplankton functional groups are based on Reynolds et al. (2002), Reynolds (2006), and Padisák et al. (2009).

Data analysis

After dimensionalizating the biomass data of phytoplankton functional group and environmental data other than pH through $Lg^{(X+1)}$, detrended correspondence analysis EQ showed that the lengths of gradient value were 2.622 (between 2 and 3). So after employing Monte Carlo simulation to test the significance of physico-chemical variable in explaining the phytoplankton functional groups under unrestricted model of 499 permutations. RDA (Redundancy Analysis) was used to further analyze the correlation between functional group characteristics and environmental factors. Seasonal variations of physical-chemical factors and phytoplankton biomass were tested by one-way ANOVA using the SPSS 19 software. Pearson's correlation analysis was also used to explore the relationships between phytoplankton functional groups and environmental factors. Meanwhile, the Q index using to evaluate the water quality of the lake was calculated by *Equations 1 and 2* (Padisák et al., 2006). According to the different value of Q index, water condition can be divided into five grades: 0-1: poor ecological status; 1-2: tolerable; 2-3: medium; 3-4: good; 4-5: excellent. On the whole, statistical analysis and figures drawing was done using SPSS 17.0 (SPSS, 2008), Microsoft Excel window and Origin Pro 8.

$$Q = \sum_{i=1}^{n} p_i F \quad \text{(Eq.1)}$$

$$p_i = n_i / N \quad \text{(Eq.2)}$$

In *Equation 2*, n_i is the biomass of the *i*-th fuctional group, N is the total biomass, and F factor is established for each *i*-th functional group in the given lake type which shows in *Table 2.*

Results

Seasonal variation of environmental factors

The variation of water environmental factors in Hulun Lake are recorded in *Table 2.* During the investigation, most of the environmental factors displayed significant seasonal differences (p<0.01, by ANOVA). Water temperature (WT) got the minimum value in October (6.25℃) and the maximum value in July (24.13℃), this huge temperature differences had a strong impact on the growth and distribution of phytoplankton. Following the same pattern, the dissolved oxygen (DO) value fluctuated greatly (p< 0.01, by ANOVA) with the seasons too. pH was always higher than 8.9 which means the whole area is alkaline. Mean conductivity (Cond) in the Hulun Lake did not vary seasonally (p>0.*05*, by ANOVA) but relative low value was recorded in August (1522.2 μs/cm).

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Chemical oxygen demand (COD) values were higher in autumn (September and October) and lower in Spring (May). The change of ammonia nitrogen (NH_4^+-N) value was also significant which the peak value was observed in July (0.613 mg/L). Total nitrogen (TN) showed an increasing trend from May to August. Total phosphorus (TP) fluctuated around 0.2 mg/L from May to October which indicating the severe eutrophication in Hulun Lake. Morever, statistical differences among seasons were recorded for SD too (p<0.01, by ANOVA), which the lowest value of SD was recorded in May (19 cm).

Table 2. *The seasonal variations (mean ± standard error) of environmental factors comparing by One-way ANOVA. Environmental parameters including water temperature(WT), dissolved oxygen(DO), pH, conductivity(COND), Chemical oxygen demand(COD), NH_4^+-N, total nitrogen(TN), total phosphorus(TP), water transparency (SD) and phytoplankton biomass*

	May	**June**	**July**	**August**	**September**	**October**	***p-value***
WT/ (℃)	16.27±3.05	23.92±0.95	24.13±0.90	21±0.00	14.92±1.82	6.26±0.56	0.000**
DO/ (mg/L)	9.56±0.43	8.21±0.30	7.85±0.33	7.73±0.42	9.92±0.65	11.7±0.24	0.000**
pH	8.93±0.05	8.93±0.05	9±0.09	9±0.09	9.06±0.08	9±0.07	0.002**
COND/ (μs/cm)	1670.2±109.05	1746.4±152.76	1630.8±212.2	1522.2±279.78	1568.5±411.78	1664±126.27	0.369
COD/ （mg/L）	54.6±4.70	74.4±24.08	59±4.47	78.9±19.07	106.73±29.49	134.5±15.13	0.000**
NH_4^+-N/ （mg/L）	0.057±0.02	0.03±0.03	0.613±0.94	0.105±0.07	0.0496±0.01	0.051±0.03	0.008**
TN/ (mg/L)	1.93±1.02	2.08±0.96	2.12±1.11	2.498±1.35	1.701±0.46	1.982±0.16	0.559
TP/ (mg/L)	0.25±0.10	0.18±0.02	0.255±0.04	0.179±0.08	0.2±0.04	0.1856±0.03	0.008**
SD/ （cm）	19±8.78	19.42±6.52	32.25±4.96	49.9±3.73	40±11.29	34.7±4.08	0.000**
Biomass/ (mg/L)	1.54±0.52	2.22±1.44	2.22±1.44	0.90±0.40	1.84±0.48	1.67±2.67	0.027

**The environmental factors were significantly different at the level of 0.01

Phytoplankton community and functional groups

From May to October in 2018, 86 genera belonging to 7 taxonomic categories were identified at ten sampling sites in Hulun Lake. Among them, 41 species (47.67%) were Chlorophyta, followed by Bacillariophyta (25 species) (29.07%), Cyanophyta (12 species) (13.95%), Euglenophyta (3 species) (3.49%), Chrysophyta (3 species) (3.49%), Cryptophyta (1 species) (1.16%) and Pyrrophyta (1 species) (1.16%). With the passage of time, the number of species in the whole area showed a trend of August (66 species) > October (58 species) > June (57 species) > May (56 species) > July(53 species) ＞ September (50 species).

In total, phytoplankton were divided into 21 functional groups namely C, D, E, F, G, H1, J, L0, M, MP, N, P, S1, T, TB, W1, W2, X1, X2, X3, Y (*Table 3*). However, 7 functional groups (J, X2, W1, F, H1, M, MP) contributing more than 10% of total phytoplankton abundance per sample were classified as dominant groups.

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Table 3. *Division of phytoplankton functional groups and their biomass proportion. F factor corresponding with each functional group in Hulun Lake*

Functional groups	Phytoplankton Species	Taxonomic	Biomass(%)	F factor
C	*Asterionella formosa* *Cyclotella meneghiniana* *Synedra acus*	Bacillariophyta	5.37%	5
D	*Synedra tabulata* *Synedra ulna*	Bacillariophyta	1.17%	2
E	*Dinobryon divergens* *Westella botryoides*	Chrysophyceae	0.01%	2
F	*Selenastrum gracile* *Kirchneriella lunaris* *Quadrigula chodatii* *Dictyosphaerium pulchellum* *Stichococcus bacillaris* *Oocystis elliptica*	Chlorophyceae	7.31%	5
G	*Eudorina elegans* *Pandorina morum*	Chlorophyceae	0.37%	1
H1	*Anabaena circinalis* *Anabaena variabilis*	Cyanophyceae	19.55%	1
J	*Tetrastrum elegans* *Tetraëdron trigonum* *Chodatella quadriseta* *Tetraëdron pusillum* *Tetrastrum elegans* *Scenedesmus bijuga* *Scenedesmus dimorphus* *Scenedesmus quadricauda* *Scenedesmus spinosus* *Scenedesmus platydiscus* *Coelastrum microporum* *Pediastrum birtadiatum* *Pediastrum duplex* *Pediastrum tetras* *Pediastrum simplex* *Crucigenia tetrapedia* *Crucigenia quadrata* *Actinastrum fluviatile*	Chlorophyceae	14.45%	1
L0	*Merismopedia minima* *Merismopedia marssonii* *Synechocystis minuscula* *Chroococcus minutus*	Cyanophyceae	2.32%	5
	Gyrosigma acuminatum *Amphora ovalis*	Bacillariophyta		
M	*Microcystis wesenbergii* *Microcystis firma*	Cyanophyceae	5.00%	0
MP	*Coelosphaerium kutzingianum* Nag *Navicula exigua* *Navicula anglica* *Navicula radiosa* *Navicula dicephala* *Gomphonema constrictum var.capitatum* *Cocconeis placentula* *Cymbella ventricosa* *Surirella angustata* *Cymatopleura solea* *Pinnularia major* *Diatoma vulgare*	Cyanophyceae Bacillariophyta	7.37%	5
	Ulothris variabilis	Chlorophyceae		

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Functional groups	Phytoplankton Species	Taxonomic	Biomass(%)	F factor
N	*Cosmarium obtusatum* *Staurastrum gracile*	Chlorophyceae	4.14%	5
	Fragilaria brevistriata *Fragilaria virescens* *Fragilaria ca pucina*	Bacillariophyta		
P	*Melosira granulata* *Melosira granulata var. angustissima*		1.44%	5
	Closterium acerosum *Closterium gracile* *Closterium moniliforum* *Closterium gracile parvulum*	Chlorophyceae		
S1	*Phomidium okenii* *Phormidium allorgei* *Phormidium lismorense*	Cyanophyceae	0.90%	0
T	*Mougeotia Agardh*	Chlorophyceae	0.63%	5
TB	*Melosira varians*	Bacillariophyta	0.08%	5
W1	*Euglena oxyuris* *Lepocinclis steinii*	Euglenophyceae	6.18%	0
W2	*Trachelomonas granulosa*	Euglenophyceae	0.12%	0
X1	*Ankistrodesmus angustus* *Ankistrodesmus acicularis* *Schroederia setigera* *Schroederia nitzschioides*	Chlorophyceae	1.57%	4
X2	*Chlamydomonas ovalis* *Chlamydomonas globosa*	Chlorophyceae	20.07%	3.5
X3	*Chromulina elegans* *Kephyrion planctonicum*	Chrysophyceae	0.02%	4
Y	*Cryptomonas ovata* *Glenodinium pulvisculus*	Cryptophyceae Euglenophyceae	1.92%	2

Temporal and spatial variation of phytoplankton functional groups

The biomass of phytoplankton functional groups showed seasonal and spatial variation (*Fig. 2*). At the beginning of the study (May), functional groups J and X2 occupied the main dominant position with the relative biomass of 29.78% and 23.14%. From the perspective of the functional groups' biomass distribution at each sampling point, group W1 accounted for a large proportion (46.58%) in site 9#. Furthermore, in this month's investigation, all the dominant functional groups were distributed in all sampling points except group M. In June, the biomass of each sampling site varied greatly. The highest biomass was in site 7# (4.54 mg/L) and the lowest was in site 2# (0.50 mg/L). The dominant functional groups were H1 and X2. Group H1 showed a very high biomass in site 5#, 7#, 8#, 9#, accounting for 63.63%, 49.12%, 53.03% and 59.50% of the total biomass of these sampling sites respectively, and the total biomass of functional group H1 reached the highest value (8.59 mg/L) in this month during the whole survey. In July, the dominant functional groups were H1 and J. Group J got the peak biomass in site 5# and the biomass distribution of group H1 was the largest among the site 9#. The dominant position of these two functional groups in July indicated that eutrophication was very serious at this time. With the increasing of the temperature, the cyanobacteria boom in Hulun Lake becoming more serious. From the beginning of August, the biomass of group M began to increase abruptly, with H1 together, became the dominant functional group in August. It was noteworthy that group M did not distribute at 3#-7# sampling points, but it occupied a considerable proportion in site 1#, 2#and 10#. In September, H1 functional

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group no longer occupied the dominant position, even did not distribute in site 4#. Group X2/J became the dominant functional group. X2 functional group accounting for 63.07% of the total biomass in site 3#. Group J was less distributed in site 9# than in other sampling points. In October, the dominant functional groups were still J and X2. Group X2 accounted for a large proportion in site 2#, 3#, 4# and functional group M was only distributed in site 10#. Overall, the dominate seasonal variation of phytoplankton functional groups showed a trend of J+X2 (May) → H1/X2(June) → H1/J(July) → M/H1(August) → X2/J(September) → J/X2/MP(October).

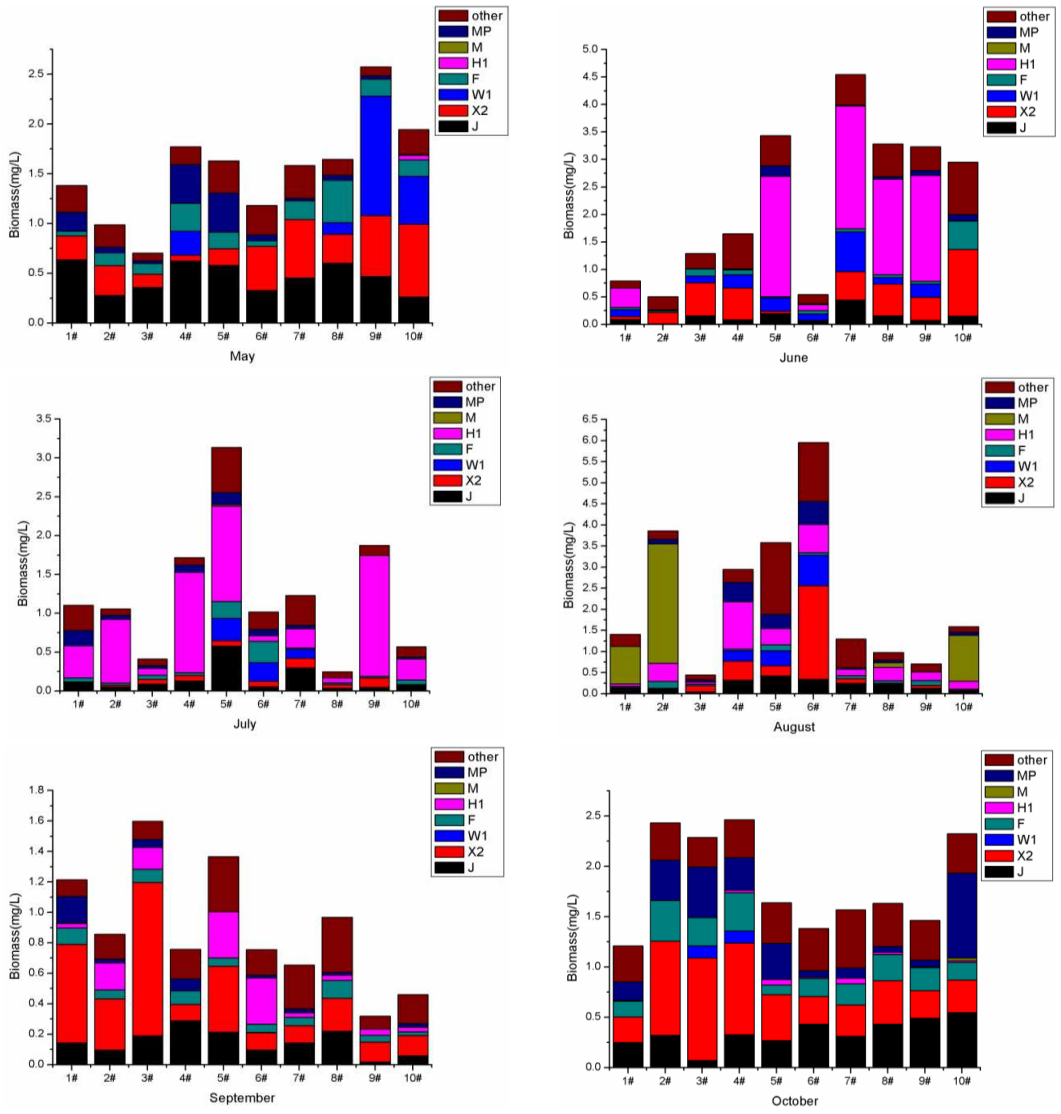

Figure 2. *Temporal and spatial distribution in biomass of phytoplankton functional group*

Q index

According to the Q index monitoring ecological status of lakes recommended by Padisák et al. (2006), factor F weights for each phytoplankton functional group identified appear in *Table 3*. The results are showed in *Fig. 3*. Differences of Q index between seasons and sampling sites are very significant.

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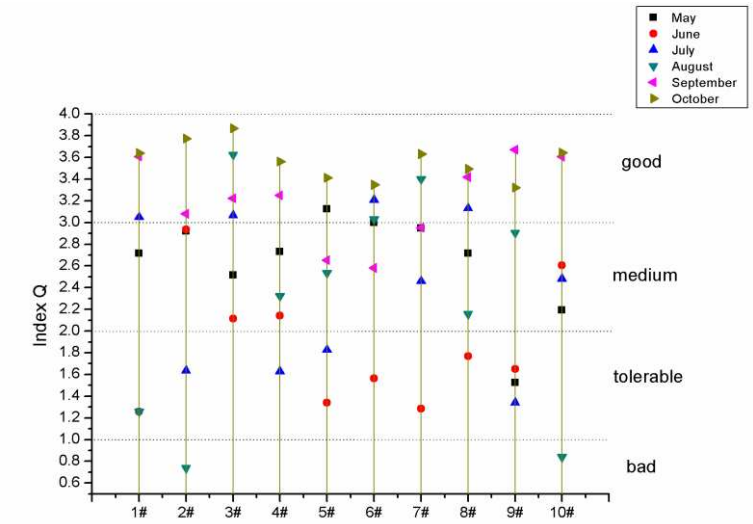

Figure 3. *Ecological status evaluation by Q index in Hulun Lake*

During the whole investigation, the Q index fluctuated between 0.74 and 3.87, and the two sampling points with the lowest value also means having the worst water quality both appeared in August, one was in site 2# and the other was in site 10#, which having a "bad" water quality. The highest value of Q index was in site 3# in October which had the lowest TP value (0.148 mg/L). According to the comprehensive evaluation on the average value of Q index in each month, the water quality in May, July and August was the same, all of which were "medium". The water quality in the lake was "good" in September and October, while the overall water quality in June was the worst, showing a "tolerable" level. As can also be seen from *Fig. 3*, sampling sites which on the level of "tolerable" and "bad" were mostly occured in June, July and August when Hulun Lake was having a severe cyanobacteria boom.

Correlation analysis

Redundancy analysis (RDA) was performed to reveal the relationship between phytoplankton functional groups and environmental parameters. The analysis involved seven phytoplankton functional groups and nine physiochemical data. The eigenvalues for RDA axis 1 (0.089) and axis 2 (0.053) explained 14.2% (axis 1: 8.9%, axis 2: 5.3%) of variance in phytoplankton functional groups data and 63.1% (axis 1: 44.9%, axis 2: 26.6%) of functional groups-environment variables (*Table 4*). Variables like DO (0.7076), COD (0.5238), TN (0.2717), SD (0.0596) and Cond (0.0120) were positively related to axis 1, whereas it was negatively related with WT (-0.8493), NH_4^+-N (-0.3978) TP (-0.1567) and pH (-0.0163). Axis 2 was positively correlated with TN (0.5188), WT (0.2698), pH (0.0981), COD (0.0289) and negatively correlated with TP (-0.5280), SD (-0.3698), DO (-0.3564), Cond (-0.2695) and NH_4^+-N (-0.0360) (*Fig. 4*).

The Pearson correlations among the biomass of the dominant phytoplankton functional groups and environmental variables are presented in *Table 5*. Most of phytoplankton functional groups were significantly correlated with the environmental variables. The biomass of functional J and F were all significantly positive correlated with WT ($p<0.01$) while negatively correlated with DO ($p<0.01$). Moreover, Cond had a positive correlation with group F ($p<0.05$). Conversely, group H1 was positively correlated with WT ($p<0.01$),

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while negatively correlated with DO ($p<0.01$). Group W1 was positively correlated with WT ($p<0.05$), while negatively correlated with SD ($p<0.05$). Besides, group MP was both positively correlated with DO ($p<0.05$) and COD ($p<0.05$), while negatively with WT ($p<0.01$).

Table 4. *CCA analysis of dominant phytoplankton functional groups and environmental factors in Hulun Lake*

Axes	Eigenvalues	Speccies-environment correlations	Cumulative percentage variance of species data%	Cumulative percentage variance of species-environment relation%
1	0.089	0.581	8.9	44.9
2	0.053	0.463	14.2	71.5
3	0.036	0.577	17.8	89.5
4	0.01	0.413	18.8	94.5

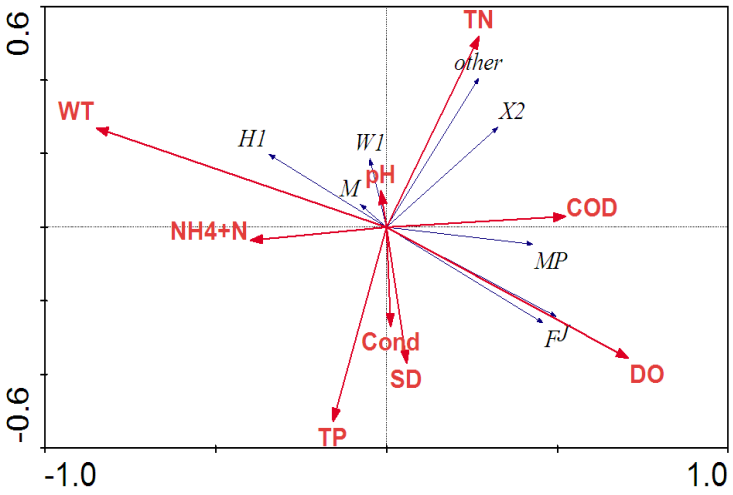

Figure 4. *Redundancy analysis (RDA) ordination diagram of the dominant functional groups and physical-chemical variables in Hulun Lake*

Table 5. *Pearson correlation between phytoplankton functional group biomass (J, X2, W1, F, H1, M, MP, other) and environmental factors, water temperature(WT), dissolved oxygen(DO), pH, conductivity(COND), Chemical oxygen demand(COD), NH_4^+-N, total nitrogen(TN), total phosphorus(TP), water transparency (SD)*

	J	X2	W1	F	H1	M	MP	other
WT	-.389**	-0.211	.270*	-.396**	.444**	0.103	-.377**	0.012
DO	.362**	0.24	-0.173	.407**	-.353**	-0.159	.257*	-0.148
pH	-0.123	-0.071	-0.142	0.019	-0.047	0.033	0.054	0.112
Cond	-0.044	-0.121	-0.107	.262*	-0.052	0.021	-0.219	-0.154
COD	0.031	0.123	-0.223	0.223	-0.245	-0.108	.265*	0.153
NH_4^+-N	-0.21	-0.151	-0.088	-0.147	0.046	-0.044	-0.103	-0.159
TN	0.001	0.049	0.103	0.01	-0.088	-0.03	0.057	.375**
TP	0.101	-0.246	0.122	0.065	-0.091	-0.12	-0.05	-.280*
SD	-0.158	-0.02	-.264*	-0.115	-0.077	0.226	0.153	0.146

**$p<0.01$; *$p<0.05$

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Discussion

Temporal succession of phytoplankton functional groups

Phytoplankton community structure responds to trophic and spatio-temporal gradient through changes in species composition and quantitative ecological traits such as biomass, species richness, and diversity (Watson et al., 1997). Since the concept of functional group was proposed, on account of functional group has high potential as an indicator of variation in limnological conditions, people began to use this new method to reflect and monitor water quality. Our results showed the temporal and spatial environmental changes of the lake ecosystem and the response of phytoplankton functional groups to this environmental heterogeneity. In such a special cold area, high alkalinity, turbidity and eutrophication water environment, the most important thing to be concerned about was the succession process of phytoplankton functional groups.

The same population is maintained when the extent of environmental parameter does not exceed the morphological adaptative capacity of that single population; if environmental changes are strong enough, species replacement takes place offering further adaptation at a higher organization level (Fonseca and de M. Bicudo, 2008). In the investigation of the time succession characteristics of phytoplankton functional groups in Hulun Lake, temperature plays a decisive role in the change of environment. We found that phytoplankton functional groups biomass was dominated by chlorophyta (group X2/J) in spring (May) corresponding to relatively low temperature (16.27C) and high nutrients. Group X2, represented by *Chlamydomonas ovalis* and *Chlamydomonas globosa*. According to the report (Tian et al., 2018), it was mainly dominant during seasons with high concentrations of total photosynthesis and total nitrogen. Because of their small size, rapid reproduction and relatively high surface-volume ratio, they can rapidly absorb nutrients from the surrounding environment for growth and reproduction (Jones, 2000). Flagella also allowed vertical migration between water layers with optimal light conditions and nutrient concentrations, which further enhanced the group's competitiveness in the month when light are not very abundant (Jansson et al., 1996; Tian et al., 2018). In addition, group X2 was also very sensitive to filter feeding. Owing to the content of nitrogen and phosphorus in Hulun Lake exceeds the standard seriously, lead directly to a significant reduction of large filter feeding zooplankton in the lake, which is more suitable for the growth of X2 functional group. As for group J, it was mostly found in shallow, high pH, highly enriched lakes (Sommer et al., 1986; Padisák et al., 2009). The advantage position of two functional groups at the same time indicates that the eutrophication is very serious in the lake, which has become the most direct flashpoint of cyanobacteria bloom. The same situation occurred before the cyanobacteria boom in Xiangxi Bay (Wang et al., 2011) in July, the biomass of functional group J remained high during the investigation. In summer (June/July and August) cyanobacteria (mainly group H1/M) took over the dominant position gradually which also resulting in the cyanobacteria bloom in the lake. *Anabaena circalinalis* and *Microcystis* are the two main culprits for the cyanobacteria boom in Hulun Lake, which belongs to group H1 and M respectively, dominated in summer. Group H1, which was suitable for living in the water environment with low nitrogen content, eutrophication and stratification. Group M, growing in small and medium-sized lakes with rich to super nutrition, low light tolerance and sensitivity to disturbance. As the main functional group causing cyanobacteria bloom, they have been reported in many reservoirs and lakes (Varol, 2019; Yao et al., 2020; Jin et al., 2020). In eutrophic lakes, cyanobacteria tend to dominate in the season with the

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highest temperature due to their optimal growth temperature (Paerl, 1988; Nalewajko and Murphy, 2001; Yang et al., 2009). In the meanwhile, increased WT will decrease the surface water viscosity, which can accelerate the sinking of large immovable species. Owing to cyanobacteria have so good ability to adjust their buoyancy that dominate the communities (O'Neil et al., 2012), they can also reduce the biomass of other phytoplankton species through an allelopathic mechanism (Sarma et al., 2005), which may account for their advantages in freshwater ecosystems. In addition to the appropriate temperature and eutrophic water conditions, light also played a critical role in the cyanobacteria bloom. In the study of the transformation mechanism of *Anabaena* and *Microcystis* (Wang, 2017), we found that compared with *microcystis*, *Anabaena* needed more light energy in the growth process, which is also the vital reason why group M was dominant in August while group H1 was dominant in June and July. Autumn (September and October) caming as the temperature droping rapidly to 10.59℃ on average, chlorophyta (group X2/J) and bacillariophyta (group MP) which adapted to lower temperature occupied a dominant position.

The predominant functional groups demonstrated strong seasonal variations which showed a trend of J/X2(May) → H1/X2(June) →H1/J(July) → M/H1(August) → X2/J(September) → J/X2/MP(October). The whole succession mechanism not only accords with the PEG model of freshwater ecosystem, but also shows the same succession trend with the groups in Dali Lake, which is also a water area with high alkalinity in the cold region (Ma et al., 2019). For the special water body like Hulun Lake, what we concerned about is the distinctive feature. As can be seen, group H1 and M were the main culprits for the cyanobacteria bloom in the lake which had significant difference with groups in tropical areas. Moreover, cyanobacteria bloom in June and July was mainly dominated by functional group H1, while in August, it was dominated by group M. From the perspective of biomass changes, the cyanobacteria bloom in Hulun Lake began in June, reached its peak in July and August, basically disappeared in September. The duration of bloom in Hulun Lake was significantly shorter than that of other tropical and subtropical regions. The unique phytoplankton succession mechanism and characteristics of cyanobacteria bloom also showed that the constantly fluctuating physical and chemical factors in the water played a vital role in regulating the distribution of phytoplankton.

Phytoplankton functional groups and their correlation with environmental factors

Results of previous studies have showed that the spatiotemporal pattern of diversity indices of functional groupings is dependent on the environmental factors (Becker et al., 2010; Weithoff et al., 2015). Understanding how environmental variations affect the biodiversity and succession of phytoplankton is a key challenge (Wang et al., 2020). Generally speaking, the environmental factors that dominate the distribution of phytoplankton functional groups in different regions are quite different. Transparency, salinity and chlorophyll a are considered to be crucial factors affecting the distribution of phytoplankton in tropical areas such as Li'an Bay (Wang et al., 2016a) in Hainan Province. The results of investigation in subtropical areas such as Baihua reservoir (Chen et al., 2018) in Guizhou Province have showed that nutrients are the main factors affecting the distribution of phytoplankton functional groups. Studies in temperate regions such as the Nanshui reservoir in Guangdong Province have found that nutrients, water stability and transparency are the main environmental factors affecting the dynamic changes of phytoplankton functional groups (Huang et al., 2014). However, it is obviously different from other areas in the cold region with notable water temperature change. The main

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environmental factor affecting the distribution of phytoplankton functional groups in the cold region is water temperature (Cao et al., 2019).

In the RDA correlation analysis of phytoplankton functional groups and water environmental factors in Hulun Lake, it can be seen that water temperature (WT) has the highest correlation, which is consistent with the research results of other cold water areas (Lin et al., 2017; Yu et al., 2008; Wu et al., 2015). Temperature can directly affect the growth of phytoplankton by controlling the intensity of enzymatic reaction or respiration of photosynthesis, by controlling the solubility, dissociation degree or decomposition rate of various nutrients in water affects the growth of phytoplankton indirectly (Song and Yu, 2009; Peng et al., 2009; Wu et al., 2010). Group F, J and MP were negatively correlated with temperature, while group H1 and M just on the contrary. Therefore, in spring and autumn with lower temperature, groups J and MP are more likely to gain dominant position in resource competition, while group H1 and M take advantage of the high temperature environment in summer to be dominant species in water area. Once their biomass starts to increase rapidly with the rise of water temperature, a large area of cyanobacteria bloom begins to form, which will not only make the dissolved oxygen content in water drop rapidly, but also have many negative effects on the water quality (Elliott et al., 2006; Edwards et al., 2016).

Dissolved oxygen (DO) is also a pivotal factor affecting the distribution of phytoplankton, which plays a vital role in maintaining the ecological security of the water body. Phytoplankton are the suppliers of dissolved oxygen in water, but if phytoplankton increase excessively, it will also become the booster of dissolved oxygen reduction (Zhao et al., 2011). The formation of cyanobacteria bloom in summer by functional groups M and H1 is a distinct proof of this view. Therefore, there is a significant negative correlation between group M, H1 and dissolved oxygen in RDA analysis diagram, which is consistent with the conclusion on Dianshan Lake (Yang et al., 2018). Moreover, the higher the dissolved oxygen content, the better the water quality. In Pearson correlation analysis, it can be seen that there is a significant positive correlation between dissolved oxygen and functional group F, which is represented by *stichcoccus bacillaris* and *oocystis elliptica* of Chlorophyta, suitable for growing in even and clear deep-water lakes and is very sensitive to high turbidity. It also shows that dissolved oxygen plays a significant role in phytoplankton.

The effect of nutrients on phytoplankton functional groups in eutrophic waters has been received considerable attention. For Hulun Lake, in recent years, due to the impact of climate and human activities, coupled with a large number of surface runoff and hay into the lake, the content of total nitrogen and total phosphorus in the lake has been increasing. With the continuous increase of evaporation and decline of water level, water in lake cannot be circulated effectively, the nutrients, salts and organic pollutants in the lake have been concentrated continuously. Up to now, the water quality of Hulun Lake has been polluted by different degrees of eutrophication. RDA analysis showed that TN and TP were one of the most significant factors affecting the distribution of phytoplankton functional groups in Hulun Lake which is the same as that of other eutrophic cyanobacteria bloom area (Wang et al., 2016b, 2018; Xia et al., 2019). However, there was no significant correlation between TN/TP and H1, the main functional group causing the cyanobacteria bloom in Hulun Lake. On the one hand, the correlation between group H1 and nutrients is closely related to the degree of cyanobacteria bloom in the lake. Generally speaking, the high content of nutrients in water is a vital factor to induce cyanobacteria bloom. Phosphorus content often appears as an important limiting factor.

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The measuration of total phosphorus includes dissolved phosphorus and granular phosphorus. In the process of digesting, the granular phosphorus contained in cyanobacteria itself will be determined as part of the total phosphorus content. When the cyanobacteria bloom is severe, the proportion of particulate phosphorus in the total phosphorus content of cyanobacteria is very high. Therefore, when monitoring Hulun Lake in 2015, due to the serious outbreak of cyanobacteria, there is a significant positive correlation between nutrients and group H1. However, the bloom in 2018 is more slight compared with 2015, so the proportion of cyanobacteria to particulate phosphorus is relatively low in the total phosphorus content, there is no significant positive correlation. On the other hand, this result is consistent with Liebig Law of Minimum, which states that any specific factor below the minimum required by a certain organism is the fundamental factor determining the survival and distribution of that organism. The results showed that when the concentrations of total phosphorus and total nitrogen reached 0.03 mg/L and 0.60 mg/L respectively, there is a trend of cyanobacteria bloom in the lake. *Anabaena* can fix nitrogen in the air, so the threshold of total nitrogen is much lower. The average value of TN and TP in Hulun Lake during the whole investigation period (TN: 2.05; TP: 0.21) is far beyond this threshold, which provides sufficient nutrients for the growth of phytoplankton, especially cyanobacteria. Therefore, TN and TP are not the main limiting factors for the outbreak of cyanobacteria in Hulun Lake.

Chemical oxygen demand (CODcr) can measure the content of organic matter in water scientifically, reflect the degree of water pollution by reducing substances directly. The water area of Hulun Lake is not only polluted by external organic pollutants, but also polluted by the release of organic substances in the sediment. Some studies have shown that diatoms are more suitable to grow in the environment with higher chemical oxygen demand than other phytoplankton. Pearson correlation analysis in this study indicates that CODcr is positively correlated with group MP which is consistent with the conclusion of Chai (Chai et al., 2020).

Transparency (SD) is a major indicator of water cleanliness, which can reflect the state of water intuitively. Hulun Lake not only has the emergence of cyanobacteria bloom, but also due to the lack of large aquatic plants on the lake, is unable to play a role in purifying water quality, leading to low transparency in the lake as a whole. The results of Pearson correlation analysis showed that there was a significant negative correlation between the transparency and the biomass of W1 functional group, which was also consistent with the characteristics of W1 suitable for growing in sewage. Moreover, for the water area with high alkalinity, calcium ion will be consumed and precipitated, resulting in the turbidity of the water in Hulun Lake. This is also the reason why transparency and pH are significantly negatively correlated, which is the same as the monitoring result of Hulun Lake in 2017 (Pan et al., 2017).

Q index

Q index was originally developed to assess ecological status of different water types without geographic limitations (Padisák et al., 2009). So far, many scholars have successfully used Q index to evaluate the ecological status in different regions. For instance, Crossetti and de M. Bicudo (2008) have successfully applied this index in an urban tropical reservoir, Becker et al. (2009) have used Q index in a subtropical water-supply reservoir. It can also be used in assessing the water quality of lake (Pasztaleniec and Poniewozik, 2010). In this study, through the calculation of Q index by using

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phytoplankton functional group, we have successfully indicated the water quality of Hulun Lake from the perspective of time and space.

From the temporal aspect, the ecological status was correspondent with the cyanobacteria bloom process. During the whole investigation period, the ecological status in the lake took on a declining and followed improving trend, which was the same as that in the process of cyanobacteria bloom in Xiangxi River (Wang et al., 2011). From June, the biomass of H1 functional group reached the highest value, the lake began to show a trend of cyanobacteria bloom, Q index fell to "tolerable" level with the worst water quality in the whole investigation. In July and August, with the gradual increase of temperature, a large area of cyanobacteria began to break out in the lake under the joint efforts of group H1 and M, the water quality was also worrying. The best water quality occured in October, the biomass of MP functional group increased due to the decreased of temperature, water quality states rose once more to a "medium" ecological level. As to the spatial pattern, there were obvious differences between ten sites in Hulun Lake. On the whole, the worst average water quality points appeared in site $9^{\#}$ and $5^{\#}$. In August, site $2^{\#}$ and $10^{\#}$ were the sampling points with the lowest Q index value, which was also having the worst water quality. All of these could be related to the cyanobacteria bloom in the lake and the large increase of biomass of H1 and M functional groups, which had been reported in the research of Silva and da Costa (2015).

It is concluded that this method of using Q index to assess the ecological status in a eutrophic seriously, with cyanobacteria blooming lake is available. It has been showed a high sensitivity to changes in species composition and functional groups. Revealing itself as a suitable tool for monitoring the water quality in the studied area. However, it remains to be further studied whether the other indicators for evaluating water quality and eutrophication are the same as those indicated by Q index.

Conclusion

This study shows that the monitoring method of phytoplankton functional group is also applicable in the cold area with high alkalinity and eutrophication, and it is also a favorable method to monitor the phenomenon of cyanobacteria bloom in the lake. The Q index emerged as the times require can reflect the water quality in the lake accurately. During the study period, the time succession of phytoplankton functional groups was obvious which showed the variation trend of J+X2 (May)→H1+X2 (June)→H1+J (July)→M+H1 (August)→X2+J (September)→J+X2+MP (October). The cyanobacteria bloom in lake began in June, reached its peak in July and August, basically disappeared in September. The main phytoplankton functional groups causing cyanobacteria bloom in the lake were H1 and M. It also turned out that water temperature, dissolved oxygen, total phosphorus and total nitrogen were the most significant environmental factors affecting the distribution of phytoplankton functional groups in Hulun Lake studied by using RDA and Pearson correlation analysis. Meanwhile, the persistently high content of nitrogen and phosphorus in Hulun Lake provides rich nutrients for phytoplankton. Therefore, total nitrogen and total phosphorus are not the main limiting factors of cyanobacteria bloom in Hulun Lake.

Our research has provided a new idea for the study of cyanobacteria bloom and water quality monitoring in cold regions. However, how to eliminate and control severe cyanobacteria bloom is the most significant thing we need to pay attention to currently. Our team has also been monitoring the population dynamics of zooplankton in this area,

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particularly the large filter-feeding zooplankton, with a view to long-term monitoring to find a new approach of controlling cyanobacteria bloom by means of plankton. Further studies should establish a more intuitive connection between phytoplankton especially Cyanobacteria, water environmental factors and zooplankton bases on data collected over a longer period with more frequent sampling and analysis in different regions, to evaluate the impact of top-down effects on phytoplankton and cyanobacteria bloom. Further analyze, solve and prevent the phenomenon of cyanobacteria bloom.

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EFFECTS OF GENIPOSIDE ON THE REGULATION MECHANISMS OF PHOTOSYNTHETIC PHYSIOLOGY, ROOT OSMOSIS AND HORMONE LEVELS IN MAIZE UNDER SALINE-ALKALI STRESS

LIU, Z. Y.[1#] – XU, W. H.[2#] – GU, W. R.[1*] – LI, J.[1] – LI, C. F.[3] – WANG, M. Q.[4] – ZHANG, L. G.[4] – PIAO, L.[4] – ZHU, Q. R.[5] – WEI, S.[1]

[1]*College of Agronomy, Northeast Agricultural University, Changjiang Road 600, 150030 Harbin, Heilongjiang, China*

[2]*CAS Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, 110016 Shenyang, China*

[3]*Institute of Crop Science, Chinese Academy of Agricultural Sciences, No.12 Zhongguancun South Street, Haidian District, 100081 Beijing, China*

[4]*Maize Research Institute, Heilongjiang Academy of Agricultural Sciences, No.11 Scientific Research Street, 150086 Harbin, Heilongjiang, China*

[5]*Guangxi Shan Yun Biochemical Science and Technology Co., Ltd, Luzhai County Industrial Park, 545600 Liuzhou, Guangxi, China*

[#]*Liu, Z. H. and Xu, W. H. are co-first authors.*

[*]*Corresponding author*
e-mail: wanronggu@163.com

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Abstract. The aim of the study was to monitor the effects of exogenous geniposide (GD) on maize (*Zea mays* L.) and explore the mechanisms through which it mitigates saline-alkali stress to provide a theoretical basis for revealing the growth physiology and chemical regulation processes in maize. The control (CK) received nutrient solution culture while the group under saline-alkali stress(SAS) received 150mmol L^{-1} saline-alkali solution (molar concentration ratio NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3=1:9:9:1). 'Jilong2' (saline-alkali tolerant) and 'Xinxuan58' (saline-alkali sensitive) were chosen as experimental materials and were cultivated with hydroponic method. Maize received the saline-alkali solution 12 hours after GD treatment and saline-alkali solution was administered 3 times, every 12 hours. The GD and saline-alkali solution group constituted the S+G treatment which promoted maize and a significant increase was observed in fresh and dry weights, plant height and photosynthetic enzymes in the leaf along with a significant decrease in the root to shoot ratio compared with saline-alkali treatment. The indoleacetic acid (IAA), zeatin-riboside (ZR), gibberellin (GA), superoxide radical ($O_2^{\cdot-}$) generation rate, malonaldehy (MDA) and hydrogen peroxide (H_2O_2) levels in roots were significantly lower than in the S+G treatment under saline-alkali stress, while the level of abscisic acid (ABA) and the activity of antioxidative enzymes increased.
Keywords: *maize seedlings, photosynthetic enzyme, osmotic substance, antioxidant enzyme, hormone*

Introduction

Enrichment of soil soluble saline-alkali can change soil physical and chemical properties, even brings about land salinization. The salinization and alkalization in soil often occurs together in natural conditions. High concentrations of salts and alkali causes injury to plants not only with ionic, osmotic and oxidative stresses to plants, but also through high

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pH leading to a block in the transportation of substances required for nutrition (Mukhtar et al., 2019). Salinization is a severe trouble that threatens agricultural development worldwide, the area of saline-alkali soil is increasing year by year, most of which are not exploited and utilized (Zhao et al., 2020). The total area of saline-alkaline soils is 9.5×10^8 ha^2 worldwide. Especially in China, saline-alkaline soils were widely distributed in several provinces of Northwest China, Northeast China, North China and Central China. Saline-alkaline land with agricultural development potentially occupies over 10% of the total cultivated area of China. Songnen Plain is one of the most widely distributed area in saline-alkali soils all over the world and it is located in the northeast China. The saline-alkali area is estimated at 3.73 million hectares and was still increasing at a speed of 1.4% per year (Litalien et al., 2020).

Maize is considered to be particularly sensitive to saline-alkali stress during seedling stage. Therefore, revealing the adaptive and protective mechanisms of maize to saline-alkali stress is of great theoretical significance and practical value. Assimilation of inorganic carbon by photosynthesis is the main source of CO_2 organic matter in plant assimilation, plant growth and biomass accumulation (Huang et al., 2019). The primary pathway of photosynthetic carbon fixation in higher plants contains three kinds that is C_3 pathway, C_4 pathway and Crassulacean acid metabolism (CAM) pathway. C_4 pathway is the physiological basis of C_4 crops such as maize of high photosynthetic efficiency, biomass potential and crop yield formation under saline-alkali stress (Hughes et al., 2019).

The main physiological function of PEPCase in C_4 plant photosynthesis is to use PEP as substrate to fix CO_2 and produce oxaloacetate, and oxaloacetate(OAA) is catalysed to malic acid by MADP-ME. Malate is metabolized by NADP-MDH to produce CO_2 and pyruvate, PEP use pyruvate as a substrate to be produced by PPDK catalysis and returned to the C_4 cycle. Studies have shown that plants convert carbon dioxide via C_4-pathway and Calvin cycle immobilization under the influence of the activity of related metabolic photosynthetic enzymes, such as phosphoenolpyruvate carboxylase (PEPCase), pyruvate dikinase (PPDK), NADP-malatease (NADP-ME), malate dehydrogenase (NADP-MDH) and ribone glycol diphosphate carboxylase (RuBPCase) (Lei et al., 2017). This indicated that saline-alkali stress slowed down the CO_2 assimilation rate in crops, resulting in decreased enzyme activity, which further disrupted the balance of consumption and accumulation of metabolites in the C_4 pathway and Calvin cycle, and reduces the content of assimilates transported to roots, which affect root growth (Zhao et al., 2019). The production of reactive oxygen species is affected by saline-alkali stress, which affect hormone metabolism and distribution, and destroys hormone balance. There are synergistic or antagonistic effects between endogenous hormones. Hormones can be used as an endogenous signal in response to abiotic stress. Plant hormones can be signals involved in physiological functions and metabolism. IAA play a major role in root growth of maize seedlings, GA induces cell elongation, ZR is an important form of CTK transport in xylem, and ABA is a growth inhibitor to slow seedling growth under saline-alkali stress. The production of reactive oxygen species (ROS) was led by salt-alkali stress, which in turn affects hormone metabolism and distribution, and destroys hormone balance (Li et al., 2017). The accumulation of soluble sugar and proline can effectively maintain the normal metabolism of plant roots because of reducing the osmotic potential of plant cells, eliminating free radicals and ROS, thus relieving oxidative stress (Amira et al., 2020). At present, some progress has been made in the application of physiological control measures of exogenous substances in agricultural production, such as amendments (Wang et al., 2016), trehalose (Rohman et al., 2019), abscisic acid (Lu et al., 2019), humic acid (Kaya et al., 2018) and

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salicylic acid (Taha et al., 2019), which can effectively alleviate the injury of crops under saline-alkali stress. GD is a class of cyclic ether terpenoids (Iridoids) that exhibit a wide variety of biological activities and are widely distributed in the plant kingdom. GD content in gardenia is about 5%. As a natural extract, GD is non-toxic and has high biological activity, soluble in water, safe and convenient to use, mainly includes Gentiopicroside, Vanilloylcatalpol and geniposide. GD has wide-ranging beneficial applications in the avoidance of neuroprotection and anti-diabetes as well as GD has the effect of protecting the liver and the gallbladder (Wu et al., 2019). In agricultural production, GD also plays an important role in the growth of crops, and the compound product-enhancing agent prepared by GD can effectively increase the yield of cucumber, cowpea, wheat and cotton (Zhang et al., 1998a,b; Zhang et al., 1999a,b). It can also promote the growth and development of maize seedlings, improve the chlorophyll content and chlorophyll light energy conversion efficiency in the leaves of green vegetables, promote the accumulation of soluble sugar and soluble protein, and enhance the photosynthetic ability of plant leaves. GD could promote root growth, thus increasing plant biomass under saline-alkali stress.

At present, effects of GD on growth and development of maize seedlings under saline-alkali stress have not been reported. Effects of exogenous GD on growth morphology, seedling biomass, leaf photosynthetic enzyme activity, root osmotic regulation, antioxidant system and endogenous hormones under saline-alkali stress of different saline-alkali tolerant maize seedlings were investigated.

In this study, the effects of exogenous GD on growth morphology, seedling biomass, leaf photozyme activity, root osmotic regulatory substance content, antioxidant system and endogenous hormones of saline-alkali resistant maize seedlings under saline-alkali stress were investigated with two varieties as test materials, and it provides theoretical and experimental basis for the application of exogenous GD in maize field stress tolerance production.

Materials and methods

Study design and experimental procedures

In the present study, we choose maize (*Zea mays* L.) Jilong2 and Xinxuan58 cultures to be transplanted into nutrient solution after seedling stage. The maize cultures were provided by Heilongjiang jiulong seed industry co., LTD and Shenyang xinxuan agricultural science and technology co., Ltd. The concentrations and treatment duration of the agents used in this study were determined after preliminary experiments. GD was provided by Guangxi Shan Yun Biochemistry Technology Co., Ltd, the treatment concentration was 5 mg L^{-1}, and its molecular weight was 388.37, and the purity was > 98%. The saline-alkali solution was prepared, in which the major constituents were NaCl: Na_2SO_4: $NaHCO_3$: Na_2CO_3 with a molar ratio of 1:9:9:9:1. Light absorbance was measured by a UV-visible spectrophotometer (UV-5500, manufactured by Shanghai Metash Insruments Co., Ltd.).

A hydroponic experiment was performed under different treatment conditions in the maize cultivation physiology laboratory of the Agricultural College of Northeast Agricultural University in June 2019. Maize seeds were surface-sterilized by rinsing them for 2 min in a 10% NaClO solution, then the seeds were rinsed with distilled water for several times, and soaked in distilled water for 8 h. Germination tests were carried out in an incubator at 25±1℃ with 75% humidity. The uniformly germinated seeds were selected after 48 h incubation and cultivated in a test pot (60 cm in length, 30 cm in width, 12 cm in high) containing 150 L 1/2 Hoagland nutrient solution (pH 6.0±0.1) with 25 seedlings in

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each pot with nutrient solution supply (CK), saline-alkali solution supply (SAS), saline-alkali solution and GD supply (S+G) or GD solution supply (GD). The cultures were maintained in standard conditions of day room temperature (28±1°C), night room temperature (25±1°C) with 65%-75% humidity, on a 12-h light and 12-h dark cycle with a light intensity of 4000 lx. The nutrient solution was changed in every 3 days, and water was supplied with oxygen by air pump every day. When the plants had 3 true leaves, the seedlings were divided into four groups before further treatment. Fifty seedlings were used for each set of experiments. The seedlings were randomly assigned to one of the four groups: control group (CK), saline-alkali group (SAS), GD and saline-alkali group (S+G) or GD group (GD). The CK treatment received 1/2 Hoagland nutrient solution. The SAS treatment received 1/2 Hoagland nutrient solution and 150 mmol L^{-1} saline-alkali solution. The S+G treatment received 1/2 Hoagland nutrient solution, 150 mmol L^{-1} saline-alkali solution and 5 mg L^{-1} GD. The GD treatment received 1/2 Hoagland nutrient solution and 5 mg L^{-1} GD. To avoid the impact of saline-alkali stress on maize seedlings, saline-alkali solution was added to the maize 12 hours after GD treatment and saline-alkali solution was administered 3 times, every 12 hours. Saline-alkali solution reaching 150 mmol L^{-1} marked Day 0. Seedlings were sampled at 4th d of germination for biochemical and physiological measurements. All experiments were repeated a minimum of 3 times.

Agronomic characters of maize seedlings

Three plants of each treatment were harvested on the 4th day. Plants were rinsed with distilled water and, after removing the excess water with filter paper the fresh and dry weight of the seedlings was measured by a balance. After the plant samples were heated for 30 min at 105°C to halt metabolism, and dried at 80°C to constant weight for assays of dry weight and the average value was taken as the final result, the fresh and dry weight has units of g $3plant^{-1}$ (take the total weight of three seedlings as the unit). Height was measured from the base of the seedlings to its highest point and the average value was taken as the final result, the plant height has units of cm. Root dry weight was divided by stem dry weight to calculate the root-shoot ratio (R/S).

Measuring photosynthetic activity of maize leaves

Photosynthesis was measured during the study at day 4. The third true leaves from three plants of maize seedlings were cut for measurements. The enzyme extraction and activity of PEPCase and RuBPCase was performed according to a modified method of Sayre (1979). The concentration of the standard substance and the OD value at 490 nm of each sample were determined sequentially on the enzyme-linked immunoassay spectrophotometer. RuBPCase determination: 0.1 ml of the enzyme solution was mixed with 0.3 ml 0.1 mol L^{-1} Tris-HCl (pH 8.0), 0.3 ml 0.1 mol L^{-1} $MgCl_2$, 0.3 ml 50 mmol L^{-1} adenine nucleoside triphosphate (ATP), 0.3 ml 50 mmol L^{-1} dithiothreitolol (DTT), 0.3 ml 2.0 mmol L^{-1} NADH, 0.3 ml 1.0 mmol L^{-1} EDTA-Na, 0.1 ml 200 μmol L^{-1} $NaHCO_3$, 1.0 ml distilled water and 0.1 ml 3-phosphoglycerate kinase (PGK)/ 3-glyceraldehyde phosphate dehydrogenase (GAP-DH) (15 u/15 u) buffer preheated in 30 constant temperature water bath for 10 min. The absorbance E_0 of the samples was measured at a wavelength of 340 nm with distilled water as a reference wavelength. Then 0.1 ml 9 mmol L^{-1} 1,5-ribose diphosphate (RuBP) was added to initiate the reaction, the changes in light absorption was determined in 15 s intervals. PEPCase determination: the enzyme solution 1 ml was mixed with 1 ml 0.1 mol L^{-1} H_2SO_4 (pH 9.2), 0.1 ml 10 mmol L^{-1} $MgCl_2$, 0.1 ml 10 mmol L^{-1} $NaHCO_3$, 0.2 ml 40 mmol L^{-1} phosphoenolpyruvate (PEP), 0.3 ml 1 mg ml^{-1} NADH, 0.2 ml malic dehydrogenase (MDH)

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then pre-heated by water bath at 28℃ for 10 min, the reaction was initiated with 0.2 ml PEPC extract, and the decrease of sample absorbance was measured rapidly at 340 nm. To determine the activity of NADP-MDH, NDAP-ME and PPDK a kit was used according to the operating procedures of the instructions. At least three replicates for each assay were performed. The average of test result was calculated and analyzed. Experiments were repeated thrice and three repeats were performed each time.

Measuring endogenous hormone content of maize roots

Endogenous hormone content was measured during the study at day 4. The contents of IAA, ZR, GA and ABA in maize root were analyzed using an enzyme-linked immunosorbent assay. 0.5 g tissue samples (maize leaves) were ground in 2 ml sample extract under ice bath to form homogenate, and samples were transferred into the test tube and extracted under 4℃ for 4 h, then centrifuged at 3500 r/min for 8 min at 4℃, and the supernatants were collected into a 10 ml centrifuge tube. 1 ml of extract was added in the precipitate and laid still at 4℃ for 1 h, then centrifuge, the supernatant was taken merge with the previous supernatant and record the volume, the residue is discarded. Use a C-18 solid phase extraction column to purity the supernatant. After passing through the column, the sample was transferred into a 5 ml plastic centrifuge tube. The methanol in the extract was removed by vacuum, concentrated and dried or blown dry with nitrogen and the volume was fixed with the sample diluent. After extracting the hormone, the maximum concentration of the ABA is 500 ng ml^{-1}, IAA and ZR is 100 ng ml^{-1}, GA is 10 ng ml^{-1}, and then diluted 2 times ,4 times ,8 times ,16 times ,32 times ,64 times until there are 8 concentrations (including 0 ng/ml). The series of standard samples were added to the first two rows of a 96-well enzyme standard plate, add samples to be tested to the remaining holes, and add the same sample every two holes, 50 μl per hole. A certain amount of anti-mixing was added to the 5 ml sample diluent, then the enzyme label plate was put into the wet box to start the competition. The competitive conditions were 37℃ and 0.5 h. Washing boards: get rid of the reaction fluid on the board and pat it clean on paper. Get rid of it immediately after adding washing liquid to the board, repeat four times. Appropriate enzymatic II antibodies were added in 10 ml sample diluents, each hole was100 μl, then the enzyme labeling board was incubated in wet box at 37℃ for 0.5 h. Wash again. 10 mg o-phenylenediamine (OPD) was completely dissolved in 10 ml substrate buffer and mixed with 4 μl 30% H_2O, each hole was 100 μl, and the enzyme label plate was placed into the wet box until appropriate color development, then 50 μl 2 mol L^{-1} sulfuric acid was added to each hole to terminate the reaction. The concentration of the standard substance and the OD value at 490 nm of each sample were determined sequentially on the enzyme-linked immunoassay spectrophotometer. Experiments were repeated thrice and three repeats were performed each time.

Osmotic content in maize roots measurement

Osmotic content was measured during the study at day 4. The anthrone colorimetric method was used to determine the soluble sugar content in maize roots. The standard curve was established using sucrose. The 0.3 g root was cut into pieces and put into the scale test tube together with 5 ml distilled water and sealed with plastic film. Samples were extracted in boiling water for 30 min the extraction was performed two times. The extract was filtered into 25 ml volumetric flasks, the test tube was washed with distilled water, and the residues were transferred into volumetric flasks and diluted to 25 ml. 0.5 ml extract was transferred by 1.5 ml of distilled water to a 20 ml scale tube, 0.5 mL anthrone and ethyl acetate were

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added into the mixture. The samples were placed in a boiling water bath for 1 min then cooled to room temperature. The absorbance of the samples was measured at a wavelength of 630 nm with blank space as a reference wavelength. Soluble sugar content was calculated with the following formula:

$$\text{Soluble sugar content [mg g-1]} = (2500 \times C)/0.3 \text{ g},$$

where C is soluble sugar content of determination solution on standard curve. Soluble sugar content. Proline content in maize root was determined according to ninhydrin coloring method. Before sample measurement, standard curves were made with different concentrations of 2 ml standard solutions of proline. 0.5 g of fresh leaves and 5 ml 3% sulfosalicylic acid were added to the test tube and then transferred to boiling water for 10 min. After cooling, the extract was filtered. After that, 2 ml filtrated extraction mixed with a 2 ml ninhydrin reagent and 2 ml glacial acetic acid. The mixture was heated in a boiling water bath for 30 min. 4 ml of toluene was added after cooling and shaken for 30 s and the supernatants were collected into a 10 ml centrifuge tube. The supernatant was poured into a colorimetric cuvette and water was used as blank control. The absorbance values were read at 520 nm. Proline content was calculated with the following formula:

$$\text{Proline content [μg g-1]} = 2500 \times C,$$

where C is 2 ml proline content of determination solution on standard curve.

Determination of superoxide anion production rate and hydrogen peroxide content in maize root system and antioxidant enzyme system

Photosynthesis was measured during the study at day 4. O_2^- generation rate was measured using a hydroxylamine oxidization method. For determining O_2^- generation rate, 5 g tissue samples (maize root-tip) were ground in 6 ml of 65 mmol L^{-1} phosphate buffer solution (PBS) (pH 7.8) into a homogenate, after filtration through four layers of gauze, centrifuged at 5000 r/min for 10 min at 4°C. Then, 1 ml (about 0.5 mg protein) supernatant was mixed with 0.9 ml PBS and 0.1 ml 10 mmol L^{-1} hydroxylamine chloride for reaction for 20 min at 25°C. Supernatant (0.5 mL) was mixed with 0.5 ml 7 mmol L^{-1} p-aminophenylsulfonic acid and 0.5 ml 7 mmol L^{-1} α-naphthylamine, and incubated for 20 min at 25℃. After the reaction, the same volume of n-butanol was added and shaken vigorously, and laid still for layer separation. The intensity of the chromogenic agent in the butanol layer was determined at 530 nm and sample solution was replaced with phosphate buffer. Both supernatants were combined and centrifuged for 5 min at 1500 × g and absorbance was measured at 530 nm.

The H_2O_2 content was determined spectrophotometrically. First, 2 g of fresh sample was weighed, precooled at 4°C, acetone with quartz sand was added at a ratio of 1:1, and the homogenate was ground. Then, it was centrifuged at 3000 rpm for 10 min and the supernatant (V_t) was taken. Finally, 5% titanium sulfate and ammonia water were added to the supernatant. When the solution became turbid, it was centrifuged again 5000 rpm for 10 min. After discarding the clear supernatant, the precipitates were washed with acetone to remove plant pigments. Subsequently, 5 mL of concentrated sulfuric acid was added to the precipitates. When the precipitate dissolved, the sample volume was made up to 10 ml, the absorbance was measured at 415 nm. H_2O_2 content was calculated with the following formula:

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$$H_2O_2 \text{ content } [nmol^{-1} FW] = (C \times V_t)/(2\ g \times 1\ ml),$$

where C is the H_2O_2 content from the standard curve, V_t: total volume of extracting enzyme solution.

The crude enzyme extraction for SOD (Superoxide dismutase), POD (Peroxidase), CAT (catalase), MDA was similar: 4 ml 0.1 mol L^{-1} PBS (pH 7) was added to 2 g of each sample to crush in precooled mortar and pestle. The final volume was made up to 200 ml. The 1.5 ml homogenate was centrifuged at 10,000 rmp at 4℃ for 15 min, and the crude enzyme extract was the supernatant. SOD activity was assayed by measuring the ability of inhibit photochemical reduction of NBT. 0.6 ml 50 mmol L^{-1} PBS, 0.12 ml 130 mmol L^{-1} Met, 0.12 ml 750 μmol L^{-1} NBT, 0.12 ml 100 μmol L^{-1} EDTA-Na_2, 0.12 ml 20 μmol L^{-1} riboflavin, 0.02 mL of an enzyme solution (sections applied with PBS instead of enzyme solution were studied as the control) were mixed with 0.1 mL of distilled water in a 10 mL centrifuge tube and mixed gently. One control centrifuge tube was placed in the dark and the other samples reacted at 25℃ under 4000 lx sunlight for 20 min (each tube was light consistent). One unit of SOD activity was defined as the quantity of enzyme required for 50% inhibition of NBT reduction at 560 nm. POD activity was estimated using the guaiacol method. The reaction mixture contained 0.05 ml of enzyme extract, 2.2 ml PBS, 0.15 ml of 1% H_2O_2 and 0.6 mL 1% guaiacol solution and were warmed for 5 min at 37℃ in a water bath. Reaction buffer without crude enzyme solution was used as the control. The change in absorbance at 470 nm was measured over a period of 3 min at 1 min intervals. We determined enzyme activity by measuring the change in absorbance per minute, and one unit (U) of enzyme activity was defined as the amount of the enzyme that changes the absorbance by 0.01 per minute. CAT activity was assayed using hydrogen peroxide as substrate. A 0.1 ml enzyme sample and 2.865 mL PBS were added to a test tube, mixed, and placed in a boiling water bath for 10 min. All the reaction mixture was incubated at 25°C for 5 min in the water bath. Then 0.035 ml 3% H_2O_2 was added and mixed well, the tube was quickly poured into the colorimetric cup and start to calculate the time. The colorimetric reaction was read at 450 nm, each record lasted 3 min and was set at 1 min intervals, and enzyme volume of 0.01 absorbance per minute was regarded as one unit (U) of enzyme activity. The content of MDA was determined by thiobarbituric acid (TBA) reaction chronometry. Two milliliter of the enzyme solution was mixed with 2 ml of 0.6% TBA, and the mixture was placed in a boiling water bath for 10 min. The absorbance of the resulting supernatant was measured respectively at the wavelength of 532, 600, and 450 nm after cooling and centrifuge.

For SOD activity determination the absorbance values of the control tube and the measuring tube were measured by a spectrophotometer at 560 nm to be A0 and A, respectively. Percentage of inhibition was calculated as $P\ [\%] = (A0 - A)/A0 \times 100$, and SOD activity as $[U\ g^{-1}] = 114 \times P/(1 - P)$. For POD activity determination the absorbance value A1 at 1 min and the absorbance value A2 at 2 min at 470 nm were measured with a spectrophotometer. POD activity was calculated as $[U\ g^{-1}] = 20{,}000\ \Delta A$, where $\Delta A = A2 - A1$. CAT activity was calculated as $[U\ g^{-1}] = \Delta 240 \times V/Va/W$. For MDA content determination the absorbance value D_{600} at 600 nm, value D_{532} at 532 nm and value D_{450} at 450 nm were measured with a spectrophotometer. MDA content was calculated as (nmol g^{-1} FW) = $(6.45 \times (D_{532} - D_{600}) - 0.56 D_{450}) \times 0.015/W$. Experiments were repeated thrice and three repeats were performed each time.

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Data calculation and analysis

The experimental data were statistically analyzed by Excel2010 software, variance analysis by SPSS17.0 software, and difference significance analysis by LSD method (Wu et al., 2014).

Results

Effect of GD on maize growth phenotype under saline-alkali stress

As shown in *Table 1*, a significant growth inhibition in maize seedlings has been caused by saline-alkali stress. Compared with the seedlings under the CK treatment, the seedlings under the SAS treatment had short plants and significantly decreased dry and fresh weight and root-shoot ratio increased. And compared with the seedlings under the SAS treatment, the seedlings under the S+G treatment had long plants and significantly increased dry and fresh weight and root-shoot ratio decreased. This shows that exogenous GD had a better alleviating effect on dry-fresh weight and height of maize seedlings. Especially the GD treatment can have significant promoting effect on the shoot and the change of Xinxuan58 greater than that of Jilong2. It was observed that GD treatment greatly promoted dry-fresh weight, plants height and the growth of both maize cultivars compared with the CK. Seedling growth of Jilong2 and Xinxuan58 was significantly inhibited by saline-alkali stress (*Fig. 1*). Compared with the seedlings under the CK treatment, the seedlings under the SAS treatment had smaller leaves and roots, significantly decreased height and seedlings under the SAS treatment were wilting and some mature leaves showed chlorosis, the fibrous roots reduced, and seedlings growth was relatively inhibited. In both maize cultivars leaves of ecotype yellowed, and the area of yellowing in Jilong2 was larger than that in the Xinxuan58. The S+G treatment delayed leaf wilting in maize seedlings subjected to saline-alkali and fibrous roots increased. GD can effectively promote seedling growth, increase leaf width, promote root whitening, and increase fibrous roots.

Table 1. *Effects of geniposide on the dry and fresh weight, plant height and root-shoot ratio of maize under saline-alkali stress on the fourth days*

Variety	Treatment	Fresh weight (g 3^{-1} plant)		Dry weight (g 3^{-1} plant)		Plant height (cm)	Root-shoot ratio
		above ground	underground	above ground	underground		
Jilong2	CK	4.81±0.37^{b}	2.59±0.13^{b}	0.31±0.074^{ab}	0.22±0.039^{b}	52.40±2.48^{b}	0.54±0.04^{b}
	SAS	1.63±0.18^{d}	1.95±0.06^{c}	0.20±0.059^{b}	0.21±0.054^{b}	41.90±1.93^{d}	1.21±0.17^{a}
	S+G	2.44±0.15^{c}	2.55±0.13^{b}	0.34±0.058^{a}	0.37±0.032^{b}	48.40±1.40^{c}	1.04±0.02^{b}
	GD	5.89±0.19^{a}	2.95±0.12^{a}	0.37±0.036^{a}	0.22±0.042^{a}	71.60±1.70^{a}	0.50±0.04^{b}
Xinxuan 58	CK	2.70±0.17^{b}	1.89±0.12^{b}	0.18±0.060^{c}	0.41±0.061^{a}	51.80±1.69^{a}	0.70±0.08^{b}
	SAS	0.66±0.13^{c}	1.50±0.13^{b}	0.11±0.038^{c}	0.31±0.042^{a}	43.50±1.94^{b}	2.19±0.48^{a}
	S+D	2.59±0.25^{b}	2.34±0.31^{a}	0.28±0.066^{b}	0.20±0.044^{b}	54.50±2.12^{a}	0.92±0.20^{b}
	GD	4.96±0.12^{a}	2.64±0.21^{a}	0.39±0.020^{a}	0.21±0.056^{b}	54.40±1.91^{a}	0.53±0.04^{b}

Different letters within the same variety and column indicate significant difference at 0.05 level. The same as below

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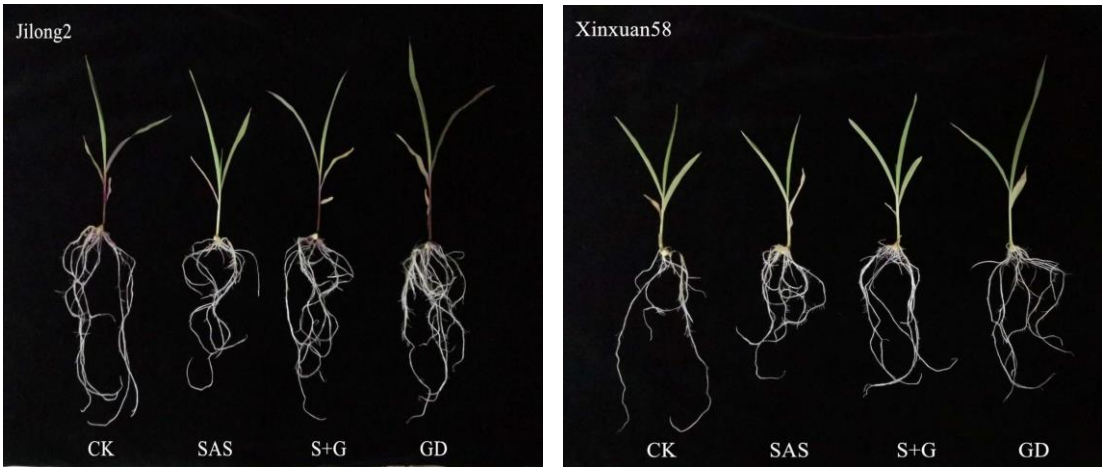

Figure 1. *Effect of GD on growth morphology of maize under saline-alkali stress on the fourth day*

Effect of GD on photosynthetic enzyme activity of maize leaves under saline-alkali stress

The main physiological function of PEPCase in C_4 plant photosynthesis is to use PEP as a substrate to fix CO_2 and produce oxaloacetate, and oxaloacetate(OAA) is catalysed to malic acid by MADP-ME. Malate is metabolized by NADP-MDH to produce CO_2 and pyruvate, PEP use pyruvate as a substrate to be produced by PPDK catalysis and returned to the C_4 cycle. An important carbon fixation in photosynthesis is the Calvin cycle. CO_2 reacts with RuBP under RuBPCase catalysis in the Calvin cycle. Thus, photosynthetic enzyme reflects the photosynthetic activity of maize seedlings under saline-alkali stress (*Fig. 2*). Compared with the seedlings under the CK treatment, in the seedlings under the SAS treatment the activity of PEPCase, RuBPCase, NADP-ME, NADP-MDH and PPDK significantly decreased, the change of Xinxuan58 was greater than that of Jilong2. Compared with SAS treatment, there was no significant change in NADP-ME activity in Jilong2 under S+G treatment, while NADP-ME activity in Xinxuan58 increased. Compared with SAS treatment, in the seedlings under the S+G treatment the activity of NADP-MDH, PPDK and RuBPCase significantly increased. This suggests that saline-alkali stress inhibits the photosynthetic pathway and GD treatment resulted in increased C_4 photosynthesis activity.

Effect of GD on endogenous hormone content and its ratio in maize roots under saline-alkali stress

The plant adaptations to saline-alkali stress is reflected in changes of endogenous hormones and their ratios in maize under saline-alkali stress. Compared with CK, SAS treatment significantly inhibited the content of IAA in Jilong2, the relative content of endogenous IAA in Xinxuan58 was significantly increased and reached a notably high level (*Table 2*). The IAA concentration in Jilong2 and Xinxuan58 significantly increased under S+G treatment compared to the SAS treatments, respectively. Compared with CK, SAS treatment significantly inhibited the content of GA and ZR, the change of GA in Xinxuan58 was greater than that in Jilong2. After S+G treatment, the content of GA and ZR in roots of Jilong2 and Xinxuan58 was significantly greater than that in the SAS treatment. Compared with CK, SAS treatment significantly

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increased the content of ABA in Jilong2, and in Xinxuan58, ABA content decreased. The ABA content in S+G treatment decreased relative to SAS treatment. Saline-alkali stress reduced IAA, GA and ZR contents in root of maize and inhibited plant growth. Under saline-alkali stress, increased levels of ABA may help to improve saline-alkali tolerance of plants. The accumulation of ABA content is conducive to plants to adaptation to stresses, and GD treatment can effectively alleviate the harm of saline-alkali stress and promote hormone balance recovery.

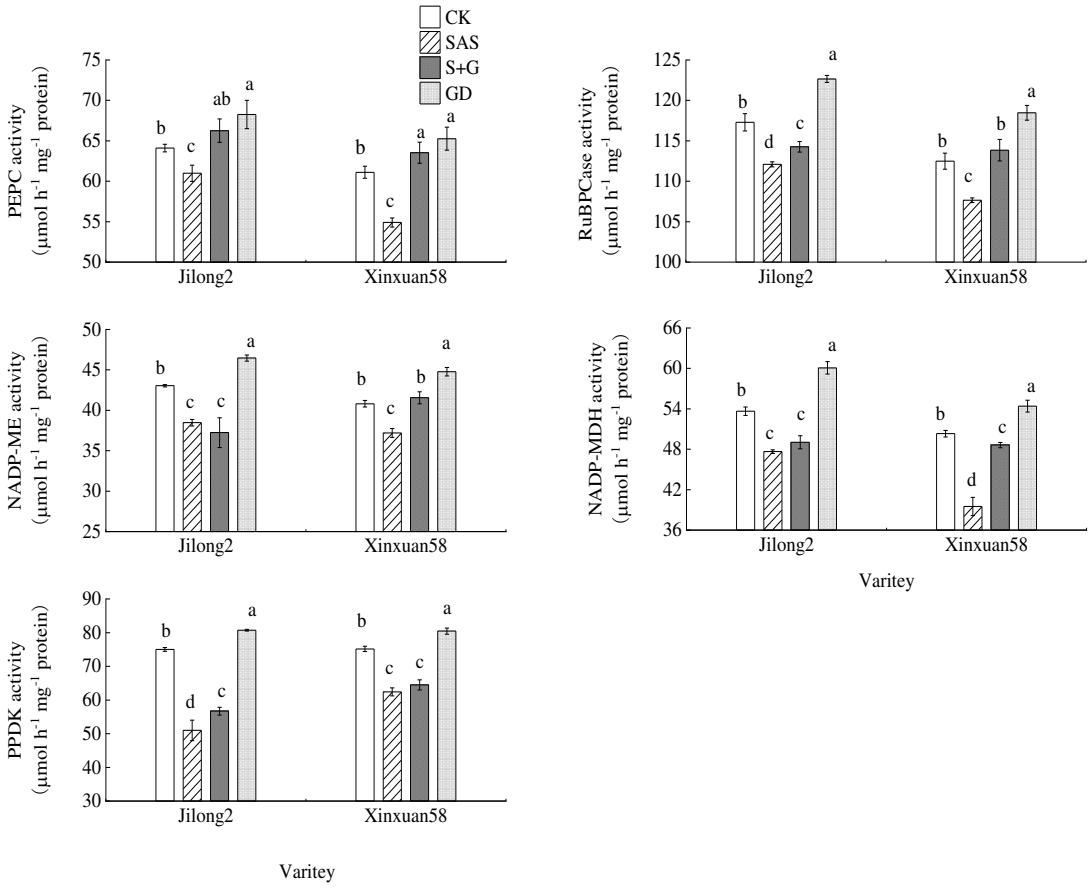

Figure 2. *Effect of GD on photosynthetic enzyme activity in leaves of maize under saline-alkali stress on the fourth day. Different letters within the same variety and column indicate significant difference at 0.05 level*

Hormone interaction in plants shows synergism or antagonism, the ratio of IAA/ABA, ZR/ABA and GA/ABA indicate different hormone impact on each other. The IAA/ABA and GA/ABA ratio in Jilong2 in SAS treatments decreased relative to CK, that in Xinxuan58 increased. And ZR/ABA ratio has no significant change. Compared with SAS, S+G treatment the ratio of IAA/ABA, ZR/ABA and GA/ABA significantly increased in Jilong2 and Xinxuan58. The ratio of IAA/ABA, ZR/ABA and GA/ABA under SAS treatment was greater, indicating that growth-promoting hormone inhibits ABA action. Plants regulate energy distribution through hormones to support growth and development under stress. GD can change the hormone content under saline-alkali stress and keep the relative balance of hormone content in plants.

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Table 2. *Effect of GD on endogenous hormones and hormones ratio in roots of maize under saline-alkali stress on the fourth day.*

Variety	Treatment	IAA (ng g^{-1})	GA (ng g^{-1})	ZR (ng g^{-1})	ABA (ng g^{-1})	IAA/ABA	GA/ABA	ZR/ABA
Jilong2	CK	253.15± 10.23^{b}	376.47± 2.65^{b}	125.44± 2.46^{b}	146.21± 3.97^{b}	1.73± 0.04^{c}	0.89± 0.02^{c}	2.58± 0.09^{c}
	SAS	196.98± 3.96^{c}	364.16± 4.96^{c}	116.26± 5.30^{b}	155.48± 1.78^{a}	1.27± 0.03^{d}	0.75± 0.04^{d}	2.34± 0.03^{c}
	S+G	249.37± 3.40^{b}	395.31± 10.33^{a}	148.29± 11.20^{a}	106.43± 1.68^{d}	2.34± 0.13^{a}	1.39± 0.11^{a}	3.71± 0.08^{a}
	GD	274.07± 16.89^{a}	372.58± 2.11^{bc}	146.69± 4.03^{a}	128.70± 2.08^{c}	2.13± 0.06^{b}	1.14± 0.04^{b}	2.90± 0.06^{b}
Xinxuan58	CK	189.37± 4.84^{c}	409.56± 5.62^{b}	160.86± 3.79^{b}	161.24± 5.60^{a}	1.17± 0.03^{d}	1.00± 0.04^{c}	2.54± 0.05^{c}
	SAS	244.33± 3.32^{b}	385.94± 10.25^{c}	142.38± 6.25^{c}	144.48± 15.37^{b}	1.70± 0.15^{c}	0.99± 0.11^{c}	2.69± 0.21^{c}
	S+G	269.25± 10.44^{a}	407.80± 9.61^{b}	159.03± 4.01^{b}	123.77± 1.21^{d}	2.18± 0.01^{b}	1.29± 0.04^{b}	3.30± 0.09^{b}
	GD	250.81± 10.53^{b}	453.18± 1.63^{a}	202.28± 2.30^{a}	101.76± 2.83^{c}	2.47± 0.17^{a}	1.99± 0.03^{a}	4.46± 0.13^{a}

Different letters within the same variety and column indicate significant difference at 0.05 level. The same as below

Effects of GD on antioxidant system and osmotic regulation of maize roots under saline-alkali stress

In order to maintain the normal physiological function of the cells, the water potential of the cells was reduced by the accumulation of proline and soluble sugar under saline-alkali stress. The proline content in SAS treatment increased relative to CK, the change of GA in Xinxuan58 was greater than that in Jilong2 (*Fig. 3*). And S+G treatment could further increase proline content. Compared with SAS treatment, soluble sugar content in two maize cultivars under S+G treatment significantly increased. This indicates that the accumulation of proline and soluble sugar content can effectively alleviate the saline-alkali stress on the root of Jilong2, relatively, Xinxuan58 saline-alkali tolerance was weaker. GD treatment is beneficial to the accumulation of proline and soluble sugar content to maintain the normal function of root system under saline-alkali stress. The H_2O_2 contents and O_2^- generation rate in the root of Jilong2 and Xinxuan58 under SAS treatment increased compared with that of CK. The S+GD treatments had lower H_2O_2 contents and O_2^- generation rate than the CK. SOD, POD and CAT activity and MDA content under SAS treatment higher than that of CK. Under SAS treatment, the POD and CAT activity of maize roots were consistent with the trend of SOD activity. And S+G treatment significantly increased SOD, POD and CAT activity and MDA content compared with SAS treatment. GD can significantly alleviate the damage caused by saline-alkali stress on two maize cultivars, the change of antioxidant capacity Jilong2 was greater than that of Xinxuan58.

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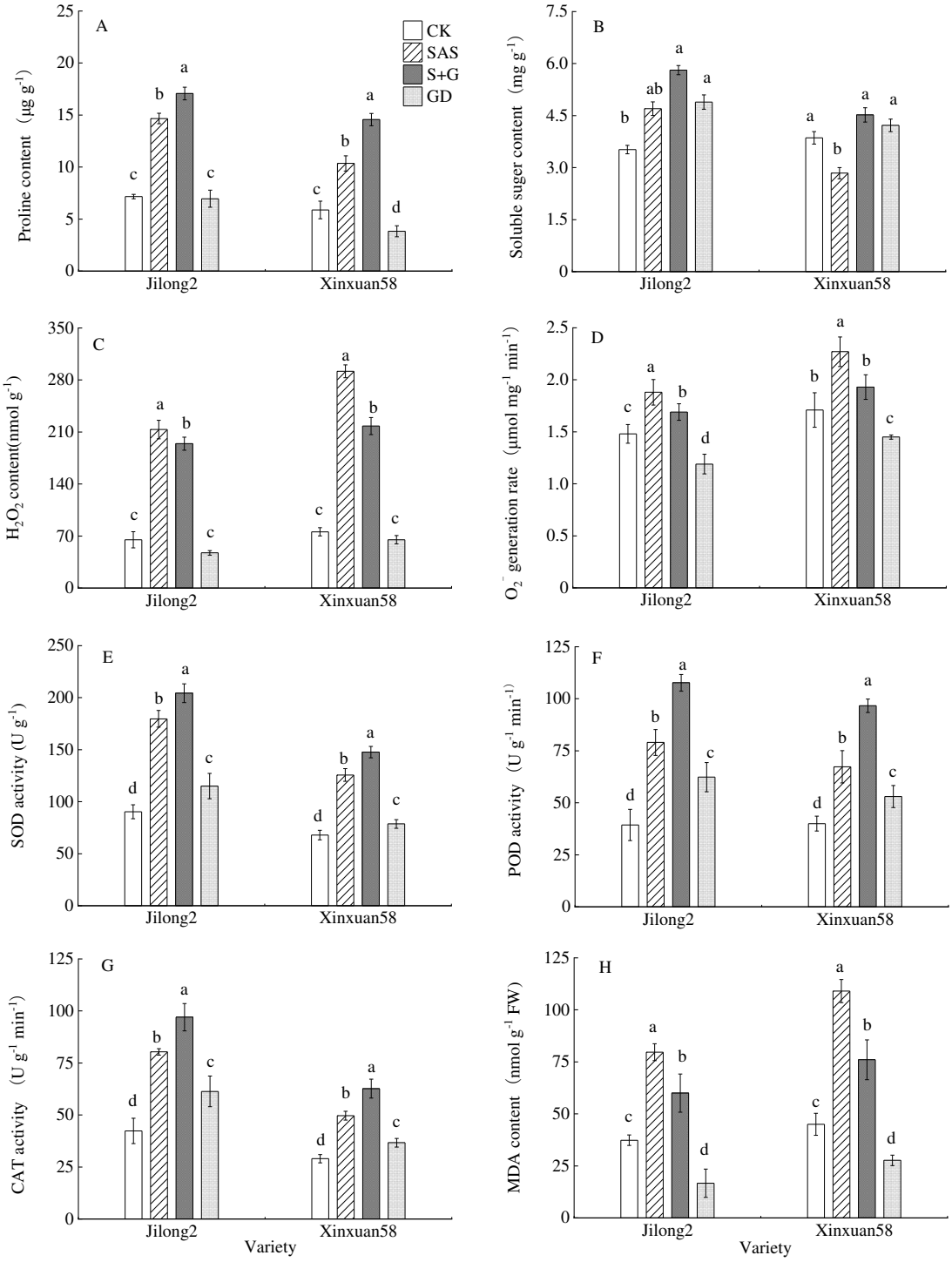

Figure 3. *Effect of GD on permeating substances, antioxidase activity and MDA content in roots of maize under saline-alkali stress on the fourth day. A: Proline content; B: Soluble sugar content; C: H_2O_2 content; D: O_2^- generation rate; E: SOD activity; F: POD activity; G: CAT activity; H: MDA content. Different letters within the same variety and column indicate significant difference at 0.05 level*

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Discussion

The accumulation of biomass is the mitigation and adaptation of plants to saline-alkali stress, and the difference of accumulation reflects the resistance of different plants (Razieh et al., 2019). Maize seedling dry-fresh weight and plant height decreased during saline-alkali stress, root-shoot ratio increased. This indicates that the growth of maize seedlings was inhibited under saline-alkali stress, the photosynthate produced was preferentially transferred to the root to maintain absorption of water and nutrients. Normal physiological function of root system is the basis of seedling growth. GD treatment markedly improved seedling growth under saline-alkali stress conditions, increased dry-fresh weight and plant height and decreased root-shoot ratio. Photosynthates in the growing period were normally allocated to each organs to increase the saline-alkali resistance of maize seedlings. Simple GD can promote root growth, increase plant dry-fresh weight and plant height, that can be used as growth promoters. Former study has shown that GD and its compound can promote dry-fresh weight of cucumber and cowpea (Zhang et al., 1998a). 25 mg L^{-1} GD could increase dry-fresh weight of radish, and improve the root-shoot ratio (Qian et al., 2016). It is proved that GD was propitious to increase plant biomass accumulation, and promote roots growth by promoting the transport of photosynthetic products to roots. Our conclusions are in line with these studies.

Photosynthesis is the physiological metabolism that provides energy for plants, photosynthetic efficiency is closely linked with biomass accumulation in plants. And photosynthetic efficiency is the foundation of plant growth. Photosynthetic enzyme activity can reflect the effect of saline-alkali stress on carbon assimilation pathway in maize plants. PEPCase play important roles in the regulation of responses to abiotic stresses and organic acid metabolism (Ueno et al., 2020). NADP - ME not only can catalyse reversible reactions of oxidative decarboxylation of malate, but also regulate pyruvate sources in plants through synergism between PEPCase and NADP-MDH (Borba et al., 2018). PEPCase has the function of maintaining the stability of cytoplasmic pH and osmotic potential. Research shows that NADP - ME activity of plants is increased by saline-alkali stress, and improve stress tolerance (Zhao et al., 2019). The present study shows that PEPC, PPDK, NADP-ME, NADP-MDH and RuBPCase activities were significantly decreased under saline-alkali stress compared to CK. Carbon assimilation rate in C_4 photosynthesis was inhibited by saline-alkali stress, carbon assimilation rate reduced and assimilates translocation decreased (Kandoi et al., 2018; Dong et al., 2019). GD treatment significantly increased photosynthesis activity in maize leaves under saline-alkali stress, and GD could enhance the activity of photosynthesis to alleviate the inhibition of photosynthetic metabolism of maize seedlings under saline-alkali stress.

Plant endogenous hormone refers to the trace organic matter in plants, which can regulate plant physiological metabolism and directly or indirectly regulate plant growth. The level and distribution of endogenous hormones in plants follow the changes of external environment, so plants adapt to stress by regulating hormones. Regulation of hormone levels under abiotic stress can mediate physiological metabolic responses in plants and improve plant adaptability to adversity (Joshua et al., 2019). Studies have shown that saline-alkali stress may increase the level of reactive oxygen species (ROS), and ROS was capable of inhibiting the hormone synthesis, which leads to decreased hormone content (Jan et al., 2019). ABA is an endogenous signal to regulate stomatal closure to avoid water loss during saline-alkali stress treatment (Zhang et al., 2019b).

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The IAA, ZR and GA contents in SAS treatment decreased relative to CK, and the ABA content was higher in Jilong2 and lower in Xinxuan58. There was different saline-alkali tolerance among cultivars. Because the energy distribution of physiological metabolism in plants to osmotic regulation and antioxidant regulation is suppressed by ABA (Li et al., 2017, 2020), Jilong2 can alleviate their own osmotic stress and antioxidant stress injury through induced ABA synthesis, and then improve maize resistance under stress. IAA/ABA, ZR/ABA and GA/ABA ratios directly reflect the relationship of endogenous hormones, and changes of endogenous hormone levels under saline-alkali stress affect the balance between hormones (Amany et al., 2019). According to the change of IAA/ABA, ZR/ABA and GA/ABA ratios under CK treatment and SAS treatment is explained ABA in Jilong2 is more sensitive to saline stress than IAA and GA, and ZR and ABA sensitivities are similar. Root system of Jilong2 by secreting ABA slows root growth and adaptation to saline-alkali stress. The response sensitivity of Xinxuan 58 to IAA was higher than that of Jilong 2, and ABA content decreased, so the tolerance was poor (Xie et al., 2019). This suggests that GD treatment effectively regulates endogenous hormone balance and promotes IAA, ZR and GA content. IAA, ZR and GA as ABA antagonists increased, which inhibited the production of ABA, and alleviated the inhibition of root growth, improved the saline-alkali tolerance of maize roots.

The saline-alkali stress significantly decreased water potential and osmotic pressure, but increased cell expansion pressure in seedlings, which resulted in osmotic stress. Osmotic stress caused direct damage to maize plants and affected various physiological activities in plants (Amany et al., 2019). Studies have shown that accumulation of proline and soluble sugar content can reduce the cell permeability potential in maize roots and play an important role in maintaining water absorption in plant roots under saline-alkali stress. This study showed that the content of proline and soluble sugar was significantly higher than that of control in Jilong2 roots and lower than that in Xinxuan 58 root under saline-alkali stress, saline-alkali tolerant varieties can maintain the osmotic balance of plant cells by accumulating osmotic substances. GD can effectively promote the content of osmotic substances in maize roots under saline-alkali stress and improve the capacity of osmotic adjustment, which plays an important role in maintaining the water balance in roots of maize. Former study shows that 100 mg L^{-1} GD significantly increased soluble sugar content in willow leaves, which was consistent with the conclusion of this study (Zhang et al., 2018, 2019a). Plant cells accumulate a large amount of reactive oxygen species under saline-alkali stress, which leads to membrane lipid peroxidation and destruction of cell membrane structure and function, which leads to dysregulation of intracellular metabolism. Membrane lipid peroxidation under saline-alkali stress is closely related to active oxygen accumulation, indicating a disruption in the structure and function of the cell membrane, that leads to disturbance of lipid metabolism (Ahmad et al., 2019). SOD, PDD and CAT are a system of antioxidant enzymes that scavenge ROS in plants, the change of enzyme activity showed the plant resistance (Li et al., 2020).

The main function of SOD is to clear O_2^- generate to form H_2O_2, and then H_2O_2 was decomposed into O_2 and H_2O under POD and CAT catalysis. The antioxidant enzymes maintain a certain balance with ROS, the product of membrane lipid peroxidation is MDA that can reflect the degree of oxidative stress damage to plants (Jing et al., 2019). Compared with CK, O_2^- generation rate, H_2O_2 content, MDA content, the activity of SOD, POD and CAT increased significantly under SAS treatment. During saline-alkali stress condition, the root of maize was affected by oxidative stress, the change of

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antioxidant enzyme activity in Jilong2 was greater than that in Xinxuan58. ROS accumulation cause damages to membrane structure and the imbalance between pro-oxidant and anti-oxidant systems and then following oxidative stress (Liu et al., 2017). Compared with SAS treatment, O_2^- generation rate, H_2O_2 content, MDA content, the activity of SOD, POD and CAT decreased significantly under GD treatment. GD treatment alleviates the oxidative stress damage caused by saline-alkali stress on maize roots, and effectively improves the saline-alkali tolerance of maize, Xinxuan58 is more sensitive to GD effects. The increased activity of antioxidant enzymes can effectively scavenge ROS (Abdelgawad et al., 2016; Yao et al., 2016; Elrys et al., 2020). 25 mg L^{-1} GD significantly reduced SOD and POD activity in green vegetable leaves (Yan et al., 2016). 1 mg L^{-1} GD effectively reduced SOD activity in maize leaves (Qian et al., 2015). In this study the GD treatment could significantly improve the activity of SOD, POD and CAT, which appear to be at odds with that conclusion, due to different GD concentrations on antioxidant oxidase activity.

Conclusions

In conclusion, this study demonstrated that exogenous GD increased tolerance to saline-alkali stress in maize. Under saline-alkali stress, GD could reduce the harmful effects on the growth of maize by regulating osmotic metabolism, activating photosynthesis, enhancing the antioxidant system and increasing endogenous content. These results revealed a key role of GD in the relief of saline-alkali stress and indicated that it can be used as root-promoting agent. The physiological mechanism of GD on maize seedling growth promotion and saline-alkali stress tolerance is not clear. The next step is to determine the effect of GD on carbon and nitrogen metabolism of maize seedlings to determine the role of GD under saline-alkali stress.

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Author contributions. Liu, Z., Gu, W. R., Li, J., and Wei, S. conceptualized and planned the study. Liu, Z., Zhu, Q. R., Li, C. F., and Xu, W. H. conducted the experiments and prepared samples. Liu, Z., and Wang, M. Q. conducted the laboratory analyses. Liu, Z. interpreted the results. Liu, Z., and Gu, W. R. wrote the manuscript with contributions from all authors.

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Conflict of interests. The authors declare that they have no conflicts of interests.

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THE IMPACT OF HUMAN ACTIVITIES, NATURAL FACTORS AND CLIMATE TIME-LAG EFFECTS OVER 33 YEARS IN THE HEIHE RIVER BASIN, CHINA

GAO, N. N. [1,2*] – LI, F.[2] – ZENG, H.[1] – ZHENG, Y. R.[3*]

[1]*School of Urban Planning and Design, Peking University Shenzhen Graduate School, University Town, Nanshan District, Shenzhen, Guangdong 518055, China*
(phone: +86-186-1156-1826)

[2]*Shenzhen Institute of Building Research Co., Ltd., No. 29 Mei'ao 3rd Road, Futian District, Shenzhen, Guangdong 518049, China*
(phone: +86-185-1166-5630)

[3]*Key Laboratory of Resource Plants, West China Subalpine Botanical Garden, Institute of Botany, Chinese Academy of Sciences, No. 20 Nanxin Village, Xiangshan, Beijing 100093, China*
(phone: +86-135-5232-2091)

**Corresponding authors*
e-mail/phone: bnugaonannan@163.com/+86-186-1156-1826; zhengyr@ibcas.ac.cn/+86-10-6283-6508

The two corresponding authors contributed equally to this work

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Abstract. Vegetation is a key factor adapting to and mitigating climate change. The quantitative analysis of vegetation dynamics including climate and human activities is necessary to take appropriate actions to adapt to climate change and optimize vegetation distribution to mitigate it. This article integrates the relationship between climate, soil, socioeconomic factors and vegetation change using redundancy analysis (RDA) and partial redundancy analysis (pRDA): which also considers the time-lag effect of climate change. The correlation between vegetation and dynamics of the three periods at land-use and vegetation group levels in the 14 counties in Heihe River basin (HRB) of China was revealed. Results showed that the most important driving factor was groundwater depth and mean annual temperature with 15-year lag times. More variation of vegetation change was determined at land-use level (54.7%) than that at vegetation group level (42.0%): Climate change factors explained more variations than human activities both at vegetation group and land-use level, so did the time-lag effect. Land use planning not is only necessary in urban area but also in rural area in HRB. To increase resilience of agriculture, we suggest appropriate grazing management strategy. Meanwhile time-lag effects are quite important for better evaluating vegetation dynamics under climate change.
Keywords: *vegetation dynamics, climate change, climate adapting, mitigating climate risk, agriculture resilience*

Introduction

Global warming will happen faster than we think, the time for rapid adaptation has arrived (Xu et al., 2018). Vegetation such as forest and agriculture play a key role in mitigating and adapting to climate change. Restoring natural forests is the best ways to remove atmospheric carbon (Lewis, 2019). We should make adaptive management strategies for agriculture which is vulnerable to climate change (Havstad et al., 2018). How to adjust or protect vegetation distribution in an effective way is a suspended problem because important human activities and natural factors vary from place to place. To solve the problem, it's necessary to find out major factors that caused changes in

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vegetation distribution at research site, which may be human activities, climate or other natural factors such as soil.

For climate fundamentally controls the distribution of ecosystems, species ranges, and process rates (Grimm et al., 2013), ecological systems are being altered by climate change throughout the world (Lawler et al., 2010). Meanwhile, 75% of the area of Earth's ice-free terrestrial biomes is affected by humans (Ellis and Ramankutty, 2008), and currently in many parts of the world, human activities are the main forces shaping land-use and ecosystem changes (Foley et al., 2005; Serra et al., 2008). Further, the extent of human-induced land-cover change is increasing dramatically, which threatens biodiversity globally (Watson et al., 2014). Adaptation to climate change is getting more and more attention, Therefore, the impact of multiple stressors on vegetation has become one of the most pressing questions in ecology and biodiversity conservation (Sirami et al., 2017).

Quantifying vegetation change relationship with human activities and climate change is necessary to make suggestion to take action to mitigate or adapt to climate change. Vegetation changes can be triggered by climatic factors and human activities, separately or together, which influence vegetation on regional and global scales (Linderman et al., 2006; Wu et al., 2015). However, most studies on vegetation dynamics have only focused on the effects of climatic change (Nemani et al., 2003; Woodward and Lomas, 2004), despite repeated calls for better integration of multiple drivers (Didham et al., 2007; Mantyka-pringle et al., 2012; Oliver and Morecroft, 2014). In the absence of integrative multi-driver approaches, limited understanding of how interactions among drivers affect observed changes will likely hamper reliable projections and relevant conservation recommendations (Titeux et al., 2016). Thus, understanding vegetation dynamics and its response to both climatic change and human activities is important.

Ordination is an effective method to solve complicated ecological multivariate analysis. In quantitative analysis of vegetation distribution causal factors, many simulation models and statistical analysis methods have been used, such as the Sheffield Dynamic Global Vegetation Model (Woodward and Lomas, 2004), biome-specific production efficiency model (Nemani et al., 2003), Principal Component Analysis and Ordination. During Ordination Analysis, Forward Selection via the Monte Carlo permutation test is typically used to select suitably representative factors that explain most vegetation variations (Lepš and Šmilauer, 2003).

This article integrates multiple drivers such as climatic, soil and the socioeconomic factors, to accurately simulate spatiotemporal changes of vegetation in the Heihe River basin (HRB) over a recent 33 years (1980–2013) and to distinguish the effects of various causal factors on vegetation dynamics at land-use and vegetation group levels. The main objectives of this study were: 1) quantitative analysis of vegetation dynamics and responses to climatic change and human activities; 2) comparison of the difference of these correlations at land-use and vegetation group levels; and 3) the time-lag effects of temperature and precipitation on the variation of vegetation distribution. The results will be useful to promote protection of the ecological environment and making agricultural adaptive management strategies in the study region.

Materials and methods

Site description

The Heihe River basin is the second largest inland river basin of China. It is a relatively independent and diversified geographic unit in the northwestern part of the

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country at the intersection of the northeast margin of the Tibetan Plateau and southern slopes of the Gobi-Altay Mountains, and covers an area of 143,000 km^2, which includes 14 counties (*Table 1; Fig. 1*). The basin is an important grain producing region because of its relatively abundant water resources, with a mean annual runoff of 3.73 × 109 m^3 at high points in the basin. Precipitation and evaporation have strong spatiotemporal heterogeneity under the influence of geographic factors and atmospheric circulations in this basin.

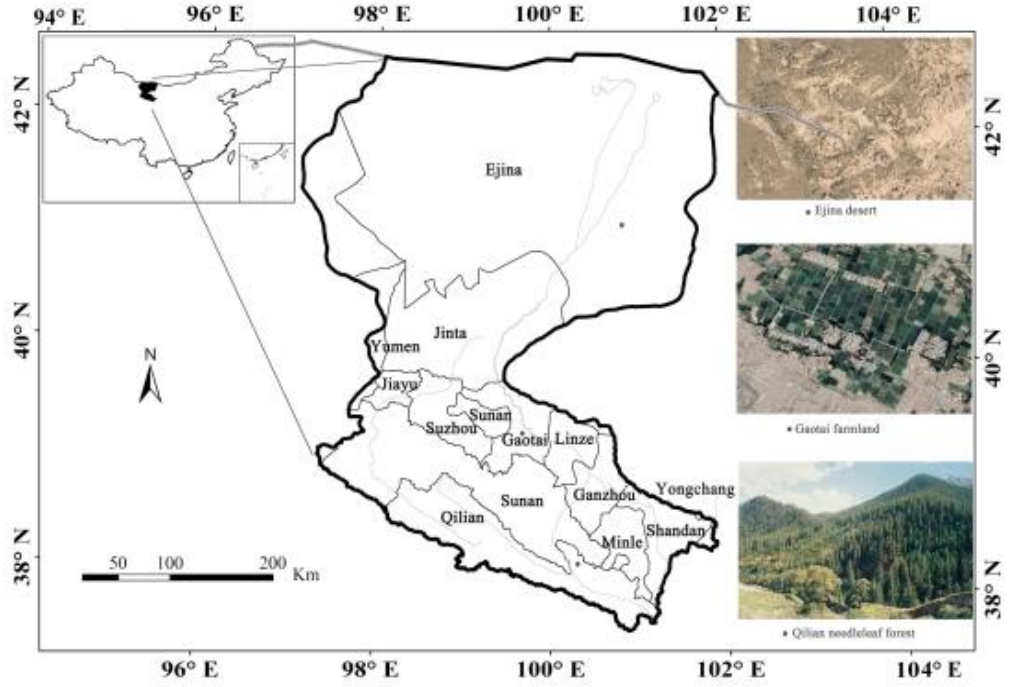

Figure 1. *Location of Heihe River basin (HRB) and counties in the basin*

The HRB has three major divisions, namely, the upper, middle and lower reaches, which are marked by various natural conditions and socioeconomic development states. The upper reaches, with elevations from 2180 to 5547 m, have a humid cold climate. This is the main runoff portion of the watershed and the main land cover is pasture. The middle reaches are between the Qilian and Beishan mountains, at elevations 1289 to 3920 m. This is the main water consumption zone, where most of the land has been reclaimed for oasis agriculture. It accounts for 95% of the 8471 km^2 cultivated land, 91% of the 26 billion population, and more than 80% of the GDP of the16.5 billion-dollar GDP of the entire Basin (Cheng et al., 2014). The area has a continental arid temperate climate with mean annual precipitation from 100 to 250 mm. The lower reaches form the tail-end zone, mainly occupied by the barren Gobi Desert, with a huge evaporative capacity and very fragile ecological environment. Elevation in this area is about 1000 m. Mean annual precipitation is less than 50 mm.

The HRB is severely affected by both climate change and human activities (Cheng et al., 2014; Wu, 2011). Change of the hydrologic processes of HRB has substantially modified the local environment over recent decades, including environmental degradation, salinization and desertification (Luo et al., 2016). Further, around 2000, the local government started an ecological project, returning cultivated land to grassland

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and diverting water to the lower reach to recover a disappeared lake in the HRB. Therefore, the basin may be an ideal area to understand the effects of climate and human activities on vegetation distribution.

Table 1. *Basic information of climate, social economy and vegetation of counties in the HRB*

County	Altitude (m)	Temperature (°C)	Precipitation (mm)	GDP (million RMB)	Population (capita)	Main land use	Main vegetation group
Ejina	1142	7	46	2024	19846	Barren land	Desert
Jinta	1313	8	48	2862	144442	Barren land	Desert
Subei	1703	5	49	1800	12449	Barren land	Desert
Jiayu	1768	6	65	12147	200557	Barren land	Desert
Yumen	1544	7	51	8364	174039	Barren land	Desert
Suzhou	1566	7	77	8448	367793	Barren land, farmland	Desert, cultural vegetation
Gaotai	1488	8	84	2380	158077	Barren land, farmland	Desert, cultural vegetation
Linze	1494	8	91	2201	147168	Barren land, farmland	Desert, cultural vegetation
Ganzhou	1692	7	149	7536	492928	Barren land, farmland	Desert, cultural vegetation
Shandan	2533	4	325	2263	200353	Desert, steppe, farmland	Desert, steppe, cultural vegetation
Minle	2423	4	372	1810	232632	Barren land, farmland, pasture	Desert, cultural vegetation, meadow, steppe, alpine
Yongchang	2346	4	242	3274	253248	Barren land, pasture	Desert, steppe
Sunan	3214	-1	290	1256	36237	Pasture, barren land	Steppe, alpine, meadow
Qilian	3831	-4	491	822	48050	Pasture, barren land shrub	Meadow, alpine, shrub

Altitude (m), temperature (°C), and precipitation (mm) in this table presented are mean data of the individual counties

Data and preprocessing

Temporal and spatial scale affect analysis results of the relationship between vegetation and driving factors (Sirami et al., 2017). Considering that China began to compile development statistical yearbooks where socioeconomic data was from in 2000, and sequenced climate and soil data of HRB were updated from 1980 to 2013, the study period was 1980 to 2013 when climate time lag effect when studied.

Satellite imagery data and pre-processing

The satellite imageries were relatively cloud-free (<5% cloud cover) Landsat Thematic Mapper (TM), Enhanced Thematic Mapper Plus (ETM+) and Operational Land Imager (OLI) image data from 2000, 2007 and 2013 in July during growing season, with a 30-meter spatial resolution. All images were downloaded from the Geospatial Data Cloud (available at http://www.gscloud.cn/) following the same criteria, i.e., cloud cover, quality and date of acquisition in the late dry season. Eleven Landsat scenes were selected to cover the study area. All scenes for each year were used after radiometric calibration and fast atmospheric correction processing in ENVI 5.3.1 software. Google Earth image and map were used as ancillary data.

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Satellite imagery data classification

Several kinds of data were analyzed by discriminant analyses using image random forest (RF) classification (Cutler et al., 2007) in which (1) Landsat data (2000, Landsat ETM+, seven data channels; 2007, Landsat TM, seven data channels; 2013, Landsat 8 OLI, nine data channels); (2) geographic data (elevation, slope, aspect and terrain ruggedness index, four data channels); (3) spectral index data (NDVI, difference vegetation index, enhanced vegetation index, water index, and normalized difference built-up index, five data channels) were included. RFs are decision tree ensembles for the classification or regression of categorical and continuous data (Breiman, 2001). the RF classifier is a very useful analytical tool and is considered very desirable for multisource classification of remote sensing and geographic data (Gislason et al., 2006).

Finally, Land-use and Vegetation group types were got. Land-use types included forest, shrub, water, farmland, settlements, pasture and barren land (*Fig. 2; Table 2*). Vegetation group types were desert, meadow, needleleaf forest, broadleaf forest, alpine vegetation, shrub, steppe, cultural vegetation and land without vegetation. Classification system was according to Editorial Committee of Vegetation Map of China (CAS, 2007).

Table 2. *Characteristics of land use and vegetation group in the HRB*

Types		2000	2007	2013	Area change (ha)*	Area change (%)*
Land use	Forest	167,589	170,684	189,018	21,429	12.79
	Shrub	393,729	428,016	533,305	139,576	35.45
	Water	160,674	104,011	115,403	-45,271	-28.18
	Farmland	595,033	683,750	847,137	252,104	42.37
	Settlements	35,003	48,722	63,348	28,345	80.98
	Pasture	2,340,302	2,344,829	2,056,240	-284,062	-12.14
	Barren land	10,585,586	10,497,903	10,473,466	-112,120	-1.06
Vegetation group	Desert	10,193,292	10,026,082	9,892,044	-301,248	-2.96
	Meadow	1,312,043	1,242,152	1,100,090	-211,953	-16.15
	Needleleaf forest	132,224	138,867	158,057	25,833	19.54
	Broadleaf forest	35,365	31,817	30,961	-4,405	-12.45
	Alpine vegetation	392,294	471,821	581,422	189,128	48.21
	Shrub	393,729	428,016	533,305	139,576	35.45
	Steppe	1,028,259	1,102,677	956,150	-72,109	-7.01
	Cultural vegetation	595,033	683,750	847,137	252,104	42.37
	Land without vegetation	195,677	152,734	178,751	-16,926	-8.65

*The time interval is 2000-2013

Climate, soil and human activity data

Log-transformation was used to normalize the response variable (vegetation data) and explanatory variable (climate, soil, economic and social factors) to ensure that the response variable data were not less than zero and the explanatory variables were comparable and at least contain one positive number. It was calculated by *Equation 1*.

$$X = \log_{10}(X_i \times a + 1) \times b \quad \text{(Eq.1)}$$

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Here X*i* is original value of variable X*i*, X is log-transformed value of X*i*, a and b are constants to make sure all X are comparable in the same axis.

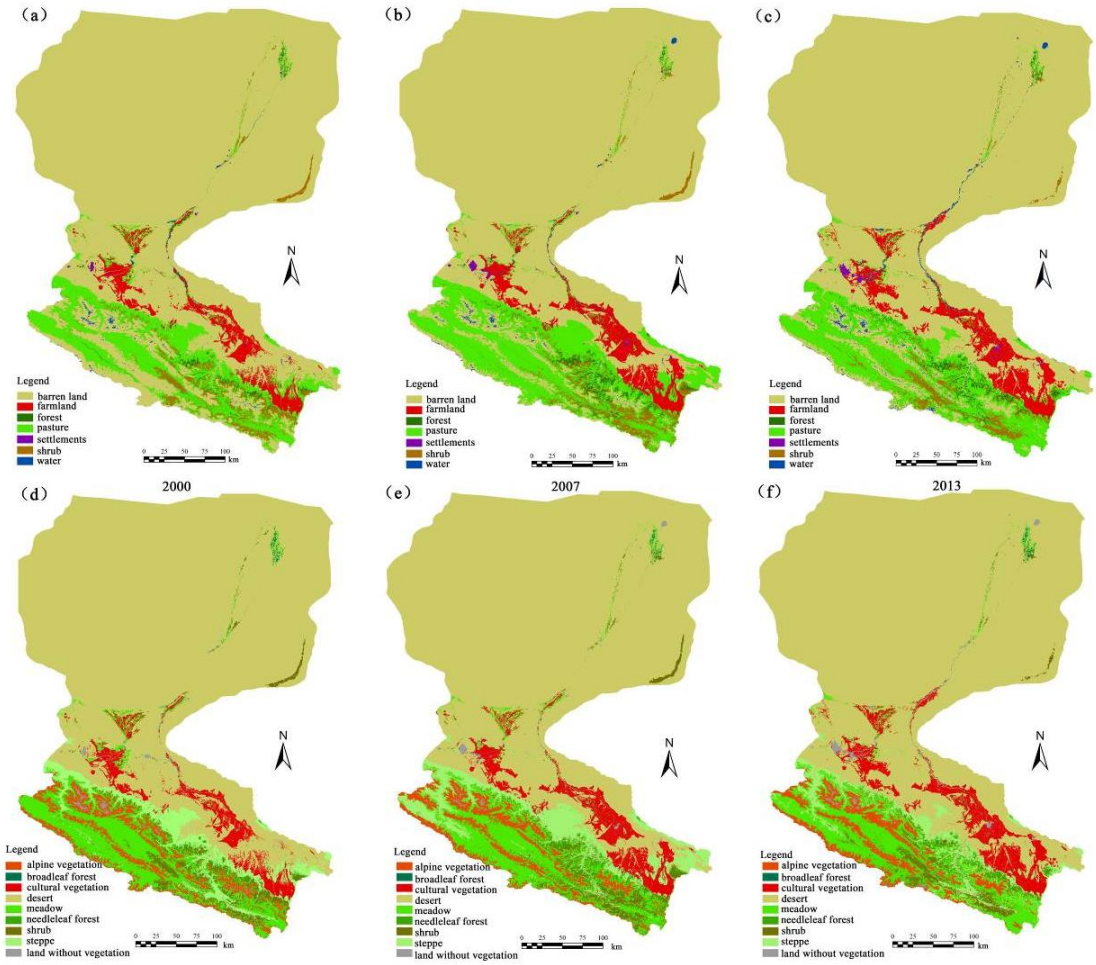

Figure 2. *Classification of land use (a, b, c) and vegetation group (d, e, f) in the HRB*

Climate and soil data, such as mean annual temperature (T), evapotranspiration (EVAP), soil moisture measured at 100 cm from surface (SM100), groundwater depth (ZWT), and other factors were considered. The explanatory variables were analyzed and showed in figures below after removing the collinear factors (collinearity exists if VIF > 10) (Borthwick et al., 2020). These data were provided by the Data Cloud of the Chinese Academy of Sciences (*Fig. 3*).

Time-lag effect of climatic factors was also included as considering that vegetation distribution reaction to climate change may last several years (*Figs. 3, 4* and *5*). Time-lag series was set as follow, 0 year (abbreviation 0yr, the same as following, 2000's), 1 year (1yr, 1999's), 2 years (2yr, 1998's), 3 years (3yr, 1997's), 4 years (4yr, 1996's), 5 years (5yr, 1995's), 10 years (10yr, 1990's), 15 years (15yr, 1985's), and 20 years (20yr, 1980's). To match the vegetation and land use data of 2000, 2007 and 2013, the time-lag year was set as 0yr (2000, 2007, 2013), 1yr (1999, 2006, 2012), 2yr (1998, 2005, 2011), 3yr (1997, 2004, 2010), 4yr (1996, 2003, 2009), 5yr (1995, 2002, 2008), 10yr (1990, 1997, 2003), 15yr (1985, 1992, 1998) and 20yr (1980, 1987, 2003), respectively.

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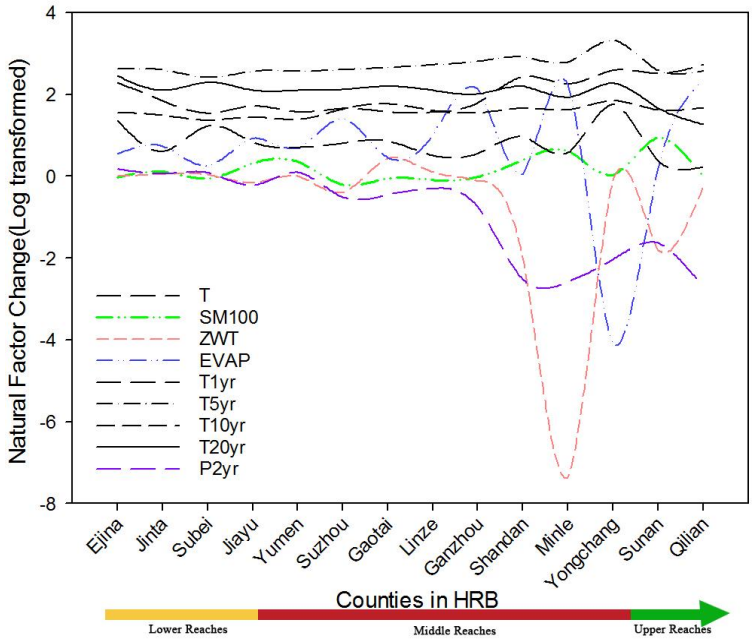

Figure 3. *Natural factors change during 2000-2013 by counties in the HRB. The abbreviations are mean annual temperature (T), evapotranspiration (EVAP), oil moisture measured at 100 cm from surface (SM100), groundwater depth (ZWT). The time-lag year was set as 0yr (2000, 2007, 2013), 1yr (1999, 2006, 2012), 2yr (1998, 2005, 2011), 3yr (1997, 2004, 2010), 4yr (1996, 2003, 2009), 5yr (1995, 2002, 2008), 10yr (1990, 1997, 2003), 15yr (1985, 1992, 1998) and 20yr (1980, 1987, 2003), respectively*

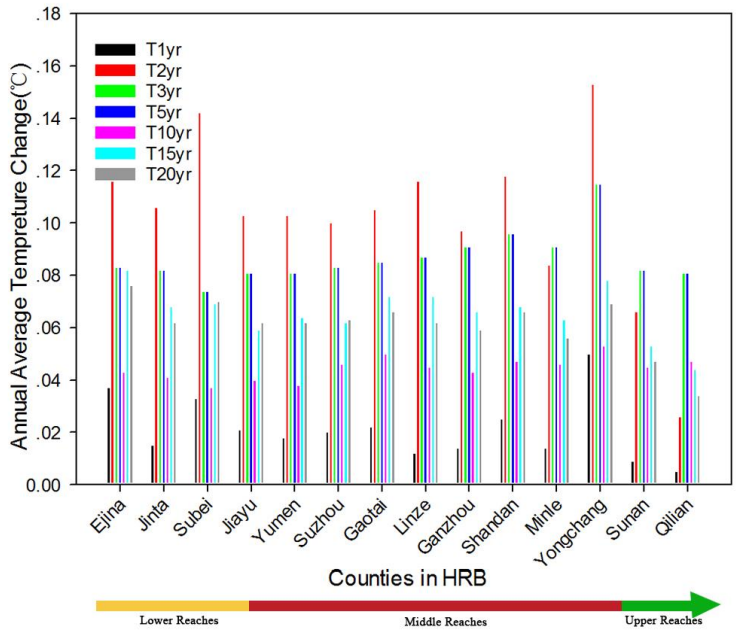

Figure 4. *Annual Average Temperature change of various lag time by counties in 1980-2013. The abbreviations are temperature (T) of the time-lag year. The time-lag year was set as 0yr (2000, 2007, 2013), 1yr (1999, 2006, 2012), 2yr (1998, 2005, 2011), 3yr (1997, 2004, 2010), 4yr (1996, 2003, 2009), 5yr (1995, 2002, 2008), 10yr (1990, 1997, 2003), 15yr (1985, 1992, 1998) and 20yr (1980, 1987, 2003), respectively*

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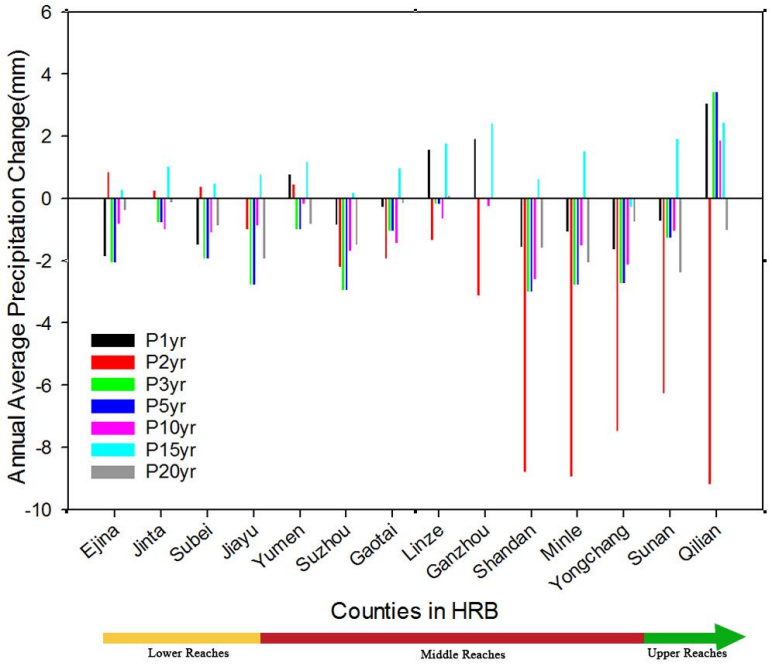

Figure 5. *Annual average precipitation change of various lag time by counties in 1980-2013. The abbreviations are precipitation (P) of the time-lag year. The time-lag year was set as 0yr (2000, 2007, 2013), 1yr (1999, 2006, 2012), 2yr (1998, 2005, 2011), 3yr (1997, 2004, 2010), 4yr (1996, 2003, 2009), 5yr (1995, 2002, 2008), 10yr (1990, 1997, 2003), 15yr (1985, 1992, 1998) and 20yr (1980, 1987, 2003), respectively*

Social and economic statistics data, such as agricultural GDP per capita (GVAP/AP), agricultural GDP per GDP (GVAP/GDP), agricultural GDP (GVAP), urban population (UP), pasturage density (PD), and other factors were considered. Only variables pass forward selection were showed in figures below. They were derived from the statistical yearbook, the government bulletin of counties in the HRB (*Fig. 6*).

Policy impact was considered qualitatively. An ecological water diversion project diverting water to lower reach in the Heihe River basin (Cheng et al., 2014) was implemented in 2000, the project of returning farmland to forest and grass in the upstream area of the basin was implemented in 1999 and an afforestation project was implemented in the 1990s. However, there was a difference in the timing and enforcement of policies in each county.

Analysis of vegetation distribution change

Vegetation classification levels have effect on vegetation distribution (Gao et al., 2017). We analyzed the relationship between vegetation distribution and drivers at land-use and vegetation group level, respectively. Land cover is predominantly human-oriented and is more likely to be reasonably planned for farmland and residential areas. The vegetation group represents plant biomes, which is conducive to the protection of species, scientific division of nature reserves, and development and utilization of ecological resources. The vegetation dynamic index K (Wang and Bao, 1999) was used to quantitatively describe the vegetation distribution change rate in a certain period in the research area, was calculated by *Equation 2*.

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$$K = \left[\frac{(U2-U1)}{U1}\right] \times \left(\frac{1}{T}\right) \times 100\% \quad \text{(Eq.2)}$$

Here, K is the dynamic index of land-use (vegetation group) type in the research period. U1 is the area of land type i at the beginning time and U2 is the area of land cover (or vegetation group) type i at the final time. T is the research period, with the year as its basic unit. K is the annual change rate of land-use type i. This model can be used to analyze and compare the change rate of different land-use types in the research area, with $|K| \leq 0.5\%$ (the absolute of K was not less than 0.5%) indicating small change, $0.5\% \leq |K| \leq 1.5\%$ moderate change, $1.5\% \leq |K| \leq 2.5\%$ large change, and $|K| \geq 2.5\%$ great change.

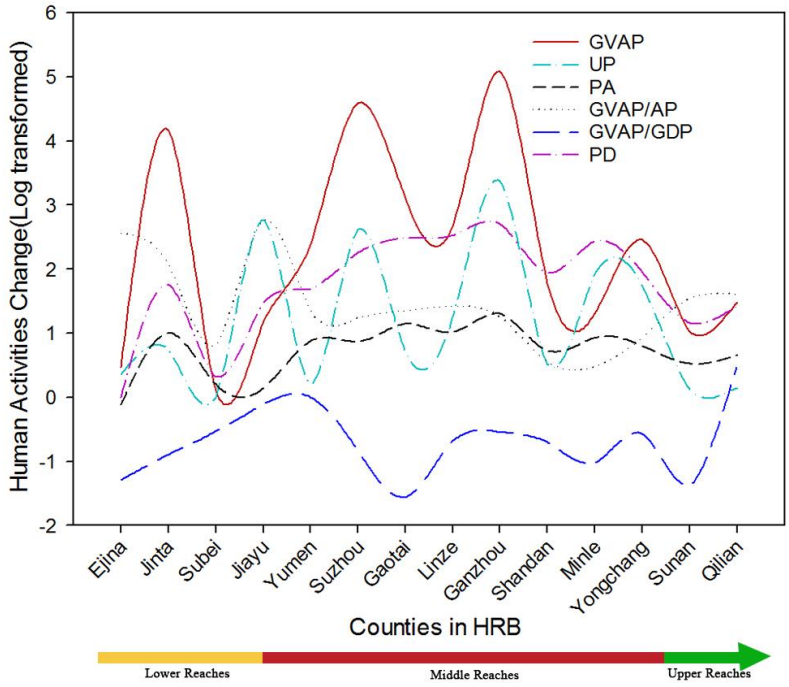

Figure 6. *Social and economic factor change during 2000-2013 by counties in the HRB. The abbreviations are agricultural GDP per capita (GVAP/AP), agricultural GDP per GDP (GVAP/GDP), agricultural GDP (GVAP), urban population (UP), pasturage (PA), pasturage density (PD)*

Correlation analysis

Correlation between land cover, vegetation group and causal factors data were analyzed by constrained ordination via the Canoco for Windows program (version 4.5) (ter Braak and Smilauer, 2002). Dynamic index data of 2000–2007, 2007–2013 and 2000–2013 of the 14 counties in the Heihe River basin were used as species data.

Detrended correspondence analysis (DCA) was conducted for response variables to detect the length of the species gradient, to decide whether to use unimodal or linear methods. After that, RDA was used because the lengths of gradients were smaller than 3 in the study. The Monte Carlo permutation test was used to test significance of the relationship with explanatory variables (Lepš and Šmilauer, 2003). "Automatic selection" and "Monte Carlo permutation tests" were used in the forward selection of

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environmental variables, removing explanatory variables with the smallest explanatory value (p-value > 0.05) and leaving the retained explanatory variable with p-value ≤ 0.5 and variance inflation factor < 10. Forward selection of variables (Montgomery et al., 2012) was used to determine the relative importance of environmental variables and variance explained by them.

The results of the RDA were plotted as two-dimensional graphs using CanoDraw (Version 4.5). The continuous environmental variables were plotted as arrows originating from the center of the graph. Although RDA analysis can identify the main effects on land-use or vegetation group change, it is difficult to distinguish the overall contribution of human and climate factors to variance of such change, so a partial RDA method was also used to distinguish the effect of human activity and climate change.

Results

Climate, soil and human activity change

As shown in *Figure 3,* natural factors do not fluctuate much in the downstream and midstream areas near the downstream of HRB, while ZWT, P2yr, EVAP, and SM100 change drastically in the upstream and near upstream areas.

As shown in *Figure 4,* temperature of HRB had increased significantly. T2yr in the middle and lower reaches is significantly higher than that of other lag time. There is not a strong correlation between changes in vegetation distribution and unusual dramatic temperature changes in a year (as T2yr), but long-term temperature changes profoundly affect vegetation changes (*Fig. 3*).

As shown in *Figures 3* and *5,* the effect of precipitation change on vegetation pattern is not as great as that of temperature change. Precipitation changes a lot in the last 20 years, and the decline is the main trend. Especially P2yr, the decline is obviously greater than 6 mm/year. Unlike temperature, vegetation distribution change only has relationship with dramatic precipitation change of 2-year lag time.

As shown in *Figure 6,* human activities are more dramatic than the natural factors. Considering the proportion of GDP, the agricultural GDP proportion decreased, while the industrial and service GDP proportion increased in all counties. PD change is higher in the middle reaches than in the upper and lower reaches. But other factors have no special trend of different regions.

Land cover and vegetation change

Main land cover type in HRB was barren land, which accounted for > 72% of the entire basin (72.84% in 2000 and 73.35% in 2013). The second largest type was pasture, accounting for 15.64% and 14.40% in 2000 and 2013, respectively, followed by farmland. Barren land, shrub, farmland, settlements (including residential, transportation, and industrial and other) increased, and forest, water and pasture decreased from 2000 to 2013. Pasture and farmland areas showed the largest changes by area of −284,062 and + 252,103 ha, respectively. Change of settlements and farmlands showed the largest changes by percentage of 80.98% and 42.37%, as their Dynamic index K was 6.23 and 3.26, respectively (*Table 2*).

Main type in HR of vegetation group was desert, which made up > 69% of the entire basin (71.39% in 2000 and 69.28% in 2013). The second largest type was meadow,

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accounting for 9.19% and 7.70% in 2000 and 2013, respectively, followed by steppe and cultural vegetation. Cultural vegetation, alpine vegetation, shrub, and needleleaf forest increased, while desert, meadow, steppe, land without vegetation, and broadleaf forest decreased from 2000 to 2013. Alpine vegetation and cultural vegetation showed large changes by area of −301247.93 and + 252103.67 ha, respectively. Changes of alpine and cultural vegetation showed the largest changes by percentage of 48.21% and 42.37%, as their K values were 3.71 and 3.26, respectively (*Table 2*).

Land cover and vegetation change, and causal factors

At the land-use level, the results show that the Monte Carlo test of the first and all canonical axes had significant correlation ($p < 0.01$). The sum of all canonical eigenvalues was 0.547 and total variance was 1.00. The explained variance contributions of the first and first two axes reached 36.7% and 78.0%, respectively (*Table 3*). Forward selection of variables indicated that change of groundwater depth accounted for 9% of the variance ($p = 0.002$), greater than that of any other variable. It was positively related to change of barren land and pasture. The change of mean annual temperature explained 8% ($p = 0.004$) of the total variance (*Table 4*) and was positively related to change of settlements. Change of pasturage explained 6% ($p = 0.038$) of the total variance and was positively related to change of farmland, and negatively related to change of shrub and forest. Change of mean annual temperature with a time-lag of one year (T1y) explained 5% ($p = 0.044$) of the total variance. Evaporation change was negatively related to barren, pasture change. Precipitation change was positively related to pasture change and negatively related to change of barren land (*Fig. 7*).

Table 3. *Summary of RDA ordination*

Level	Summary	Axes 1	Axes 2	Axes 3	Axes 4
Land use	Eigenvalues	0.201	0.171	0.095	0.044
	Land use-environment correlations	0.845	0.860	0.722	0.528
	Cumulative percentage variance of land use-environment relation	36.7	78.0	84.5	93.4
	Sum of all eigenvalues	1.000			
	Sum of all canonical eigenvalues	0.547			
	Test of significance of first canonical axis	*P-value* = 0.002			
	Test of significance of all canonical axes	*P-value* = 0.002			
Vegetation group	Eigenvalues	0.156	0.1122	0.062	0.029
	Vegetation group-environment correlations	0.830	0.725	0.747	0.575
	Cumulative percentage variance of vegetation-environment relation	37.18	66.17	80.92	87.77
	Sum of all eigenvalues	1.000			
	Sum of all canonical eigenvalues	0.420			
	Test of significance of first canonical axis	*P-value* = 0.002			
	Test of significance of all canonical axes	*P-value* = 0.002			

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Table 4. *Explained variance of variables analyzed by redundancy analyses (China Ministry of Agriculture, 2015)*

Level	Category	Variables	Explained variance (%)	*P*-value
Land use	Climate change	T	8	0.004
		T_{10yr}	4	0.050
		T_{1yr}	5	0.044
		EVAP	4	0.048
		SM100	5	0.040
		ZWT	9	0.002
	Human activity	GVAP/GDP	4	0.014
		GVAP	4	0.040
		PD	5	0.006
		PA	6	0.020
Vegetation group	Climate change	T20yr	11	0.002
		T5yr	7	0.005
		EVAP	5	0.024
		P2yr	4	0.050
		SM100	4	0.052
	Human activity	UP	4	0.048
		GVAP/GDP	4	0.050
		GVAP/AP	4	0.050

Variables abbreviations: mean annual temperature (T), mean annual temperature with a time-lag of one/ten years (T1yr/T5yr/…T20yr), mean annual precipitation with a time-lag of two years (P2yr), evapotranspiration (EVAP), soil moisture measured at 100 cm from surface (SM100), groundwater depth (ZWT) and agricultural GDP (GVAP), agricultural GDP per capita (GVAP/AP), agricultural GDP percentage (GVAP/GDP), agricultural population (AP), urban population (UP), Pasturage (PA), pasturage density (PD)

The results of partial RDA using human activity factors as the explanatory variable and climate change factors as covariates showed that correlation coefficients of the first and second axes of climate change factors and land use were 0.754 and 0.617, respectively. The human activity factors with significant effect explained 26.3% of the variance of land-use change. From the results of partial RDA using climate change factors as the explanatory variable and human activity factors as covariates, the correlation coefficients of the first and second ordinal axes of climate change factors and land use were 0.831 and 0.697, respectively, and 41.3% of the variation of land use was explained by the climate change factors with significant effect.

At vegetation group level, the results show that the Monte Carlo test of the first and all canonical axes had significant correlation ($p < 0.01$). The sum of all canonical eigenvalues was 0.420 and total variance was 1.00. The explained contributions of the first and first two axes reached 37.18% and 76.17%, respectively. Forward selection of variables indicated that change of mean annual temperature of twenty years ago since the study year accounted for 11% of the variance ($p = 0.002$), greater than that of any other variables. That change was positively related to change of desert and broadleaf forest, but negatively related to needleleaf forest, alpine vegetation, steppe, shrub, meadow and land without vegetation. Change of mean annual temperature of twenty

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years ago since the study (T5yr) and evapotranspiration explained 7% ($p = 0.005$) and 5% ($p = 0.024$) of the total variance. T5yr was negatively related to change of cultural vegetation. Agricultural GDP percentage change has a negative relationship with change of cultural vegetation and land without vegetation (*Fig. 8*).

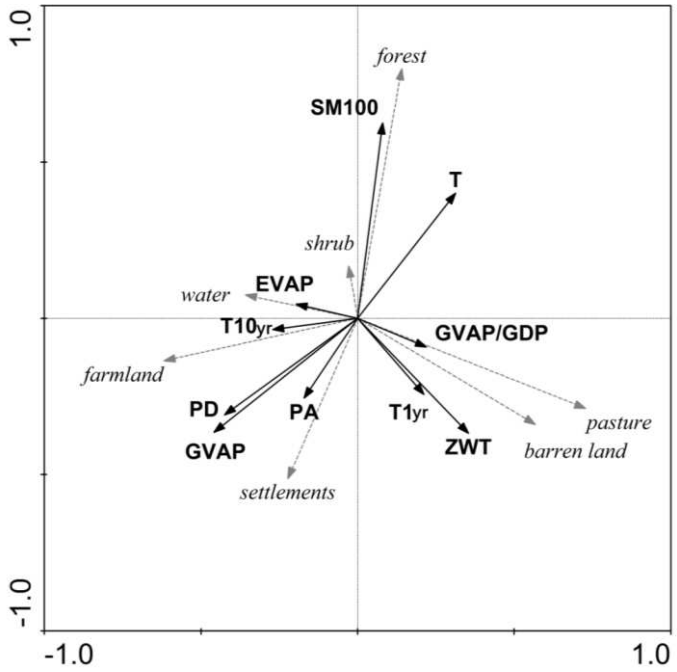

Figure 7. *Redundancy analysis diagram in the HRB for land use types and driving factors. Gray arrows represent land use types, black arrows represent driving factors. The abbreviations are mean annual temperature (T), evapotranspiration (EVAP), oil moisture measured at 100 cm from surface (SM100), groundwater depth (ZWT), T1yr (mean annual temperature of 1999, 2006, 2012), T10yr (mean annual temperature of 1990, 1997, 2003), agricultural GDP per GDP (GVAP/GDP), agricultural GDP (GVAP), pasturage (PA), pasturage density (PD), respectively*

The results of partial RDA using human activity factors as explanatory variables and climate change factors as covariates show that correlation coefficients of the first and second axes of human activity factors and land use were 0.644 and 0.511, respectively. Human activity factors with significant effect explained 17.7% of the variance of vegetation group change. The results of partial RDA using climate change factors as explanatory variables and human activity factors as covariates showed that correlation coefficients of the first and second ordinal axes of change in climate change factors and vegetation group were 0.816 and 0.726, respectively. Moreover, 34.5% of the variation of vegetation group was explained by the climate change factors with significant effect.

Discussion

Previous studies have shown that spatiotemporal heterogeneity of vegetation growth and distribution is impacted by social and natural conditions (Jalut et al., 2009; Vincens et al., 2003). In this study, the influence of society and human beings was quantitatively expressed by population, GDP of agriculture, industry and service, and amount of

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livestock of each region. Through redundant analysis (RDA) and partial redundancy analysis (pRDA), we quantitatively analyze the synergistic effects of human and natural factors and their relative independent effects. The response of vegetation distribution to climatic variations varied among vegetation types. In various regions of HRB, the economy developed rapidly and urbanization accelerated (Cheng et al., 2014). For regions that underwent intensive human landscape change, great effort is needed toward climate mitigation and adaptation (Pielke Sr et al., 2011). At land-use level, Land-use changes are strongly regionalized (Pielke Sr et al., 2011). Although settlement and croplands occupy a little of the total land area, K of them were the biggest. They are highly regionalized into concentrated landscape perturbations (Pielke Sr et al., 2011). It is suggested to do land use planning in the HRB not only in urban area but also in rural area.

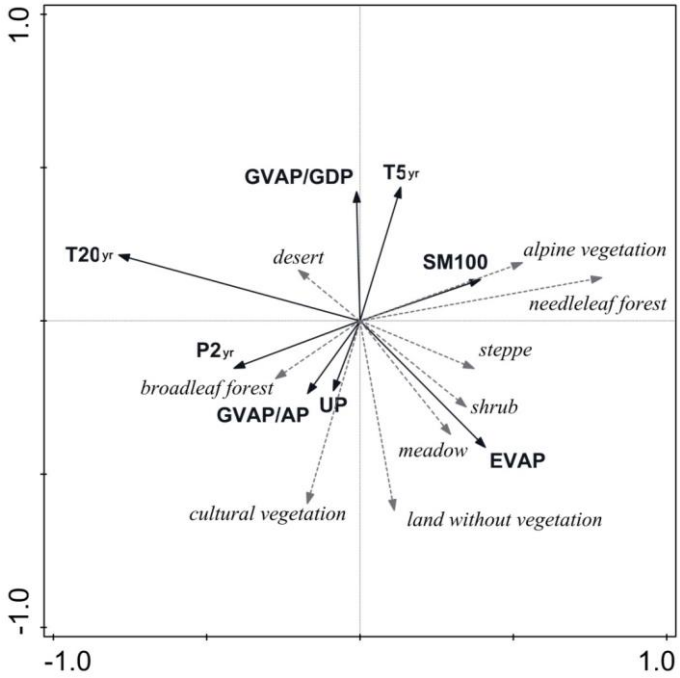

Figure 8. *Redundancy analysis diagram in the Heihe River basin for vegetation groups and driving factors. Gray arrows represent vegetation group types, black arrows represent driving factors. The abbreviations are evapotranspiration (EVAP), oil moisture measured at 100 cm from surface (SM100), 2yr (mean annual precipitation of 1998, 2005, 2011), 5yr (mean annual temperature of 1995, 2002, 2008), 20yr (mean annual temperature of 1980, 1987, 2003), agricultural GDP per capita (GVAP/AP), agricultural GDP per GDP (GVAP/GDP), urban population (UP), respectively*

The relationship between vegetation group and casual factors was weaker than that between land use and drivers. Climate change factors explained more at land-use level (41.3%) than that at vegetation group level (34.5%). Human activity factors explained less at vegetation group level (17.7%) than at land-use level (26.3%). The explanation of constrained ordination between response and explanatory variables at land-use level (54.7%) was much stronger than that at vegetation group level (42.0%). The vegetation responses to climate vary considerably with the diverse spatial patterns and the time-lag effects (Davis, 1989; Wu et al., 2015).

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In the mountainous upper reaches, climate change was the controlling factor This region is presently experiencing a century-long wet period (Qin et al., 2010). Because climate change has caused a transition from warm-dry to warm-wet conditions in northwestern China, rainfall might increase, but rising temperatures may weaken the influence of increased precipitation and runoff from the mountains (Shi, 2003). Although climate change will have a huge impact on vegetation groups and land use in the region, the influence of human activities factors cannot be ignored. Agricultural GDP percentage was an important human activities factor that affected changes of vegetation in this region.

Human activities have been dominant factor for vegetation change in the middle reaches. Increasing population and industrial development will augment greenhouse gas emissions, and continued emission of those gases will cause further warming and long-lasting changes in all components of the climate system (IPCC, 2014). This situation may bring much more pressure on relatively fragile ecosystems that depend on water. Therefore, it is imperative to control population growth and improve industrial structure to restore ecosystem. However, the ecological project started by the local government in 2000 suggested that the direction of the human activity would be an open question, whether negatively or positively influences the vegetation structure.

Analyzing correlation between human activities and natural factors can help to understand human activity changing according to natural factors, and to mitigate climate change through adjusting human activities. At the two classification levels, most relevant implications for agriculture are due to changes in temperature (Fuhrer et al., 2014). At the level of land use, trend direction of PD, GVAP, and AP is opposite to T. The higher the temperature increment is, the higher agricultural GDP, agricultural population and pasturage density decrement are. Agriculture as a primary means by which the impacts of climate change are transmitted to the poor (Hertel and Rosch, 2010). At the level of vegetation type, T5yr is most closely related to human activities. The higher the T5yr rise, the more agriculture sector accounts for GDP increase, the more UP and GVAP/AP decrease. It is good for agriculture, for global warming not so harmful for all plants (Kolanowska, 2017). To increase inherently resilience of agriculture in the HRB, and the capacity to adapt and transform as needed to the climatic changes across this region, such as changed or improved crops, reduced stocking rates, proper grazing management practices, employing animal genetics suited to environments are suggested adaptive management strategies (Havstad et al., 2018).

It also provides a research method for the quantitative study of the effects of natural and human activities on vegetation distribution change. However, it should be noted that the research on the quantitative impact of social factors on vegetation distribution is still not accurate. This is because the acquisition of spatial data of social factors is sometimes difficult. But the contemporary long-time satellite remote sensing data provides an advanced way to monitor the vegetation dynamics in relation to climate variations at different spatiotemporal scales (Huete, 2016; Zhang et al., 2013). The good news is that in June 2018 China's Luojia01 night-time satellite officially started working and provided free night-time light imagery data. The data has a resolution of up to 120 m (Jiang et al., 2018; Zhang et al., 2019; Gao et al., 2019). There is a very significant positive correlation between night-time light imagery data and GDP (Doll et al., 2006; Wu et al., 2013), and the image can provide a good data base of spatial GDP data. It is also a feasible method for quantifying the influence of social factors in the future.

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Conclusion

Vegetation is a key factor adapting to and mitigating climate change. The main goal of the current study was to determine key vegetation dynamics including climate and human activities to better adapt to and mitigate climate change. This study showed that groundwater depth and mean annual temperature with 15-year lag times are the most important factors. It also found that land use planning is necessary not only for urban area but also for rural area in HRB to guide the direction of land-use change, and ultimately affect regional climate change. The research has also provided a research method for the quantitative study of the effects of natural and human activities on vegetation distribution change. Multiscale quantitative impact of social and natural factors on vegetation distribution are suggested with access to high accuracy data. Also examining the land cover changes would be beneficial divided by reaches level because of the different climatic conditions and vegetation.

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THE PHOTOSYNTHETIC PHYSIOLOGY AND GROWTH RESPONSE OF TWO ALGAE SPECIES, *MICROCYSTIS AERUGINOSA* AND *SCENEDESMUS QUADRICAUDA*, TO DIFFERENT NITROGEN FORMS AND CONCENTRATIONS

HE, Y. J.[1] – WANG, R.[1] – MA, D. L.[1] – YIN, X. T.[1] – YANG, K. F.[1] – TIAN, Y. N.[1] – LIU, S. M.[1] – ZHOU, C. X.[2] – HOU, X. L.[1*]

[1]*College of Agronomy and Life Sciences, Kunming University, Kunming 650214, China*

[2]*Yunnan Huanlv Environmental testing technology company, Kunming 650214, China*

[*]*Corresponding author*
e-mail: hxlyn@aliyun.com

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Abstract. Organic nitrogen and inorganic nitrogen were the main nitrogen sources in the north and south of Dianchi Lake, respectively. Thus, the effect of the different concentration (0.5, 1.0, 2.0, 5.0, 10, 20, 50 mg/L) of ammonium nitrogen (NH_4^+-N) and alanine on growth of *Microcystis aeruginosa* and *Scenedesmus quadricauda* was investigated. In NH_4Cl vitro co-culture, *M. aeruginosa* grew better than *S. quadricauda*, and its chlorophyll *a* increased to 2893.94 ug/L in 2 mg/L NH_4Cl group. In vitro alanine treatments, *S. quadricauda*'s chlorophyll *a* was measured at 5034.34 ug/L in 50 mg/L alanine, which was higher than *M. aeruginosa*. *M. aeruginosa* in NH_4Cl vitro monocultures showed better cell structure in 20 mg/L NH_4Cl, and its chlorophyll *a* was higher than that of the corresponding concentration of alanine. The photosynthetic activity (F_v/F_m), the maximum electron transfers rate (ETRmax), and the saturated light intensity point (I_k) of *M. aeruginosa* increased with the ammonium concentration, and decrease with the alanine concentration, indicating that *M. aeruginosa* can tolerate higher concentrations of ammonium chloride. In high concentrations of NH_4Cl, *S. quadricauda*'s cell was seriously damaged, but of alanine alone, it was intact. F_v/F_m, ETRmax, and I_k of *S. quadricauda* increased with the alanine concentrations, showing that *S. quadricauda* makes better use of organic nitrogen.
Keywords: *ammonium, alanine, chlorophyll a, fluorescence characteristic, photosynthesis, cyanobacterial blooms*

Introduction

Nitrogen and phosphorus are key factors of eutrophic freshwater bodies, and the increase in concentrations is generally considered to be the radical cause of cyanobacteria blooms (Conley et al., 2009; Neil et al., 2013; Li et al., 2014; Wang et al., 2015). Outbreaks of cyanobacteria bloom have not been sufficiently prevented, and controlling the total concentration of nutrients is not effective in alleviating the bloom of cyanobacteria. In addition, nutrient morphology also has a corresponding impact on the growth, distribution and community structure of algae (Xu et al., 2019). Nitrogen in natural freshwater body can exist in many forms, for example, dissolved nitrogen not only exists in the form of soluble inorganic nitrogen such as nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), ammonium nitrogen (NH^+_4-N) but also in the form of soluble organic nitrogen such as amino acid, urea, amide, hypoxanthine, and guanine. The bioavailability of the nitrogen affects the absorption rate and utilization efficiency of algae. The utilization characteristics of different forms of nitrogen are closely related to the species of algae. It is generally believed that NH_4^+-N and NO_3^--N are the main forms of nitrogen utilized by phytoplankton (Zhang et al., 2011). However, the presence of NH_4^+-N in water

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may inhibit the uptake of NO_3^--N by cyanobacteria (Zhu, 2007). The high concentration of NH_4^+-N has a certain toxic effect on algae (Tang et al., 2008; Zhang et al., 2011). The main form of organic nitrogen is an amino acid, which comes from phytoplankton. Algae regeneration and death degradation are intertwined, increasing of biomass and organic matter content in the water. In the process of algal bloom, some algal death degradation occurs, which lead to an increase of dissolved organic nitrogen in freshwater (Yu et al., 2016). Therefore, understanding the relationship between amino acids and algal growth is necessary to further understand the ecological relationship between the change of organic matter concentration and algal population. However, some studies focused on the influence of inorganic nitrogen, mainly nitrate and orthophosphate, on algae and algal community structure, and extrapolated the relationship between the change in total concentration and algae growth proposing that the occurrence of cyanobacteria bloom can be controlled by limiting the content of total nitrogen and phosphorus (Wu et al., 2009; Zhu et al., 2018; Peng et al., 2018). However, through investigation of the succession of phytoplankton community in Dianchi Lake, *Microcystis* of cyanophyta and *Scenedesmus* of chlorophyta alternated with seasons. Dianchi lake was divided in 1996 by Haigeng dam into two parts, Caohai Bay in the north and Waihai lake in the south, respectively. The concentration of total nitrogen and total phosphorus in Caohai Bay is higher than that in Waihai lake. *Scenedesmus qusdricauda* becomes the dominant species in Caohai Bay in most months of the year, and their biomass is higher than that of *cyanophyta*, while the dominant species is cyanobacteria in Waihai lake (Shi et al., 2014; Hou et al., 2018). That is to say, freshwater bodies with high nitrogen and phosphorus concentrations are not the only factor for cyanobacteria bloom. The influence of nutrient concentration and form interaction on the growth of *Microcystis aeruginosa* and *Scenedesmus qusdricauda* in the freshwater body of Dianchi Lake needs further study.

M. aeruginosa and *S. quadricauda* which belongs to cyanobacteria and Chlorophyta respectively, are widely distributed in Dianchi Lake. Both of them belong to non-nitrogen fixing algae, so nutrients are one of the limiting factors for their growth. As an autotroph, the photosynthesis of the two species is closely related to the growth of algae and affects the physiological process (Jia et al., 2011). When the growth of algae is limited by available nitrogen, photosynthesis ability to catch light and energy transfer, and carbon fixation are damaged (Geider et al., 1993). Guo (2015) found that the effect of phosphorus concentration on photochemical efficiency of photosystem II (Fv/Fm) was not significant by analysis of the relation between phosphorus concentration and Fv/Fm of Cladophora oligoclora Kütz. The absorption and assimilation of inorganic nitrogen by photosynthetic organisms depends on the energy and carbon skeleton produced by photosynthesis and the metabolic process of nitrogen absorption in chloroplasts and mitochondria of algae, so nitrogen is a necessary ecological factor for energy transfer in photosynthesis (Young and Beardall, 2003). There is a certain relationship between the occurrence of nitrogen sources of different forms and concentrations with photosynthetic activity, photosynthetic efficiency and other parameters (Liu et al., 2019). Therefore, whether the form of the nitrogen source produces stress on algae or whether the ability of algae to resist adversity can be measured by using chlorophyll fluorescence parameters needs further study. Based on these discussions, this study takes *M. aeruginosa* and *S. quadricauda*, which are the dominant species of cyanobacteria and green algae in Dianchi Lake, as representative algal species. Alanine is a common amino acid in natural water, which makes up protein, while the ammonium nitrogen is an inorganic nitrogen widely existing in lake. So this study uses alanine and ammonium chloride as nitrogen sources, to explore the effects of

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different formations and concentrations of nitrogen on the growth of algae, and to study the relationship between the fluorescence parameters of algae from different nitrogen sources, and to explore the physiological response of *M. aeruginosa* and *S. quadricauda* to different nitrogen sources.

Methods

Experimental design

We chose *Microcystis aeruginosa* and *Scenedesmus quadricauda* from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-collection, Wuhan, China). These algae were cultured in medium in a light incubator under the condition of 25℃, a dark/light period of 12 h: 12 h, the light intensity of 3000-4000 lx, and shaken twice a day. The medium is BG11 which is composed of $NaNO_3$, K_2HPO_4, $MgSO_4•7H_2O$, $CaCl_2•2H_2O$, Citric acid, $EDTANa_2$, $CaCO_3$, H_3BO_3, $MnCl_2•4H_2O$, $ZnSO_4•7H_2O$, $Na_2MoO_4•2H_2O$, $CuSO_4•5H_2O$, $Co(NO_3)•6H_2O$. *M. aeruginosa* and *S. quadricauda* cells were collected in the logarithmic growth period by centrifugation (4000 rpm, 8 min). We rinsed two algae 3 times by N -free medium and then inoculated in a medium for 48 h to exhaust nutrients stored in cells. Then, alanine and ammonium nitrogen were used as nitrogen sources with a 5 g/L concentration of nitrogen and was filtered using a 0.22 μm microporous membrane. The concentration gradient of ammonium nitrogen and alanine in each treatment group was 0.5, 1.0, 2.0, 5.0, 10, 20, 50 mg/L. The experiment was divided into three groups. In the first group, *M. aeruginosa* were separately cultured in alanine and ammonium nitrogen at the above N concentration gradient. In the second group, monocultures of *S. quadricauda* were separately exposed to two nitrogen sources, alanine and ammonium, under the same N concentration gradient. In the third group, *M. aeruginosa* and *S. quadricauda* were cultured together at the above N concentration gradient with respectively ammonium nitrogen and alanine. Before combining *M. aeruginosa* and *S. quadricauda* into a co-culture, we counted the algae cells under the microscope to ensure that the number of algae cells is approximately the same value with 1.7×10^6 cells/ml. In all, each treatment group of the experiment was performed in triplicate. Each treatment group was cultured for 18 days, and 3 ml of algal solution in each treatment group was taken every 2 days for determination of physiological characteristics and chlorophyll *a*. At the end of culture period, the cell morphology of two algal was monitored under a microscope and the morphological pictures were obtained by scope photo 3.0.

Analytical methods

The chlorophyll fluorescence characteristic of algae was determined by phyto PAM (Walz, Germany). Samples need to be dark-adapted for 20 minutes. Then, chlorophyll fluorescence technology is a vivo measurement technology to detect the photosynthetic physiological status and the subtle influence of the external environment on algae which is based on using chlorophyll as an indicator of fitness. The Hansatech Flurescence Monitoring System PAM (Phyto-PAM, WALZ, Germany) was used to measure the chlorophyll *a*, chlorophyll fluorescence parameter of *M. aeruginosa* and *S. quadricauda* every two days in cultural period. The maximum photochemical efficiency (Fv/Fm) of photosystem II (PSII) is to measure the maximum chlorophyll fluorescence (Fm) of algal cells under dark adaptation. The formula described by Genty et al. (1989) is as follows:

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Fv/Fm=(Fm-Fo)/Fm, where Fm is the maximum fluorescence, which is measured when all reaction centers of PSII are completely closed and all non-photochemical processes are in the minimum under dark adaptation state. Fo is the initial fluorescence, which is the fluorescence value when all reaction centers of PSII are completely open and all non-photochemical processes are in the minimum under dark adaptation state. Fv is the maximum variable fluorescence when all non-photochemical processes are in the minimum under dark adaptation state, Fv=Fm- Fo. According to Ting and Owens (1992), the rapid light curve was measured under nine light intensity gradients (90, 162, 226, 334, 486, 707, 1075, 1586, 2343 $\mu mol \cdot m^{-2}s^{-1}$). The relative electron transfer rate of PSII (ETR) is calculated by the formula as follows: rETR = Yield×0.5×PFD, where Yield is the effective photochemical efficiency of PSII, the coefficient of 0.5 represents that 50% of all absorbed photons are allocated to PSII, PFD is the intensity of photochemical light. The maximum relative electron transfer rate (ETRmax) and photosynthetic efficiency (α) were obtained by fitting the rapid light response curve with original software (8.0) according to the exponential formula ETR=ETRmax×[1-exp(-α×PFD/ETRmax)]. The semi-saturated light intensity (I_k) reflects the tolerance of the sample to strong light, and the calculation formula is as follows: I_k=ETRmax/α. The average chlorophyll *a* and chlorophyll fluorescence parameter of *M. aeruginosa* and *S. quadricauda* were caculated by Microsoft Excel (2010) and Origin 8.0 software.

Result

Comparison of chlorophyll a content of M. aeruginosa and S. quadricauda in co-culture experiment with separately two nitrogen sources

The content of chlorophyll *a* of *M. aeruginosa* and *S. quadricauda* in a co-cultured experiment with different concentrations of alanine as nitrogen source is shown in *Figure 1*. The utilization advantage of alanine by *S. quadricauda* is higher than that of *M. aeruginosa*. The content of chlorophyll *a* of *S. quadricauda* was higher than that of *M. aeruginosa*, and increased with alanine concentration during the co-culture period. However, the chlorophyll *a* content of *M. aeruginosa* decreased with time in co-culture experiment. After co-culture in 12 days, the content of chlorophyll *a* of *S. quadricauda* in the group of 50 mg/L alanine was as high as 5034.34 ug/L, followed by that in the treatment group of 20 mg/L alanine, which was 4076.58 ug/L. At the end of co-culture period, the content of chlorophyll *a* in *M. aeruginosa* was 0 mg/L. In the range of 0.5-5 mg/L alanine concentration, the content of chlorophyll *a* of *S. quadricauda* was between 2603.93-2706.78 ug/L. The highest chlorophyll *a* of *M. aeruginosa* was 339.38 ug/L, which was far lower than that of *S. quadricauda*.

The content of chlorophyll *a* of two algae species in the co-cultured experiment with different concentrations of ammonium is shown in *Figure 2*. In different concentrations of ammonium treatment group, the chlorophyll *a* in *M. aeruginosa* was higher than that of *S. quadricauda*, which indicated that the utilization advantage of *M. aeruginosa* to ammonium chloride was higher than that of *S. quadricauda*. The *M. aeruginosa*'s chlorophyll *a* rose with the culture time, while the chlorophyll *a* of *S. quadricauda* showed the opposite trend. After 16 days of co-culture, the chlorophyll *a* content of *M. aeruginosa* in each ammonium treatment group was twice the initial content, and the highest content of chlorophyll *a* of it was 2893.94 ug/L in the 2 mg/L NH_4Cl group. In the entire culture cycle, the highest chlorophyll *a* of *S. quadricauda* was 891.14 ug/L, which was far lower than that of *M. aeruginosa*.

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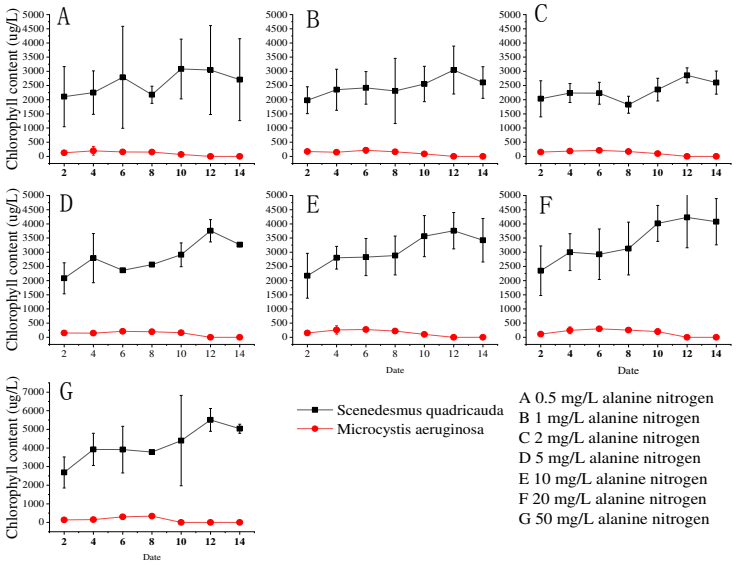

Figure 1. *Chlorophyll a of M. aeruginosa and S. quadricauda in co-cultured experiment with alanine nitrogen source. The error bars is standard deviation of Chlorophyll a*

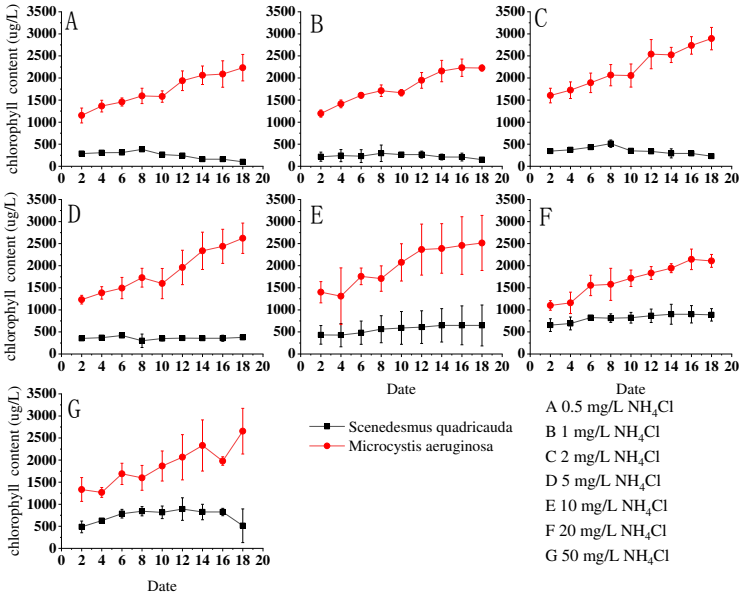

Figure 2. *Chlorophyll a of M. aeruginosa and S. quadricauda in co-culture experiment with ammonium nitrogen source. The error bars are standard deviation of Chlorophyll a*

The chlorophyll a content of M. aeruginosa and S. quadricauda under two nitrogen sources in monoculture experiment

Using alanine and ammonium as nitrogen sources, we cultured the *S. quadricauda* and *M. aeruginosa* respectively. The changing trend of chlorophyll *a* of the two algae is shown in *Figure 3*. In the ammonium nitrogen treatment group (*Figure 3A*), the chlorophyll *a* content of *M. aeruginosa* increased with time in the high concentration of 10, 20, 50 mg/L ammonium nitrogen treatment group, and the content was higher than

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that of other groups. In 0.5, 1, 2, 5 mg/L low concentration ammonium nitrogen treatment groups, the content of chlorophyll *a* of *M. aeruginosa* increased at the initial stage of culture, and decreased gradually after 11 days, which was lower than that of high concentration ammonium group. At the end of the culture period, the chlorophyll *a* content of *M. aeruginosa* was 1.74, 1.54, 1.16 mg/L separately in 50, 20, 10 mg/L ammonium nitrogen group, which was 1.7, 1.5 and 1.3 times higher than that in the initial culture. The chlorophyll *a* content of *M. aeruginosa* was 0.72, 0.73, 0.87, 0.93 mg/L separately in 0.5, 1, 2, 5 mg/L ammonium nitrogen group, which was slightly higher or lower than that in the initial culture. In the alanine treated group (*Figure 3B*), the chlorophyll *a* content of *M. aeruginosa* decreased with the culture time, and the chlorophyll *a* in each treated group was lower in the end of the experimental phase than that at the beginning phase. In the entire growth period, the *M. aeruginosa*'s chlorophyll *a* in 0.5, 1, 2 mg/L alanine group was higher than that of other high concentration groups. The results showed that the content of chlorophyll *a* of *M. aeruginosa* in low alanine concentration group was higher than that in high concentration group, especially in 20 mg/L and 50 mg/L alanine group, the chlorophyll *a* content of *M. aeruginosa* was lower than that of other treatment groups. Comparison of ammonium nitrogen and alanine treated groups, we discovered that the growth potential of *M. aeruginosa* increased with the high of the concentration of ammonium nitrogen, and the maximum growth potential happened in the treatment of 20 mg/L and 50 mg/L of ammonium nitrogen, but in the treatment of alanine, the growth trend of *M. aeruginosa* decreased with the increase of the concentration of alanine, which indicated that *M. aeruginosa* has better utilization of inorganic nitrogen than organic nitrogen.

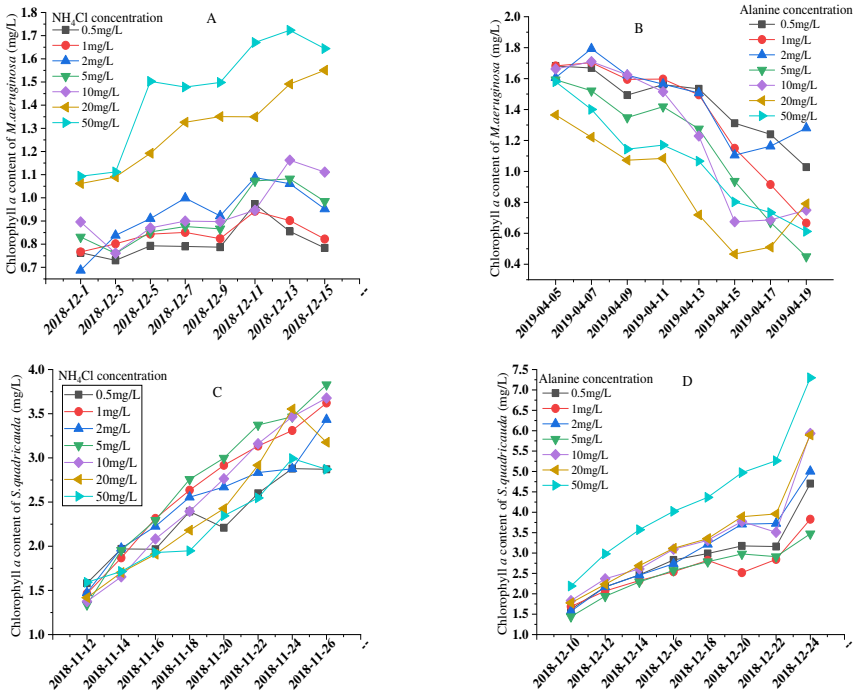

Figure 3. *Chlorophyll a of in M. aeruginosa and S. quadricauda in monoculture experiment with ammonium and alanine separately. M. aeruginosa and S. quadricauda from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-collection, Wuhan, China) were cultured in medium in a light incubator for 2 months. Then these algaes were used in the experiment*

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S. quadricauda was cultured respectively with alanine and ammonium as nitrogen sources (*Figure 3C, D*), chlorophyll *a* content is as follows, the growth of *S. quadricauda* was the highest in the treatment group of 5 mg/L and 10 mg/L concentration ammonium, that the chlorophyll *a* increased nearly three times than that in the beginning phase. With the increase of ammonium concentration, the growth of *S. quadricauda* tended to decline. The growth potential of *S. quadricauda* in 1 mg/L and 5 mg/L alanine treatment group was the worst. In 50 mg/L alanine treatment group, the *S. quadricauda*'s chlorophyll *a* increased rapidly and reached to 7.3 mg/L. Comparison to ammonium and alanine treated groups, we found that the chlorophyll *a* of *S. quadricauda* (*Figure 3D*) in alanine treatment groups was higher than that in the corresponding concentration of ammonium treatment group, which indicated that organic nitrogen (alanine) is better than inorganic nitrogen (ammonium nitrogen) for the growth of *S. quadricauda*. At the end of experimental culture phase, chlorophyll *a* of *S. quadricauda* was the same value of 2.87 mg/L in the treatment group of 0.5 mg/L and 50 mg/L ammonium nitrogen, which were the lowest among all the ammonium treatment groups. The *S. quadricauda*'s chlorophyll *a* was the highest in the treatment group of 5 mg/L and 10 mg/L ammonium, which indicated that 5-10 mg/L ammonium nitrogen was more suitable for the growth of *S. quadricauda*, and 0.5 mg/L extremely low concentration and 50 mg/L extremely high concentration ammonium did not benefit to *S. quadricauda*. In the alanine treatment group, chlorophyll *a* of *S. quadricauda* was the highest in the extremely high concentration of alanine 50 mg/L and 20 mg/L treatment groups, which were 7.30 mg/L and 5.89 mg/L, respectively, which indicated that *S. quadricauda* was more suitable for the high concentration of organic nitrogen.

Comparison of photosynthetic activity of M. aeruginosa and S. quadricauda under two nitrogen sources

The photosynthetic activity of *M. aeruginosa* in the treatment group of ammonium and alanine is shown in *Figure 4*. In the 50 mg/L ammonium group (*Figure 4A*), the average photosynthetic activity was 0.52 higher than that of other groups, which is the range of 0.47-0.60 in the whole growth period. In the 0.50 mg/L ammonium group, its value was the lowest with the average value of 0.36 and the range of 0.23-0.46. The photosynthetic activity of *M. aeruginosa* rose with the increase of ammonium concentration, indicating that *M. aeruginosa* grew strongly in the high concentration of ammonium. The average photosynthetic activity of *M. aeruginosa* in 10, 20, and 50 mg/L alanine treatment groups (*Figure 4B*) were the highest, with values of 0.49, 0.52 and 0.53, and the range value of 0.31-0.62, 0.49-0.61 and 0.47-0.60, respectively. The average photosynthetic activity of *M. aeruginosa* in the ammonium group was higher than that in the same alanine concentration group, showing that the growth potential of *M. aeruginosa* in ammonium nitrogen was higher than that in alanine organic nitrogen.

For *S. quadricauda* (*Figure 4C, D*), the mean photosynthetic activity was the highest (0.74 and 0.75, respectively) in the 5 mg/L and 10 mg/L ammonium treatment group, and the range of values was 0.68-0.80 and 0.66-0.87, respectively, the lower value appeared in the 0.5 mg/L, 1 mg/L and 50 mg/L ammonium group, which indicated that the photosynthetic activity of *S. quadricauda* was very low in very low and high concentration of ammonium nitrogen and the photosynthetic activity of *S. quadricauda* was strong in the medium concentration of ammonium nitrogen. Among the alanine treatment groups (see *Figure 4D*), the highest average photosynthetic activity of *S. quadricauda* was found in 20 mg/L and 50 mg/L alanine treatment group with the

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identical mean values of 0.75 and the range value with 0.66-0.87 and 0.67-0.81, respectively, and these values were higher than that in the corresponding same concentration ammonium treatment group, which indicated that the growth potential of *S. quadricauda* in the high concentration of alanine was higher than that in the high concentration of ammonium.

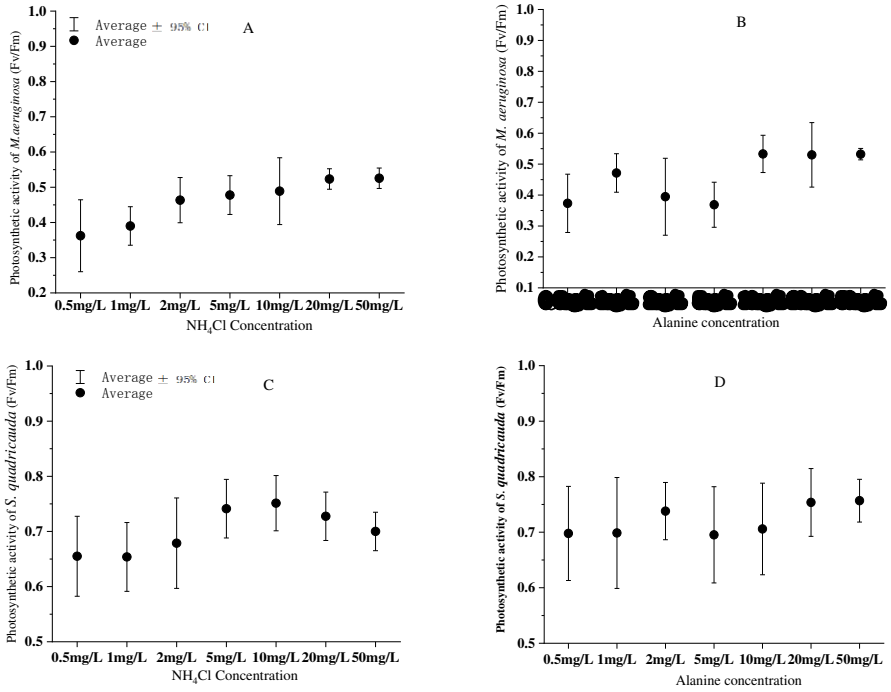

Figure 4. *The photosynthetic activities of M. aeruginosa and S. quadricauda under different nitrogen sources. These values were measured every 2 days during the 18-day culture cycle*

The maximum photosynthetic rate difference of M. aeruginosa and S. quadricauda in monoculture under two nitrogen sources

Phyto PAM was used to determine the rapid light response curve of *M. aeruginosa* and *S. quadricauda* under the separate cultivation of two nitrogen sources. The characteristic parameter of the maximum electron transport rate (ETRmax) is to characterize the photosynthesis efficiency of phytoplankton, and the data is shown in *Figure 5*. In the high of ammonium concentration such as 10, 20, 50 mg/L treatment group (in *Figure 5A*), the highest variation of ETRmax value of *M. aeruginosa* was found in the whole culture period. After 9 days, the ETRmax of *M. aeruginosa* in the 50 mg/L ammonium group increased gradually, which was higher than that in the other concentration group. And its value of *M. aeruginosa* in 10, 20 mg/L ammonium treatment group increased in the initial period, and then decreased after 9 days. In the alanine treatment group (see *Figure 5B*), the ETRmax of *M. aeruginosa* in the high concentration 10, 20, 50 mg/L treatment group was higher than that of the low concentration treatment, and increased gradually with the culture time, reaching the maximum value at 15th day, and then decreased gradually. At the end of the experiment, the ETRmax of *M. aeruginosa* in 50 mg/L alanine group is the highest, followed by 20 mg/L alanine treatment group.

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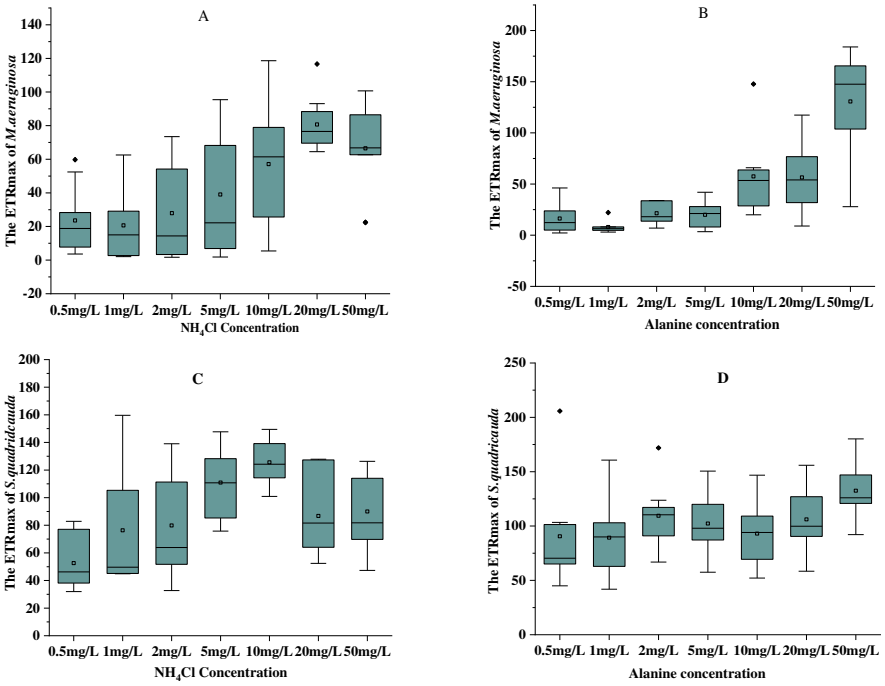

Figure 5. *The ETRmax of M. aeruginosa and S. quadricauda under different nitrogen sources. These values were measured every 2 days during the 18-day culture cycle*

The ETRmax of *S. quadricauda* treated with ammonium and alanine is as following. In the ammonium treatment group (*Figure 5C*), except for the 10 mg/L treatment group, the ETRmax of *S. quadricauda* in the other ammonium treatment groups decreased with the culture time in the growth period. The ETRmax of *S. quadricauda* in the 10 mg/L and 5 mg/L ammonium groups (see *Figure 5C*) was higher than that in the other groups, while that value in the low concentration ammonium (0.5 mg/L and 1 mg/L) treatment group was the lowest. In the alanine treatment group (*Figure 5D*), the ETRmax of *S. quadricauda* in the 50 mg/L group was higher than that in the other groups, and this value in the low concentration alanine group (0.5 mg/L and 1 mg/L) was the lowest.

The difference of saturated light intensity (I_k) between M. aeruginosa and S. quadricauda under two nitrogen sources

The saturated light intensity (I_k) indicates the adaptability of phytoplankton to light intensity. The I_k values of *M. aeruginosa* and *S. quadricauda* under the monoculture of two nitrogen sources are shown in *Figure 6*. The median value of I_k of *M. aeruginosa* in the two nitrogen source treatments grew with the increase of nitrogen concentration (see *Figure 6A, B*), which showed that *M. aeruginosa* had stronger adaptability to strong light if it was cultured in the high concentration of alanine and ammonium nitrogen. The variation ranges of I_k of *M. aeruginosa* in the high concentration of alanine group were wide, while in the high concentration of ammonium nitrogen group the variation of this was small. Among the ammonium treatment groups (*Figure 6 C*), the median value of I_k of *S. quadricauda* was the lowest in the 0.5 mg/L ammonium group, and the I_k of *S. quadricauda* in 20 mg/L and 50 mg/L treatment groups was only higher than that in 0.5 mg/L ammonium treatment group, but lower than that in other treatment groups, and the fluctuation range was large.

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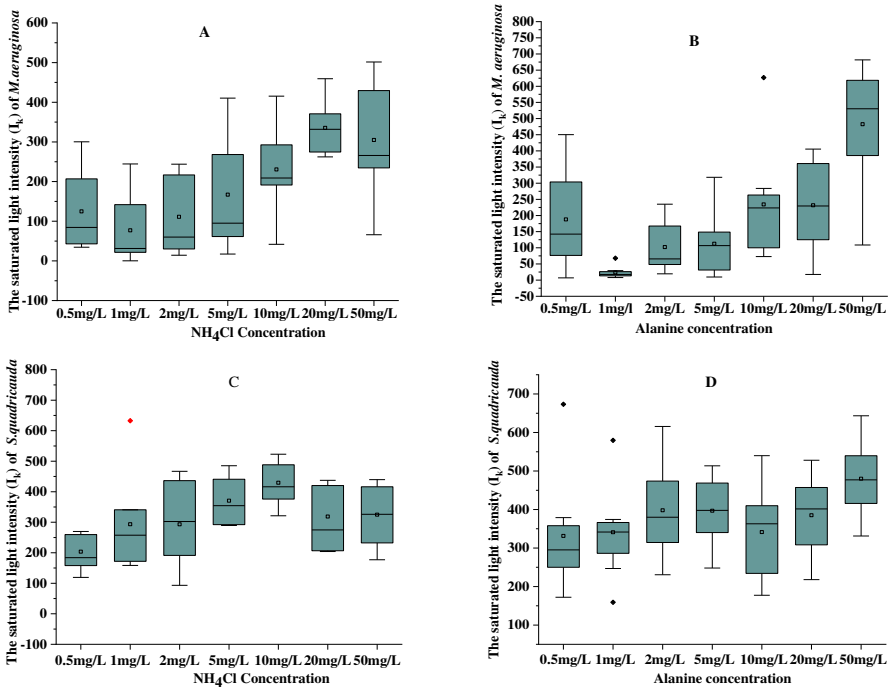

Figure 6. *The saturated light intensity (I_k) of M. aeruginosa and S. quadricauda under different nitrogen sources. These values were measured every 2 days during the 18-day culture cycle*

The I_k of *S. quadricauda* increased gradually in the alanine treatment group (*Figure 6D*), and the median I_k of *S. quadricauda* in 50 mg/L alanine treatment was the highest, and the fluctuation range of this value was very small, which indicated that the high concentration of alanine was better than the low concentration group. The ability of *S. quadricauda* in high concentration of alanine treatment to resist strong light was stronger than that in the low concentration group. In the high concentration (20 mg/L and 50 mg/L) and low concentration (0.5 mg/L and 1 mg/L) of alanine treatment group (*Figure 6D*), the saturation light intensity (I_k) of *S. quadricauda* was higher than that of the corresponding concentration of ammonium nitrogen treatment group, which indicated that the tolerance of *S. quadricauda* to strong light in organic nitrogen source was higher than that of inorganic nitrogen source.

The cell morphology of M. aeruginosa and S. quadricauda under two nitrogen sources

The plastid morphology of *S. quadricauda* under monoculture of two nitrogen sources is shown in *Figure 7*. In the ammonium treatment, *S. quadricauda* is mainly composed of two cells and four cells, and these cells were suffered seriously with the higher concentration of ammonium nitrogen. In the alanine experimental group, the content of chlorophyll of *S. quadricauda* was higher and the complete the plastid morphology was cell integrity with the increasing concentration of alanine, which indicated alanine benefited to maintain plastid integrity of *S. quadricauda*.

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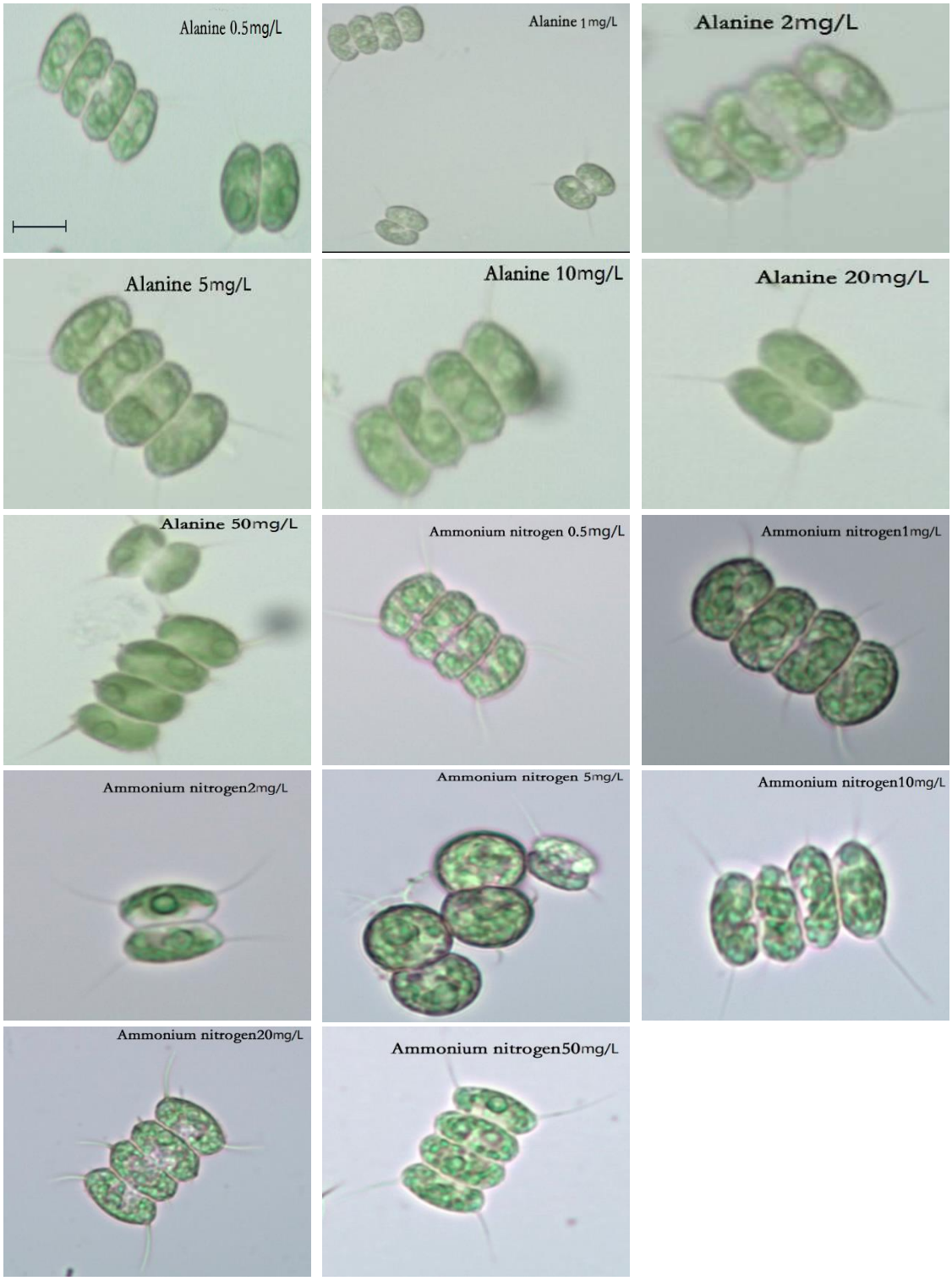

Figure 7. *Cells of S. quadricauda. Scale bars indicate 10 μm*

The plastid morphology of *M. aeruginosa* cultured with two nitrogen sources is shown in *Figure 8*. In the ammonium treatment group, the plastid of *M. aeruginosa* kept intact under the concentration of 0.5-20 mg/L NH_4Cl. When *M. aeruginosa* was cultured in 50 mg/L concentration of ammonium nitrogen, the cell chlorophyll showed a certain

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reduction. In the alanine group, the *M. aeruginosa* cells in the 0.5-5 mg/L concentration treatment group remained intact. When the alanine concentration was more than 10 mg/L, the damage of *M. aeruginosa* cells was more and more serious. It can be seen from the figure that the plastid of *M. aeruginosa* can tolerate a high concentration of inorganic nitrogen, while it suffered more serious in high concentration of organic nitrogen.

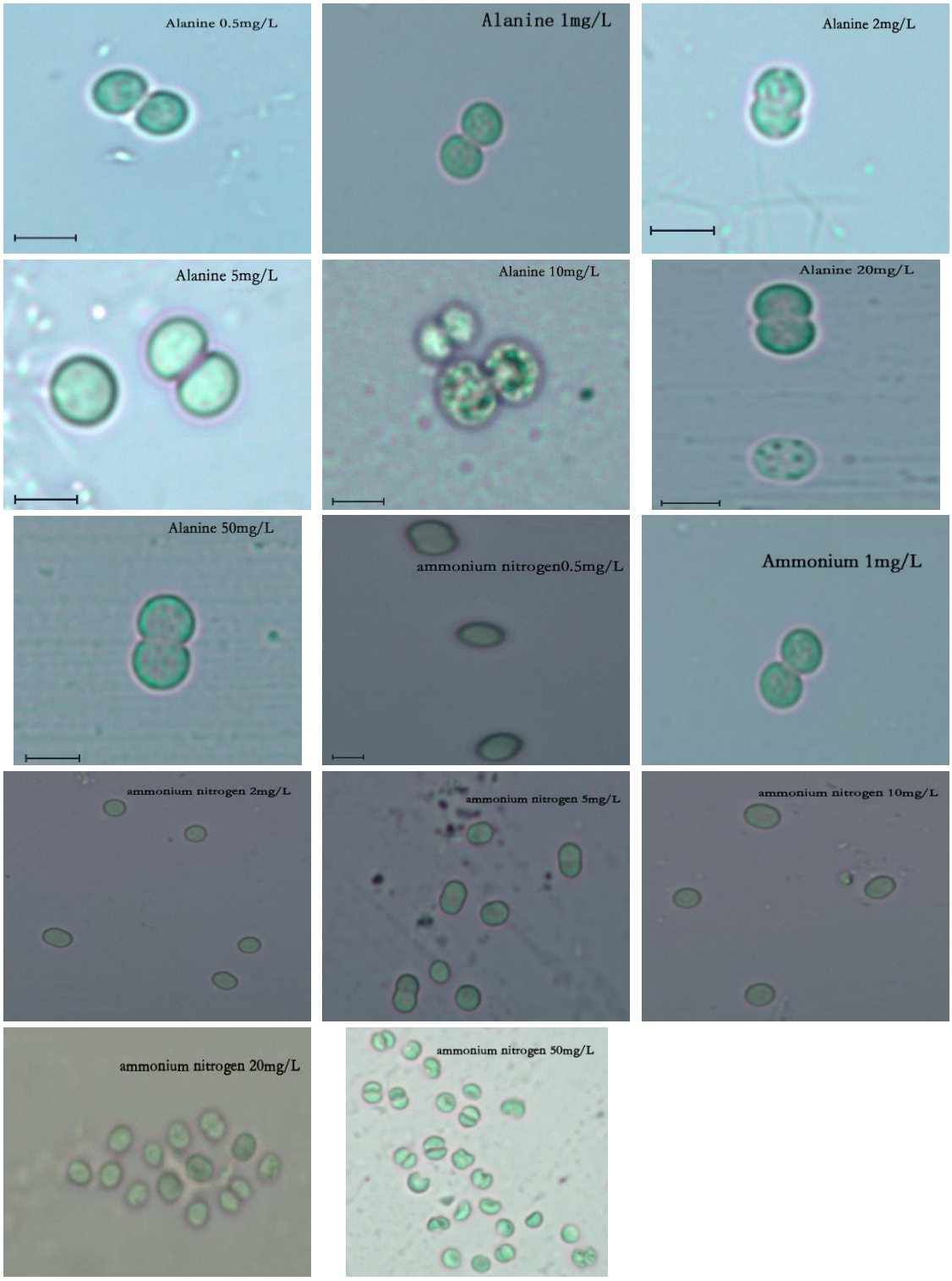

Figure 8. *Cells of M. aeruginosa. Scale bars indicate 10 μm*

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Discussion

Effect of different nitrogen on the growth of M. aeruginosa and S. quadricauda

From *Figure 1* and *Figure 2*, *M. aeruginosa* have a strong advantage in the utilization of ammonium, while *S. quadricauda* has a strong advantage in the utilization of alanine in the co-culture experiment. Because *M. aeruginosa* is a unicellular algae, it is generally believed that the utilization ability of unicellular algae to reduced ammonium salt (NH_4Cl) is better than other nitrogen forms (Muro-Pastor and Florencio, 2003). This is mainly due to other nitrogen sources needing to be reduced to ammonia by nitrate reductase and nitrite reductase before these nutrients can be used. This reduction pressure forces *M. aeruginosa* to face the energy consumption for photosynthesis (Michard et al., 1996; Giani and Delgado, 1998; Meng et al., 2015). It is generally believed that alanine, as a small molecule organic compound, can not only enter cells through passive diffusion, but also enter cells through active transport. Moreover, *M. aeruginosa*, in the alanine treatment group, decreased with culture period (see *Figure 3*), which shows that not all phytoplankton can use organic nitrogen sources efficiently. Since *M. aeruginosa* effectively prefers to utilize ammonium nitrogen, we should pay attention to the role of microorganisms in promoting *M. aeruginosa* to become the dominant species through ammonization in lake. At present, the main management measure of eutrophic water body is to reduce the input of total nitrogen. However, Wu et al. (2013) pointed out that microbial community structure is very important for the change of available nitrogen sources into different forms. So algae control should not only be limited to the control of total nitrogen, but also focus on reducing the input of various nitrogen sources due to the ammonification of microorganisms.

Different algae species have different abilities to utilize different forms of nitrogen. Compared with *M. aeruginosa*, *S. quadricauda*, as multicellular algae, needs more nitrogen to maintain cell proliferation. Each cell of *S. quadricauda* has a peripheral and protein nucleus (Hu and Wei, 2006), and light energy is converted into chemical energy depending on the photosynthetic pigment in these melanosomes (Yan et al., 2012; Chandler et al., 2014). In this study, with the increase of NH_4^+- N concentration (see *Figure 7*), the degree of damage on melanosomes in *S. quadricauda* increased gradually, which directly affected its photosynthetic efficiency. However, when alanine was used as the nitrogen source, melanosomes of *S. quadricauda* were in good condition as the alanine concentration increased. Therefore, whether cultured alone or together with *M. aeruginosa*, the growth potential of *S. quadricauda* is better with alanine nitrogen than that of ammonium nitrogen, and it has better utilization advantages with alanine nitrogen than that of ammonium nitrogen. Hou et al. (2018) studied the relationship between phytoplankton succession and nutrients in Dianchi Lake and found that *S. quadricauda* was the main dominant species in the north of Dianchi Lake (Caohai Bay) with high total nitrogen and phosphorus in Caohai Bay, and *M. aeruginosa* is the dominant species in the south of Dianchi Lake (Waihai lake). This phenomenon is because that the Caohai Bay mainly receives the treated municipal wastewater with the higher organic matter while Waihai lake receives its main nitrogen source from agricultural non-point source pollution. Therefore, in the same period, *S. quadricauda* has a strong advantage in the utilization of organic nitrogen and developed into the dominant species in Caohai Bay, while *M. aeruginosa* is the dominant species in Waihai lake. In this study, it was found that in both ammonium and alanine nitrogen sources, *S. quadricauda* forms four-cell morphological algae structures that are sensitive to settle in water. This is conducive to

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the growth of submerged plants and their strong competition for light and nutrition (Wang et al., 2009; Dong et al., 2013; Yang et al., 2015).

Different algae species not only have a distinguishable ability to use a variety of forms of nitrogen, but also have different requirements for nitrogen concentration for its growth. In our study, high concentrations of ammonium nitrogen (> 20 mg/L) in the 16 days' culture period were beneficial to *M. aeruginosa* by increasing chlorophyll *a* content. This result did not coincide with reports which state high concentrations of NH_4^+-N can produce toxic effects on *M. aeruginosa* and affect the normal algae growth (Azov and Goldman, 1982; Zhou et al., 2013). *M. aeruginosa*'s chlorophyll *a* in the stress of high concentrations of ammonium chloride is to lag, so that the toxic effect of high concentrations of ammonium chloride is not obvious in the 16 days' culture cycle. However, other studies suggested that the growth of *M. aeruginosa* is limited when the ammonium nitrogen concentration reaches 50 mg/L (Dai et al., 2017). As autotrophs for photosynthesis, the photosynthetic activity of *M. aeruginosa* is directly related to the growth potential. When the ammonium nitrogen concentration is high (> 10 mg/L), the photosynthetic activity Fv/Fm of *M. aeruginosa* is constantly rising, and higher than that of the low concentration of ammonium treatment group (see *Figure 4*). In terms of cell morphology of *M. aeruginosa*, when the ammonium nitrogen concentration is 0.5-20 mg/L, plastids remain intact. When the ammonium nitrogen concentration reaches 50 mg/L, the chlorophyll in the plastids of *M. aeruginosa* show slight damage. These results showed that *M. aeruginosa* prefers high concentrations of ammonium nitrogen because it can maintain high photosynthetic activity with sound plastid structure in ammonium nitrogen water. some other research shows that there is no significant difference in the absorption of urea (organic nitrogen) and ammonium chloride by *M. aeruginosa*, and the absorption of these nitrogen forms by *M. aeruginosa* was significantly higher than that of nitrate-nitrogen (Xu et al., 2019). However, our study found that the chlorophyll *a* of *M. aeruginosa* in the high concentration of alanine nitrogen (> 20 mg/L, organic nitrogen) was lower than that of other concentrations of alanine, and declined sharply as the culture time passed (see *Figure 3*). This indicated that not all small organic molecular forms of nitrogen can effectively promote *M. aeruginosa* growth. Alanine ($CH_3CH(NH_2)COOH$) is a carboxyl compound and an acidic amino acid, and the pH value of the alanine culture medium is unfavorable to the pH environment required for the growth of *M. aeruginosa*. In the growth process, *M. aeruginosa* absorbs CO_2 and HCO_3^-, through photosynthesis, and removes H^+ in the culture medium increasing the pH value. The high pH environment rapidly accelerates the growth of *M. aeruginosa* (Yu et al., 2016; Zhu et al., 2018).

Effects of different nitrogen on Photosynthetic Physiological Characteristics of M. aeruginosa and S. quadricauda

One of the fundamental indexes reflecting the potential maximum photosynthetic capacity (photosynthetic efficiency) is the maximum conversion efficiency (Fv/Fm) of PS Ⅱ which is the ratio of the maximum variable fluorescence to the total fluorescence. The measurement of Fv/Fm is often applied as a conventional means to investigate the response of algae to the environment because this parameter will decrease if algae is stressed by alien species or detrimental growth conditions (Björkman and Demmig, 1987; Krause, 1988; Xu et al., 1992; Han et al., 2005; Liu et al., 2019). The growth potential of algae in fresh water increases as photosynthetic activity increases, so the possibility of algae becoming the dominant species rises. In this experiment, the photosynthetic activity

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(Fv/Fm) of *M. aeruginosa* was enhanced as the level of ammonium concentration increased, and the chlorophyll *a* increased correspondingly, which indicated that there was a certain positive correlation between the photosynthetic activity and biomass. With an increase of ammonium concentration, *M. aeruginosa* had higher photosynthetic activity and grew rapidly. *M. aeruginosa* had low Fv/Fm in 0.5 mg/L and 1 mg/L ammonium culture medium, and its growth potential was weakened, which may be the result of insufficient nutrients (Young and Beardall, 2003). Although chlorophyll *a* content of *M. aeruginosa* declined sharply with of the higher alanine concentration, the Fv/Fm of *M. aeruginosa* went up to the high value in the high alanine concentration (> 20 mg/L) culture medium, which may be because the high concentration of alanine did not cause any stress on the light energy conversion rate of *M. aeruginosa*, and it was not necessary to change the parameter Fv/Fm of photosynthetic activity to adapt to the high concentration of alanine environment (Wang, 2018). For *S. quadricauda*, the Fv/Fm in the high concentration ammonium culture medium (> 20 mg/L) was lower than that of the same concentration alanine treatment group, which suggested that the photochemical activity of PSII in *S. quadricauda* may be destroyed, and the photosynthetic electron transfer may be blocked under the stress of high concentrations of ammonium nitrogen. It is generally believed that Fv/Fm will be significantly reduced under the stress of high ammonium nitrogen or low ammonium nitrogen, which will lead to the decrease of biomass and chlorophyll fluorescence parameters (Young and Beardall, 2003). Most of the plastid of *S. quadricauda* are absent due to high concentrations of non-protonation ammonium nitrogen inhibiting photosynthetic activity (Dai et al., 2017). In this study, the plastid structure of *S. quadricauda* was damaged in the ammonium nitrogen culture medium, and the photosynthetic pigments in cells that convert light energy into stable chemical energy were also damaged hindering the process of photosynthetic electron transfer (Yang et al., 2015). It is a physiological reaction process for *S. quadricauda* to adapt to the stress of nutrients through the down regulation of photosynthesis (Lin et al., 1997). However, in the alanine nitrogen culture medium, Fv/Fm of *S. quadricauda* increased with the higher alanine concentration. As a small molecular amino acid, alanine can be absorbed into the cell by the way of active transport. The protein on the cell wall functions as a carrier, accelerating the rate of alanine entering the cell (Fang et al., 2013). Therefore, the growth potential of *S. quadricauda* in organic nitrogen water body is stronger than inorganic nitrogen water.

The higher the maximum photosynthetic rate (ETRmax) indicates that algae are less susceptible to photo inhibition under strong light conditions, and the smaller the ETRmax value indicates that the electron transfer is limited. The saturated light intensity (I_k) is adaptable in algae to compensate strong light stress. Whether *M. aeruginosa* was cultured in alanine alone or in ammonium alone, *M. aeruginosa* had a higher maximum photosynthetic rate (ETRmax) and a higher I_k as the nitrogen source concentration increased in each culture. This showed that these photosynthetic parameters of *M. aeruginosa* were not sensitive to different nitrogen sources. The actual photosynthetic capacity of *M. aeruginosa* was not affected by the variety of nitrogen sources, and this undoubtedly increased the ecological range of *M. aeruginosa* to adapt to nitrogen, and also improved its survival probability and competitiveness. Some scholars suggested that temperature and light intensity were the main factors affecting the photosynthetic activity of algae rather than the chemical form of nutrients, finding that the ETRmax value of some algae decreased significantly under 12800 lx light intensity (Zang et al., 2015). The change of Fv/Fm, ETRmax and I_k values in both ammonium and alanine nitrogen groups

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was similar to that of chlorophyll *a* in *S. quadricauda*, indicating that there was no need for *S. quadricauda* to increase these parameters to adapt to the changes in ammonium nitrogen and alanine concentration gradients, while the other view was that under the stress of high ammonium nitrogen or nitrogen limitation, some chlorophyll fluorescence parameters of algae would increase to adapt to the changing living environment (Wang et al., 2012), the mechanism needs to be further explored.

Conclusion

M. aeruginosa and *S. quadricauda* have strong advantage in the utilization of ammonium and alanine, respectively. The chlorophyll *a* and the photosynthetic activity Fv/Fm of *M. aeruginosa* is constantly rising in high concentrations of ammonium nitrogen (> 20 mg/L). Whether *M. aeruginosa* was cultured in alanine alone or in ammonium alone, *M. aeruginosa* had a higher maximum photosynthetic rate (ETRmax) and the higher saturated light intensity (I_k), indicating that these parameters of *M. aeruginosa* were not sensitive to different nitrogen sources and that this undoubtedly increased the ecological range of *M. aeruginosa* to adapt to nitrogen, and also improved its survival probability and competitiveness. For *S. quadricauda,* the plastid structure was damaged in the ammonium nitrogen culture medium, and the photosynthetic pigments in cells that convert light energy into stable chemical energy were also damaged hindering the process of photosynthetic electron transfer. Then the Fv/Fm of *S. quadricauda* was lower than that of the same concentration alanine treatment group.

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FRAGMENTATION CAUSES WOODY PLANT COMPOSITION DECLINE IN SACRED GROVE PATCHES IN THE PUDUCHERRY REGION OF SOUTHEAST INDIA

RAJAMURUGAN, J.[1#] – MOHANDASS, D.[2#] – CAMPBELL, M. C.[3] – JAYAKRISHNAN, P.[1] – BALACHANDRAN, N.[4] – SHAO, S.-C.[5*]

[1]*Department of Botany, Tagore Govt. Arts and Science College, Lawspet, Puducherry 605008, India*

[2]*Novel Research Academy (A Division of New Swertia Publications), 194, Thiruvalluvar Salai, Kuyavarpalayam, Puducherry 605013, India*

[3]*Centre for Tropical Environmental and Sustainability Science (TESS) and College of Science and Engineering, James Cook University, Cairns, Queensland 4878, Australia*

[4]*Ecology Department, French Institute of Pondicherry, Puducherry 605001, India*

[5]*Gardening and Horticulture Department, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla County, Yunnan 666303, PR China*

[#]*These authors equally contributed to this work*

**Corresponding author*
e-mail: shaoshicheng@xtbg.org.cn

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Abstract. The fragmentation of tropical forests threatens plant community compositions worldwide. In the present study, we examined the impact of fragmentation on plant community compositions over 30 years in sacred forest grove fragments in southern India. For this study, we randomly selected 30 sacred grove patches (hereafter referred to as fragments) of different sizes to examine the effects of fragment size and historical changes on the plant community compositions. A total of 414 woody plant individuals consisting of 53 species belonging to 45 genera and 20 families were recorded from the 30 sites. The total area of the fragments was not significantly related to the current species richness or diversity, although there were significant negative relationships between the total fragment size and the species composition and between the total fragment size and the species evenness, indicating that fragmentation negatively impacted woody plant compositions. Interestingly, our results showed that the woody tree abundance was significantly and positively related to the total fragment size, suggesting a potential increase in recruitment due to the various forms of ongoing human disturbances. Moreover, the woody plant species compositions may be declining in these venerated forest patches due to the removal of plant resources, potentially resulting in declining fragment sizes.
Keywords: *abundance, Coromandel Coast, disturbance, diversity, species-area relationship*

Introduction

Sacred groves are holy places where local people maintain tropical dry evergreen forest patches (TDEFs) due to the many resident tree species considered to be sacred (Ramanujam and Kadamban, 2001; Kent, 2013; Pradhan et al., 2019). The majority of sacred groves are forest patches of different sizes and shapes created through previous human disturbances and forest fragmentation. Human-induced fragmentation and land use changes significantly alter forest tree species richness and compositions worldwide

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(Haddad et al., 2015; Wilson et al., 2016; Zhao et al., 2019). Fragmentation has been shown to impact species diversity and composition both positively and negatively, although the negative responses are much more prevalent (Urban and Keitt, 2001; Haila, 2002; Ethier and Fahrig, 2011; Smith et al., 2011; Munguía-Rosas and Montiel, 2014; Mohandass et al., 2018). Fragmentation is a multidimensional process because it can influence numerous patch metrics, such as patch area, shape and isolation. A decrease in patch area may result in a decrease in the amount of available habitat (Laurance et al., 2007). Moreover, a decrease in the area of a fragment may result in a decrease in the amount of forest interior relative to the edges, possibly resulting in a loss of species given that forest interiors are more similar to pre-fragmentation conditions than forest edges (Petit et al., 2004). Moreover, human-induced fragmentation may result in both direct and indirect disturbances that may influence the spatial arrangement and habitat quality of forest patches (Honnay et al., 2005; Guirado et al., 2007). Prior and ongoing disturbances of forest habitats may also increase patch isolation (Turner et al., 2001; Farina, 2006). Thus, in general, fragmentation results in negative effects on landscape-level plant diversity (Urban and Keitt, 2001; Haila, 2002), in part due to reductions in the available forest area leading to decreases in species diversity and population sizes (Dixo et al., 2009; Aguirre-Gutiérrez, 2014).

Larger tropical forest fragments support more species than small fragments do (Munguía-Rosas and Montiel, 2014; Aguirre-Gutiérrez, 2014). In addition to the available area, the species abundance per patch may also be influenced by the dispersal capacity of the resident species, which may determine the functional isolation of patches (Hubbell, 2001). The species richness of patches may influence different environmental conditions, species traits and the ecological conditions of the fragments, which can be determined by the quality of the available resources (Petit et al., 2004; Aparicio et al., 2008; Struebig et al., 2011).

On the Coromandel Coast of South India, sacred forest grove patches range across an array of sizes. There have been limited assessments of the impacts of fragmentation and patch size on the woody tree species richness and abundances in sacred groves of the Coromandel Coast of South India. In this study, we assessed the impacts of fragmentation on tree species richness and abundance in relation to the patch area among different sizes of sacred groves around the Puducherry region of South India, including the influence of historical patch sizes. Thus, this study addresses the following hypotheses: (i) the richness, abundance, composition, diversity and evenness of woody species are related to the total fragment size; (ii) the present fragment sizes differ from the past fragment sizes due to the effects of historical human disturbance factors, and these differences subsequently impact the current measurements listed in hypothesis I; and (iii) species compositional patterns differ with fragment size.

Methods

Study area

The study was conducted in recognized sacred groves (forest fragments) of the tropical dry evergreen forest of the Coromandel Coast of the Puducherry region in southeast India. In the study area, we randomly selected 30 sacred groves for analysis, and the locations of the study sites are marked on the map shown in *Figure 1*. The names of the sites and sacred groves are presented in *Table 1*. The weather data were collected in the Ecolake Estate project located 10 km away from the Puducherry seashore. The climate of the

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study area is a maritime tropical climate with an asymmetric rainfall regime. The weather is generally humid and hot; the average annual temperature was 28.68 °C in Puducherry from 2011 to 2015 (*Fig. 2*), and the daily mean temperature difference was 8 °C between summer and winter (Krishnakumar, 2018). The southwest monsoon contributes 20% of the total rainfall across the July–September period, but the retreating northeast monsoon accounts for the majority of the yearly rainfall (61% of the total) during the principal rainy season covering October, November and December. The mean annual rainfall in the Puducherry region was 1373 mm, with a mean of 57.25 rainy days (Ramanujam and Kadamban, 2001; Krishnakumar, 2018). The weather conditions of the Puducherry region consist of a tropical wet and dry season according to the Koppen-Geiger classification (Peel et al., 2007); however, the dry season comprises a prolonged period of the year, from January-September.

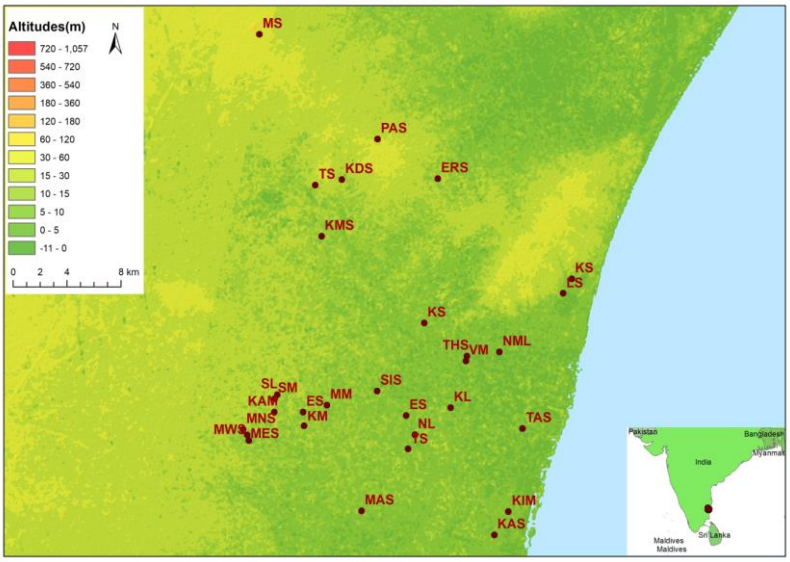

Figure 1. *Map showing the 30 sites of sampled sacred grove forest patches in the inland Puducherry region of southern India*

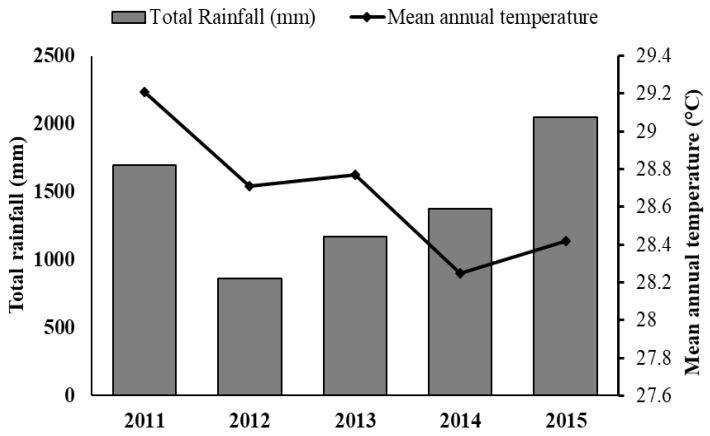

Figure 2. *The patterns of rainfall and temperature in the inland Puducherry region from 2011 to 2015 are shown. The maximum total rainfall was recorded in 2015, and the minimum total rainfall was recorded in 2012. The mean annual temperature reached a maximum in 2011 (29.2 °C), and the minimum rainfall was recorded in 2014 (28.2 °C)*

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Table 1. *Site name, latitude (°N) and longitude (°E), fragment size, total (historical) fragment size (ha), present fragment size (ha), species richness, species abundance, species composition (%), Shannon diversity index, Simpson diversity index and evenness of woody species in the sacred groves of the Puducherry region, South India*

Site number	Site name	Site code	Latitude (N)	Longitude (E)	Fragment size	Total size (ha)	Present fragment size (ha)	Reduction	Species richness	Abundance	Species composition (%)	Shannon	Simpson	Evenness
1	Villianur	VM	11°54'39.82"	79°45'19.42"	Medium	3.2 ± 0.848	0.13	3.07	17	49	34.69	2.33	0.86	0.6
2	Koodapakkam	KS	11°56'09.34"	79°43'40.70"	Small	0.45 ± 0.0617	0.04	0.41	5	8	62.5	1.49	0.75	0.89
3	Lawspet	LS	11°57'19.88"	79°49'10.74"	Small	0.17 ± 0.00667	0.07	0.1	9	12	75	2.1	0.86	0.9
4	Thennapakkam	TS	11°51'12.89"	79°43'01.52"	Small	0.35 ± 0.008	0.04	0.31	5	9	55.56	1.52	0.77	0.92
5	Embalam	ES	11°52'31.42"	79°42'57.55"	Small	0.19 ± 0.013	0.05	0.14	7	13	53.85	1.89	0.84	0.94
6	N. Manaveli	NML	11°55'01.30"	79°46'39.38"	Large	5.40 ± 0.37	0.02	5.38	4	9	44.44	1.27	0.69	0.89
7	Nathamedu	NL	11°51'46.32"	79°43'17.89"	Large	5.6 ± 0.174	0.06	5.54	11	18	61.11	2.25	0.88	0.86
8	Korkadu	KL	11°52'50.08"	79°44'43.22"	Large	6.8 ± 0.326	0.08	6.72	10	31	32.26	1.91	0.81	0.68
9	Kalmandapam	KM	11°52'07.59"	79°38'54.28"	Medium	4.5 ± 0.38	0.04	4.46	6	18	33.33	1.51	0.74	0.76
10	Kariyamanikkam	KAM	11°52'39.77"	79°37'43.27"	Medium	4.6 ± 0.263	0.04	4.56	8	12	66.67	1.98	0.85	0.9
11	Sooramangalam	SL	11°53'20.28"	79°37'50.42"	Large	6.5 ± 0.235	0.04	6.46	7	17	41.18	1.81	0.82	0.87
12	Sooramangalam	SM	11°53'10.94"	79°37'44.77"	Medium	2.8 ± 0.653	0.03	2.77	4	7	57.14	1.28	0.69	0.9
13	Mitta mandagapattu	MM	11°52'56.17"	79°39'48.87"	Medium	1.2 ± 0.133	0.03	1.17	3	5	60	1.06	0.64	0.96
14	Sivaranthagam	SIS	11°53'29.02"	79°41'48.46"	Small	0.9 ± 0.107	0.03	0.87	4	7	57.14	1.35	0.73	0.97
15	Eripakkam	ES	11°52'39.66"	79°38'52.18"	Small	0.5 ± 0.042	0.04	0.46	7	9	77.78	1.89	0.84	0.94
16	Madukarai East	MES	11°51'45.84"	79°36'39.28"	Small	0.11 ± 0.013	0.04	0.07	6	9	66.67	1.58	0.74	0.81
17	Madukarai West	MWS	11°51'32.60"	79°36'43.19"	Small	0.5 ± 0.026	0.02	0.48	5	5	100	1.61	0.8	1
18	Madukarai North	MNS	11°51'58.92	79°36'29.25"	Small	0.6 ± 0.058	0.04	0.56	7	11	63.64	1.77	0.79	0.84
19	Mayalam	MS	12.07'29.78"	79°37'08.11"	Small	0.3 ± 0.06	0.04	0.26	3	8	37.5	0.97	0.59	0.88
20	Manadau	MAS	11°48'46.97"	79°41'11.29"	Small	0.2 ± 0.04	0.03	0.17	4	6	66.67	1.33	0.72	0.94
21	K. Manaveli	KMS	11°59'33.55"	79°39'36.12"	Small	0.73 ± 0.119	0.03	0.7	5	8	62.5	1.56	0.78	0.95
22	Thiruvakkarai	TS	12°01'34.19"	79°39'20.93"	Small	0.83 ± 0.092	0.04	0.79	6	12	50	1.75	0.82	0.96
23	Thirumangalam	THS	11°54'51.55"	79°45'21.85"	Small	0.32 ± 0.054	0.03	0.29	4	7	57.14	1.28	0.69	0.9
24	Karuvadikuppam	KS	11°57'53.58"	79°49'31.69"	Small	0.44 ± 0.0562	0.06	0.38	9	16	56.25	1.99	0.84	0.82
25	Kannikoil	KAS	11°47'50.25"	79°46'27.07"	Small	0.6 ± 0.044	0.06	0.54	10	18	55.56	2.17	0.87	0.87
26	Kirumampakkm	KIM	11°48'45.50"	79°47'00.76"	Medium	1.1 ± 0.163	0.12	0.98	16	33	48.48	2.51	0.9	0.77
27	Thavalakuppam	TAS	11°52'01.08"	79°47'34.52"	Small	0.7 ± 0.076	0.08	0.62	11	18	61.11	2.26	0.88	0.87
28	Parankal	PAS	12°03'22.89"	79°41'49.38"	Small	0.5 ± 0.03	0.06	0.44	6	15	40	1.52	0.72	0.76
29	Erraiyur	ERS	12°01'49.76"	79°44'12.77"	Small	0.9 ± 0.01	0.09	0.81	10	13	76.92	2.25	0.89	0.94
30	Kadakam Pattu	KDS	12°01'47.22"	79°40'24.38"	Small	0.45 ± 0.022	0.04	0.41	7	11	63.64	1.85	0.83	0.91

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Biocultural perspectives

The majority of the examined sacred groves were located away from human habitation, although 5 sacred grove patches were located relatively close in proximity to human settlements, within a distance of 500 meters. At the edge of each sacred grove was an open shrine of Lord Siva under a banyan tree with a granite bull (Nandhi) facing the eastern side. Well-built, modern, concrete temples were located in the center of each grove. The presiding deities include Selliamman, Iyynarappan and other gods and powerful goddesses worshipped by the people, and in some temples were broken terracotta horses (*Fig. 3A, B*). The villagers claim that the entire stretch between the temples, including some of the study sites along with the intervening plantations, was a continuous forest until the 1970s, but large tracts were subsequently cleared for raising *Eucalyptus* plantations. However, most of the sacred groves had clear cutting of trees for further construction of god statues, and other temple and building construction developments were established within the temple borders (*Fig. 3C, D*). Information on the locations of the sacred groves and the temple complexes was recorded by direct observations during field visits. Traditions, beliefs, taboos, restrictions and folklore pertaining to each temple were recorded from local devotees randomly selected from the community.

Figure 3. *(A) A sacred forest grove patch that was reduced to a patchy forest area due to deity statue construction in the Suramangalam site. (B) The forest patch area was reduced due to ground floor development in the sacred grove of the Lawspet site. (C and D) Sacred forest tree individuals were removed for further temple construction in the Madukarai and Villianur sites of the inland Puducherry region of Southern India*

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Assessment of disturbances

The area of the total (historic) fragment size of each sacred grove was recorded by interviewing elderly locals > 60 years of age who were living near the sacred groves (*Table 2*). The total (historical) fragment size was estimated using their (the interviewed individuals') recollection of the size of the sacred groves in the 1980s (a minimum of 36 years ago), while the existing fragment sizes were measured through direct sampling. The incidence and series of disturbance activities of the sacred groves over the period from 1980-2016 were identified using the aforementioned interviews (*Table 2*). At each site, we interviewed 6-10 respondents, and the average size of the area was used for further data analysis. No accurate area size was available in any of the records. The outcome data of the oral interviews delivered important conservation research findings (Young et al., 2018).

Table 2. *Various disturbance attributes recorded as having occurred over the period from 1980 to 2016 in the examined sacred groves of the Puducherry region, southeast India*

S. No.[#]	Disturbance attributes
1	**Road establishment**
	a. Creation of a bridle path
	b. Vehicle parking
	c. Creation of a cement floor or siting place for devotees
2	**Land encroachment**
	a. Removal of trees in the edge zone of the forest area
	b. Agricultural land expansion and removal of trees
	c. Creation of a cement floor for crop drying
3	**Land use for general uses**
	a. Cooking construction place and cooking inside the forest
	b. Devotees' congregation place for festivals/ceremonies
	c. Trees cut for firewood prior to festival season
4	**Habitat Conversion/Resource removal**
	a. Grass and weed colonization after tree removal
	b. Secondary vegetation establishment
	c. Plantation of edible and ornamental plants
5	**Land use change for personal use**
	a. Encroachment for house construction (temporally)
	c. Creation of roads to access houses
	b. Creation of home gardens

[#]Disturbance number

Plant sampling

We counted the plant individuals and collected plant samples from 30 sites from August 2016 to February 2017 in the inland Puducherry region among different sacred grove patches. In each sacred grove patch, 10 × 10-m quadrats were laid, and all woody species above 3 cm in dbh (Diameter at Breast Height) were sampled in each sacred grove, with the total sampled areas ranging from 0.03 to 0.12 ha in the 30 sacred grove patches. The fragments were placed in size categories based on their existing areas, with those with areas < 0.01 ha considered small, > 0.01 to < 1 ha considered medium-sized, and > 1 ha considered large. Quadrats were laid contiguously as stratified sampling, but

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the continuity was broken to exclude temples and buildings whenever necessary. In particular, we sampled the area of occurrence of woody plant species that were composed of shrubs and trees. We excluded nonwoody species from this study. Species identification was counterchecked with the herbarium collections of the French Institute of Puducherry, India. The nomenclature of each species follows the Flora of Tamil Nadu (Nair and Henry, 1983; Henry et al., 1987, 1989), with the exception of updated species being incorporated from the website www.theplantlist.org. All the sampled herbarium specimens were deposited in the Department of Botany, Tagore Arts College, Puducherry, India.

Species richness, abundance, diversity and evenness

Biotic richness (at the species, genera and family levels) and stem abundance were counted in all 30 examined sacred groves. Species richness was defined as the number of species in each sacred grove, and abundance was defined as the number of individual stems above 3 cm in dbh in each sacred grove (Mohandass et al., 2018). For the floristic analyses, all collected data were pooled at the patch level, and the total number of species and individuals were tallied. Using the pooled data, the overall species richness, genera- and family-level richness, abundance, and diversity were calculated. The dominant species were considered to be those that were the most abundant in the inventory, and the dominant family was represented by the highest number of stems.

We calculated two diversity indices: the Shannon–Wiener index (H') was calculated using the following formula:

$$H' = -SP_i \ln P_i$$

where $P_i = ni/N$, n_i is the number of individuals of a species, and N is the total number of identified plants in a sacred grove.

Species evenness (E) was calculated as follows:

$$E = H'/H'_{max}$$

where H' is the Shannon–Wiener index and H'_{max} is In*S* (Magurran, 2004).

Data analyses

All the data were logarithmically transformed to allow for analytical assumptions of normality for each forest patch, including for fragment sizes and other floristic variables. The total fragment area size and existing fragment area size were analyzed by paired t-tests to compare the averages before and after the changes in fragment size. Principal component analysis was applied to test the correlations between the total fragment sizes and among different vegetation parameters, such as species richness, abundance, species composition, and the Shannon, Simpson and evenness indices. PCA was also applied to test the relationship between each species distribution among the 30 fragment sites based on presence/abundance data to determine whether there was significant positive or negative correlations in relation to fragment area. Regression analysis was used to test the effect of the existing fragment size on each species response. All statistical analyses were performed using the software Past version 3.01.

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Results

Plant communities

A total of 414 individuals from 53 species belonging to 50 genera and 30 families were recorded from the 30 examined sacred groves (*Table A1*). Of these, 51 woody trees and 2 shrubs were counted, with occasional nonwoody species also recorded (*Table A1*). Among all examined patches, the species richness ranged from 3 to 17, the abundance ranged from 5 to 49, the species composition ranged from 32.26 to 100%, the Shannon index ranged from 0.97 to 2.51, the Simpson index ranged from 0.59 to 0.90, the evenness ranged from 0.60 to 1.00 (*Table 1*), and the number of plant families ranged from 3 to 15. The means of these variables among the patches was: species richness, 7.20 ± 0.63; mean abundance, 13.80 ± 1.72; species composition, 57.37 ± 2.72; Shannon index, 1.73 ± 0.07; Simpson index, 0.79 ± 0.01; evenness, 0.87 ± 0.02; and number of plant families, 6.27 ± 0.56 (*Table 1*).

The species richness and abundance of all woody species ≥ 3 cm in dbh were recorded from 30 sacred groves. The frequency of occurrence of individuals varied among the number of species: species that showed < 6 stems had a high frequency of 38%, while species with 6 < stems < 12 had a 6% frequency of occurrence. In addition, species with 12 < stems < 18 had a 4% frequency of occurrence. In addition, the individuals with between 48 and 54 stems represented 1% of the total frequency (*Fig. 4*).

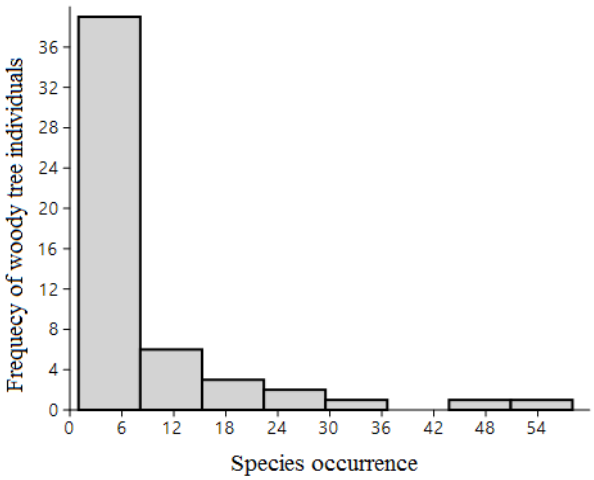

Figure 4. *Frequency of woody tree individuals based on species occurrence among 30 sacred groves in the inland Puducherry region of southern India*

The rank of the relative abundance curve is shown on the Y axis, and the abundance rank is shown on the X-axis of *Figure 5*. On the X-axis, the most abundant species was ranked 1, the second most abundant was ranked 2, the species with common abundances were ranked 10-15, the species with uncommon abundance were ranked 25-35, and rare species were ranked 45-54 (*Fig. 5*). The Y-axis shows the number of individuals, which is a measure of species abundance.

The examined sacred groves were dominated by *Azadirachta indica* A. Juss. (14%) and *Cocos nucifera* L. (12%), both of which are large trees, followed by *Ficus benghalensis* L. (8%), *Tectonia grandis* L.f. and *Borassus flabellifer* L. (6%), *Mangifera indica* (5%), *Musa paradisiaca* L. and *Tamarindus indica* L. (4%), then

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Couroupita guianensis Aubl., *Aegle marmelos* (L.) Correa., *Ficus racemosa* L., *Ficus religiosa* L., *Lepisanthes tetraphylla* Radlk. and *Sapindus emarginatus* Vahl (3%). Some rare species were *Naringi crenulata* (Roxb.) D.H. Nicolson, *Neolamarckia cadamba* (Roxb.) Bosser., *Nerium odorum* Sol, *Phoenix sylvestris* (L.) Roxb., *Sterculia foetida* L. and *Thespesia populnea* (L.) Sol. ex Corrêa. Only two genera comprised 5 species, and the other recorded genera comprised single species. *Albizia* had 2 species, and *Ficus* had 3 species recorded in the 30 sacred groves. Thus, *Ficus* was the dominant family, followed by *Albizia.*

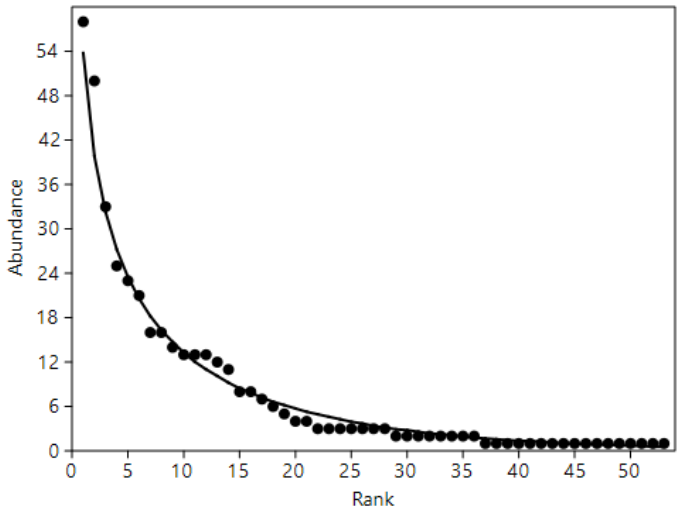

Figure 5. *Species ranked based on their tree abundances in 30 sacred groves in the Puducherry region of southern India*

Overall, abundance decreased with an increasing number of species among the 30 sacred groves. The abundance size class frequency distribution of the sacred grove stands exhibited a tendency towards a reverse J-shaped distribution (*Fig. 6*), indicating that the population was skewed towards species with fewer individuals and that species with greater numbers of individuals were disproportionally represented in the abundance of woody trees.

Arecaceae was the dominant family (19.08%) among the 30 sacred groves, followed by Moraceae (14.7%), Meliaceae (13.8%), Verbenaceae (6%), Sapindaceae (5.8%), Anacardiaceae (5.56%), Caesalpiniaceae (5.3%), and Fabaceae (4.83%). Five and seven families shared two and one species from each family, respectively (*Table A1*).

Fragment size reduced over time

All fragment size classes (small, medium and large) decreased significantly in size (small: $t = 8.327$, $N = 20$, $P = 0.0001$; medium: $t = 4.503$, $N = 6$, $P = 0.006$; large: $t = 18.14$, $N = 4$, $P = 0.0001$) over the examined time period (*Table 3*).

Impact of the total fragment size on the resident plant communities

Species richness did not show a significant relationship with total (historical) fragment size, whereas abundance showed a significant positive relationship with total fragment size. In contrast, species composition showed a significant negative

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relationship with total fragment size. The Shannon and Simpson indices were not significantly related to total fragment size, while evenness showed a significant negative relationship with total fragment size (*Table 4*).

The PCA of the fragment size and plant community composition showed a significant correlation (*Table 5*). The cumulative percentage variance of the plant community data showed that the first two PCA axes explained 95.2% of the variability among different fragment sizes. Of these, axes 1 and 2 explained 74% and 21.2% of the variance in fragment size in relation to the plant community, respectively (*Fig. 7*).

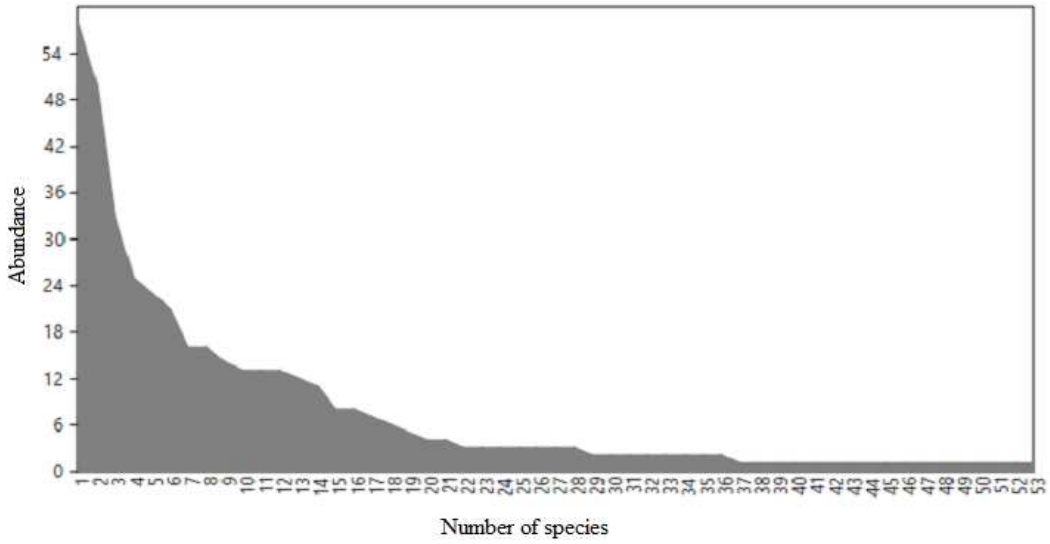

Figure 6. *Species richness and abundance patterns showed a reverse J-shaped curve from 30 sacred groves examined in the Puducherry region of southern India*

Table 3. *Average differences between past and present fragment size in the examined sacred groves of the Puducherry region, South India*

Fragment class	Fragment size (mean ha)			P-value
	Total fragment size (30 years ago)	Existing fragment size	Paired t-test	
Small fragments	0.49	0.05	8.327	0.0001
Medium fragments	2.90	0.07	4.503	0.006
Large fragments	6.08	0.05	18.142	0.0001

Table 4. *Effects of total fragment sizes on different floristic variables among the examined sacred groves of the Puducherry region, South India*

Floristic variable	Mean	Std. dev.	*r*	*P*
Species richness	7.20	3.45	0.20	0.142
Abundance	13.80	9.40	0.39	0.017
Species composition	57.37	14.88	-0.47	0.004
Shannon index	1.73	0.40	0.12	0.257
Simpson index	0.79	0.08	0.08	0.332
Evenness	0.87	0.09	-0.40	0.013

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Table 5. *Effects of total fragment size on different floristic variables according to principal component analysis (PCA) among sacred groves of different sizes in the Puducherry region, South India*

S. No.	Name of the sacred grove site	Site code	Fragment category	Existing fragment size (ha)	Total fragment size (ha)	Species richness	Abundance	Species composition	Shannon	Simpson	Evenness
1	Villianur	VM	Medium	0.13	3.2	0.610	0.696	0.590	0.575	0.571	0.576
2	Koodapakkam	KS	Small	0.04	0.45	-0.291	-0.312	-0.285	-0.283	-0.281	-0.281
3	Lawspet	LS	Small	0.07	0.17	-0.686	-0.684	-0.714	-0.701	-0.704	-0.704
4	Thennapakkam	TS	Small	0.04	0.35	-0.400	-0.407	-0.388	-0.392	-0.390	-0.391
5	Embalam	ES	Small	0.05	0.19	-0.649	-0.629	-0.650	-0.654	-0.655	-0.656
6	N. Manaveli	NML	Large	0.02	5.4	0.773	0.751	0.804	0.794	0.797	0.797
7	Nathamedu	NL	Large	0.06	5.6	0.833	0.834	0.804	0.817	0.814	0.813
8	Korkadu	KL	Large	0.08	6.8	0.912	0.970	0.919	0.899	0.898	0.901
9	Kalmandapam	KM	Medium	0.04	4.5	0.712	0.742	0.739	0.717	0.719	0.720
10	Kariyamanikkam	KAM	Medium	0.04	4.6	0.734	0.711	0.715	0.730	0.729	0.727
11	Sooramangalam I	SL	Large	0.04	6.5	0.877	0.892	0.887	0.879	0.879	0.878
12	Sooramangalam II	SM	Medium	0.03	2.8	0.489	0.448	0.508	0.509	0.512	0.512
13	Mitta mandagapattu	MM	Medium	0.03	1.2	0.110	0.057	0.140	0.138	0.144	0.143
14	Sivaranthagam	SIS	Small	0.03	0.9	-0.002	-0.032	0.018	0.017	0.020	0.018
15	Eripakkam	ES	Small	0.04	0.5	-0.231	-0.256	-0.250	-0.234	-0.235	-0.237
16	Madukarai East	MES	Small	0.04	0.11	-0.892	-0.896	-0.896	-0.893	-0.893	-0.891
17	Madukarai West	MWS	Small	0.02	0.5	-0.246	-0.314	-0.262	-0.236	-0.235	-0.237
18	Madukarai North	MNS	Small	0.04	0.6	-0.152	-0.159	-0.162	-0.156	-0.156	-0.156
19	Mayalam	MS	Small	0.04	0.3	-0.489	-0.483	-0.436	-0.465	-0.458	-0.457
20	Manapet	MAS	Small	0.03	0.2	-0.652	-0.683	-0.638	-0.636	-0.634	-0.634
21	K. Manaveli	KMS	Small	0.03	0.73	-0.082	-0.107	-0.076	-0.072	-0.071	-0.072
22	Thiruvakkarai	TS	Small	0.04	0.83	-0.019	-0.013	-0.010	-0.015	-0.015	-0.017
23	Thirumangalam	THS	Small	0.03	0.32	-0.448	-0.469	-0.428	-0.433	-0.430	-0.429
24	Karuvadikuppam	KS	Small	0.06	0.44	-0.275	-0.253	-0.290	-0.289	-0.291	-0.290
25	Kannikoil	KAS	Small	0.06	0.6	-0.137	-0.110	-0.155	-0.153	-0.156	-0.156
26	Kirumampakkam	KIM	Medium	0.12	1.1	0.146	0.206	0.113	0.112	0.108	0.109
27	Thavalakuppam	TAS	Small	0.08	0.7	-0.066	-0.045	-0.093	-0.085	-0.089	-0.089
28	Parankal	PAS	Small	0.06	0.5	-0.238	-0.205	-0.218	-0.237	-0.236	-0.233
29	Erraiyur	ERS	Small	0.09	0.9	0.038	0.029	0.004	0.024	0.020	0.019
30	Kadakampattu	KDS	Small	0.04	0.45	-0.277	-0.280	-0.286	-0.280	-0.281	-0.282

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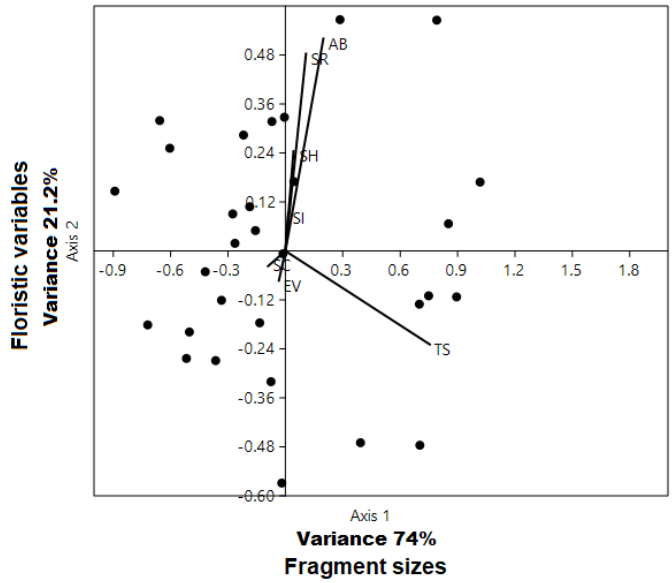

Figure 7. *Principal component analysis showed the relationships between different fragment sizes and floristic variables in Axis 1 (variance = 74%) and Axis 2 (variance 21.2%) from the examined sacred grove patches of the Puducherry region of South India*

Effect of existing fragment size on species distributions

Most species responded significantly to the existing patch size, although the direction of their response was not always consistent since a few species did not show any significant response (*Table A1*). PCA showed the species composition of the pooled data, with axis 1 describing 36.37% of the variance and axis 2 describing 17.64% based on the species presence/absence occurrence from each of the 30 sacred grove patches (*Fig. 8*).

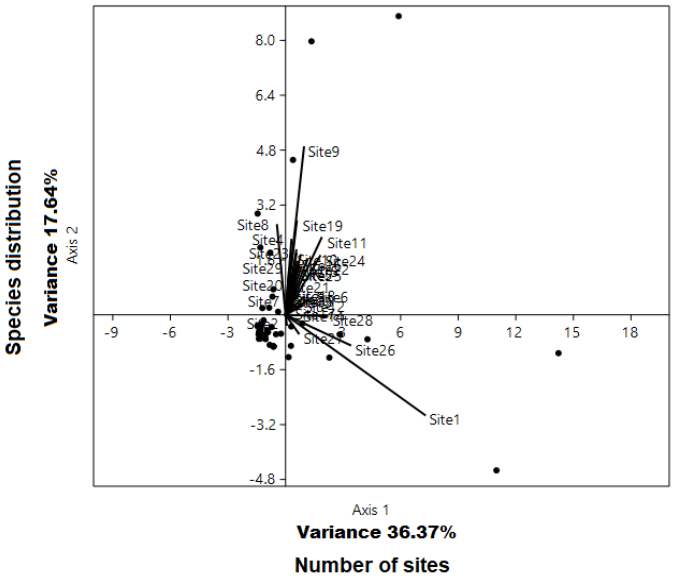

Figure 8. *Principal component analysis showed the relationships between site distribution and each species based on presence/absence data. X-axis 1 shows a variance of 36.37%, and Y-axis 2 shows a variance of 17.64% in relation to fragmented sites and to the species distributions of the sacred groves in the Puducherry region of South India*

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Species compositional patterns

The species composition pattern did not differ significantly among different fragment sizes. The overall mean rank value of different fragment sizes was 234.9 (R = 0.159; P = 0.0766). Of these, species composition also did not differ between large and medium fragments (Mean Rank Value = 25.23; R = 0.212; P = 0.101), between medium and small fragments (Mean Rank Value = 173.6; R = 0.1034; P = 0.192) or between large and small fragments (Mean Rank Value = 158.6; R = 0.205; P = 0.106).

Discussion

Current species richness was not significantly related to past fragment size or total fragment size in the forest groves of Puducherry, likely because the effective areas of the patches have often been reduced due to human disruption and encroachment. Moreover, fragment size had a larger influence on species richness than did total (historical) patch size, thus indicating that many species may have been extirpated by the identified patch size reductions. Sacred grove fragment size was also found to decline significantly in the region due to long-term human disturbance through tree harvesting at patch edges and encroachment for agricultural practices. Our results also suggest that the reduction in total fragment size has resulted in a concurrent decline in species population sizes and an increase in the number of fragments in the smaller size category. The decrease in fragment size is likely to result in degradation of the microenvironmental features in the fragments, leading to further declines in species richness (Campbell and Ortíz, 2011; Kettle and Pin Koh, 2014).

Species composition showed a significant negative relationship with total (historical) patch size. This finding is likely due to the ongoing human disturbance (such as logging and encroachment) of the forest patches. Moreover, declines in the plant community composition over time due to habitat fragmentation may have resulted from cascading effects on ecosystem properties (Tabarelli et al., 2012). In addition, the negative relationship between species composition and fragment area may also be due to factors such as matrix quality and historical legacies of human land uses in the areas surrounding the sacred groves (Driscoll et al., 2013; Ewers et al., 2013; Mesquita et al., 2015). Within the patches, however, the species composition may be influenced over time due to the colonization of disturbance-tolerant species (Mckinney and Lockwood, 1999; Tabarelli et al., 2012). Thus, the species composition of the patches may be concurrently influenced by colonization and extinction over time among the sacred groves (Jackson and Sax, 2010; Dornelas et al., 2014) in addition to gradual degradation due to ongoing human disturbances.

There was a significant impact of patch size on species composition in the examined forest fragments. Most of the examined small fragments displayed negative responses to fragment area, while medium and large fragments displayed positive responses. This finding indicates that fragment colonization may be driving compositional changes over time among the sacred grove fragments and that historical land uses may have also influenced the current plant community compositional changes. For instance, large trees (from successional climax species) may go extinct in patches after several decades due to a lack of recruitment in disturbed environments or through harvesting by locals. This, in turn, results in the creation of large gaps in the patches, leading to the further recruitment of disturbance-adapted species. Concurrently, small trees may also go locally extinct due to overharvesting for firewood, again creating more gaps that change

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the forest grove structures, leading to the patches remaining in an early successional stage. Moreover, the mentioned impacts may also lead to small fragments (as identified) that are increasingly isolated; both small fragment size and isolation contribute to depauperate resident tree communities (Laurance et al., 2018).

The identified ongoing reduction in fragment size in the study area is likely to result in a decline in the ecological functioning of the patches, such as seed dispersal, regeneration and tree stand replacement, which in turn may lead to the local extinction of species among sacred groves (Auffret et al., 2015; Berhanu et al., 2017). In the sacred grove patches, forest edges are exposed to intense and ongoing anthropogenic disturbances through practices such as agriculture and road development, which may also affect species recruitment. As a consequence, ongoing disturbances may prevent the recruitment of new seedlings to forest patches and edges or may result in increased levels of mortality due to habitat and microclimatic alterations (Comargo and Kapos, 1995; Turner and Corlett, 1996).

We used interviews to collect information of historical patch sizes and changes from local people and elucidate that long-term human intervention has highly influenced both the fragment sizes and ongoing disturbances of the examined forest grove patches (*Table 1*). Our findings suggest that the area of the examined fragments was significantly reduced, leading to negative impacts on species richness, species composition, species diversity (Shannon and Simpson indices) and evenness. However, the species abundance of the examined patches appears to have been positively influenced by disturbances, possibly due to the intervention of locals attempting to maintain woody sacred trees for religious purposes in larger fragments. Moreover, larger fragments are likely to maintain the abundance and diversity of woody species due to their fragment size (Laurance, 2008).

The sacred groves decreased in size across the period of time analyzed. In fact, the land area of sacred groves decreased by approximately 97% from the 1980s compared to that in 2016. Nevertheless, in the present study, large fragments maintained their species richness and abundances of woody species positively and consistently among sacred groves due to the slow process by which fragment areas decrease due to agricultural expansion and settlements. Even large or small fragments maintain few sacred trees due to cultural and ritualistic beliefs that species such as *Aegle marmelos* (L.) Corrêa, *Calophyllum inophyllum* L. *Couroupita guianensis* Aubl., and *Drypetes sepiaria* (Wight & Arn.) Pax & K. Hoffm. The same species found in larger fragments are also found in smaller fragments, thus indicating that the fragmentation process also maintains species specificity due to anthropogenic cultural perspectives. This suggests that changes in sacred grove landscape structure and functions across patch mosaics are influenced by human landscape alterations (Berhanu et al., 2017). In addition, losses of habitats affect interior and edge zones among patches, which influence native trees and are highly invisible as a result of human land use. Under these extreme conditions, sacred grove fragments cause small, isolated remnants of varying sizes to develop from the native original patch, which influences all margins and borders through conversion into other new landscapes by the consequences of building construction, cement floors, road establishment, agricultural expansion, and construction of temples and god statues. Similarly, an earlier study also reported that fragmentation influenced several small isolated remnant patches, which led native forests to become fragments of different small sizes due to agriculture, grazing land and human settlements (Laurance et al., 1998; Laurance, 2008).

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Conclusion

The present study represents the first investigation of the relationship between woody species richness and patch area in the sacred groves of Puducherry regions and thus shows that the species richness and diversity of woody trees are not related to fragment area. However, the abundance showed a significant positive response to fragment area due to the slow process of extinction caused by large natural fragment area sizes. Moreover, the species composition and evenness showed significant negative relationships with the total fragment size due to matrix effects. This indicates that long-term direct and indirect human intervention and disturbance might affect woody species composition, especially synanthropic species. Conventionally, the empirical evidence suggests that patch fragmentation is largely a matter of tree harvesting and thus influences habitat degradation at the edges and interiors of patches. In the studied sacred groves, human disturbances influence the spatial and temporal aspects of fragmentation that may reflect reductions in patch size. Traditionally, it is theorized that reductions in edge effects may affect patch sizes and site scales. We suggest that controlling reductions in patch size may be applicable by reducing human contact with sacred groves through closing unpaved roads and by letting woody growth close the forest edges. However, future studies are needed to examine the reproductive biology and regeneration of forest fragments. These studies may reveal the forest growth structure in relation to fragment size. Most of the fragment areas were encroached by human activities, and it may not be possible to extend these forest areas; thus, studies of seed dispersal and regeneration may facilitate sustained forest density within the existing areas of the fragments. This study concept may be conducive to understanding the enhancement of biodiversity in a narrow range of sacred groves.

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APPENDIX

Table A1. *Species names, families, habits, and abundances of woody trees in the sacred groves of Puducherry region, South India. Species responses were positive[a], negative[b] or neutral[c] to patch size alterations by human disturbances over time*

S. No.	Species	Family	Habit	Abundance	Intercept	Estimate
1	*Acacia cineraria* (L.) Willd.	Leguminosae	Tree	2	0.03[c]	-0.03
2	*Aegle marmelos* (L.) Corrêa	Rutaceae	Tree	13	-0.25[b]	7.57
3	*Albizia lebbeck* (L.) Benth.	Leguminosae	Tree	3	0.19[a]	0.29
4	*Albizia saman* (Jacq.) Merr.	Leguminosae	Tree	7	-0.88[b]	50.35
5	*Annona squamosa* L.	Annonaceae	Tree	1	-0.16[c]	3.82
6	*Areca catechu* L.	Arecaceae	Tree	2	0.12[c]	-1.03
7	*Artocarpus heterophyllus* Lam.	Moraceae	Tree	3	0.03[c]	1.35
8	*Artabotrys hexapetalus* (L.f.) Bhandari	Annonaceae	Tree	2	0.03[c]	7.31
9	*Azadirachta indica* A. Juss	Meliaceae	Tree	58	-0.03[c]	1.86
10	*Bauhinia tomentosa* L.	Leguminosae	Tree	4	0.01[c]	0.45
11	*Borassus flabellifer* L.	Arecaceae	Tree	23	-0.01[c]	0.93
12	*Butea monosperma* (Lam.) Taub.	Leguminosae	Tree	1	-0.04[c]	1.41
13	*Calophyllum inophyllum* L.	Clusiaceae	Tree	1	-0.14[c]	3.34
14	*Carica papaya* L.	Caricaceae	Tree	1	0.02[c]	0.90
15	*Cassia fistula* L.	Leguminosae	Tree	4	-0.04[c]	1.41
16	*Citrus aurantium* L.	Rutaceae	Tree	3	-0.14[c]	3.34
17	*Cocos nucifera* L.	Arecaceae	Tree	50	-0.59[b]	20.27
18	*Couroupita guianensis* Aubl.	Lecythidaceae	Tree	14	2.25[a]	-6.19
19	*Crateva adansonii* DC.	Capparaceae	Tree	3	1.62[a]	-10.20
20	*Crescentia cujete* L.	Bignoniaceae	Tree	1	0.06[c]	-0.51
21	*Dalbergia sissoo* DC.	Leguminosae	Tree	1	-0.31[b]	11.29
22	*Delonix regia* (Hook.) Raf.	Leguminosae	Tree	6	-0.41[b]	17.35
23	*Drypetes sepiaria* (Wight & Arn.) Pax & K. Hoffm.	Putranjivaceae	Tree	3	-0.62[b]	20.75
24	*Enterolobium cyclocarpum* (Jacq.) Griseb.	Leguminosae	Tree	1	0.18[a]	-1.54
25	*Ficus benghalensis* L.	Moraceae	Tree	33	-0.24[b]	6.64
26	*Ficus racemosa* L.	Moraceae	Tree	13	0.71[a]	1.19
27	*Ficus religiosa* L.	Moraceae	Tree	13	-0.16[b]	3.82
28	*Kigelia africana* (Lam.) Benth.	Bignoniaceae	Tree	1	0.18[a]	-1.54
29	*Kleinhovia hospita* L.	Malvaceae	Tree	1	-0.20[b]	5.23
30	*Lannea coromandelica* (Houtt.) Merr.	Anacardiaceae	Tree	2	-0.16[b]	3.82
31	*Lepisanthes tetraphylla* Radlk.	Sapindaceae	Tree	12	0.01[c]	0.45
32	*Madhuca longifolia* (J.Koenig ex L.) J.F.Macbr	Sapotaceae	Tree	1	0.00[c]	3.21
33	*Mangifera indica* L.	Anacardiaceae	Tree	21	0.07[c]	-0.06
34	*Mitragyna parvifolia* (Roxb.) Korth.	Rubiaceae	Tree	1	-0.32[b]	13.60
35	*Moringa pterygosperma* Gaertn.	Moringaceae	Tree	2	-1.00[b]	33.48
36	*Musa paradisiaca* L.	Musaceae	Large shrub	16	-0.14[c]	4.71
37	*Naringi crenulata* (Roxb.) Nicolson	Rutaceae	Tree	1	-0.14[c]	3.34
38	*Neolamarckia cadamba* (Roxb.) Bosser	Rubiaceae	Tree	1	-0.33[b]	11.77
39	*Nerium oleander* L.	Apocynaceae	Shrub	1	-0.07[c]	11.99
40	*Nyctanthes arbor-tristis* L.	Oleaceae	Tree	2	-0.16[b]	3.82
41	*Phoenix sylvestris* (L.) Roxb.	Arecaceae	Tree	1	-2.42[b]	64.14

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42	*Phyllanthus emblica* L.	Phyllanthaceae	Tree	8	0.02[c]	2.28
43	*Plumeria rubra* L.	Apocynaceae	Tree	3	0.43[a]	0.06
44	*Polyalthia longifolia* (Sonn.) Thwaites	Annonaceae	Tree	3	-0.04[c]	1.41
45	*Prosopis cineraria* (L.) Druce	Leguminosae	Tree	2	0.02[c]	0.90
46	*Prunus amygdalus* Stokes	Rosaceae	Tree	5	0.32[a]	-4.43
47	*Sapindus emarginatus* Vahl.	Sapindaceae	Tree	11	0.01[c]	0.45
48	*Sterculia foetida* L.	Malvaceae	Tree	1	-0.32[b]	7.63
49	*Syzygium cumini* (L.) Skeels	Myrtaceae	Tree	8	0.18[a]	-1.54
50	*Tamarindus indica* L.	Leguminosae	Tree	16	-0.10[c]	3.30
51	*Tectona grandis* L.f.	Lamiaceae	Tree	25	0.39[a]	-3.11
52	*Terminalia arjuna* (Roxb.ex DC.) Wight & Arn.	Combretaceae	Tree	2	0.68[a]	-2.92
53	*Thespesia populnea* (L.) Sol. ex Corrêa	Malvaceae	Tree	1	0.06[c]	-0.51

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RESPONSES OF SOIL RHIZOSPHERE FUNGI TO N APPLICATION LEVELS IN DIFFERENT TYPES OF SOIL

LI, Y. N.[1,2] – WANG, T. Y.[1] – WANG, C. Y.[1,2] – LI, M. S.[1,2] – WANG, Y.[1*] – LIU, S. X.[1,2*]

[1]*College of Resources and Environment, Jilin Agricultural University, Changchun 130118, China*

[2]*Key Laboratory of Soil Resource Sustainable Utilization for Jilin Province Commodity Grain Bases, Changchun 130118, Jilin Province, China*

**Corresponding authors*
e-mail/phone: wangyujlc@163.com/+86-135-0446-8360; liushuxia69@163.com/+86-155-4313-3987

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Abstract. The response of maize soil rhizosphere fungi to different N application levels (0, 168, 240, 270, and 312 kg·ha^{-1}) in sandy, meadow and alluvial soils were investigated by sequence variations in ITS rDNA determined with Illumina MiSeq sequencing. The result showed that with N application levels increased, soil available nutrients changed significantly, but total nutrients changed little, and the nutrient content site in meadow soil was higher than that in sandy soil and alluvial soil. The rhizosphere fungal community was mainly composed of Ascomycota and Basidiomycota. N application did not change the relative abundance of fungi significantly, but altered the structure of fungal communities. The relative abundance of Basidiomycota was increased with N application. Moreover, N application also increased fungal pathogens: Alternaria, Rhizopus and Waitea significantly. In addition, the soil fungal communities were clustered into two main groups, one for the meadow soil sites and another for the sandy soil and alluvial soil sites. Soil fungal community structure changed when nitrogen application level exceeded 168 kg·ha^{-1}. Our findings on the responses of fungal community to N application levels in different types of soil is important to determine rational fertilization application measures.
Keywords: *soil fungi, N fertilizer application, different types of soil, fungal diversity, fungal structure*

Introduction

Nitrogen (N) is an important nutrient element in plants, and it has a major impact on plant productivity and fungal function (Wilson, 2013). According to the National Bureau of Statistics, China's fertilizer production was 76,276,600 tons in 2015, which was an increase of more than 7.3% from 2014; The current annual nitrogen fertilizer production is 49,438,500 tons, which represents an increase of more than 6.3% from the previous year and is the largest increase in recent years (Li, 2018). The contribution rate of chemical fertilizer application to grain production is over 40% in China due to the low base soil fertility (Tian et al., 2018). Long-term fertilization can not only increase soil nutrients but also improve crop quality and promote microbial activity. In recent years, due to the neglect of the application of organic fertilizers, increasing crop yields has increasingly relied on excessive application of nitrogen fertilizers; however, excessive nitrogen application has various negative effects on soil quality, crop yield and the ecological environment (Qu et al., 2019; Zheng et al., 2013). Studies have shown that the excessive application of nitrogen fertilizer not only increases production costs but also reduces soil nutrient sustainability and nitrogen use efficiency and has a significant impact on environmental factors such as greenhouse gas emissions (Lijbert et al., 2007). Fertilizer application has a significant impact on soil fungal activities, especially soil fungal denitrification (Yamamoto et al., 2017). Different fertilization

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systems have significant effects on soil fungal population and community structure (Zhao et al., 2014)

Fungi are important components of soil communities and play an important role in agro-ecosystems (Wood et al., 2015; Joergensen et al., 2008). During decomposition, fungi can break down plant residues and difficult-to-decompose compounds such as cellulose, hemicellulose, and lignin, which are complex and closely related to vegetation, and can release nutrients needed for plant growth because of their high extracellular enzyme activity and comprehensive range of enzymes. Fungi not only affect the cycling of the nutrients C, N and S but also provide plant nutrition, promote plant growth and inhibit disease. It has been reported that between 78%～90% of the biomass in grass is decomposed by fungi (Kowalchuk et al., 1997). This indicates that fungi promote the circulation of nutrients. Fungi are more sensitive than soil bacteria to changes in the environment (Sall et al., 2006). Fungi have advantages in adapting to climate and environmental changes (Qu et al., 2019; Laura et al., 2012) due to the special structural characteristics of soil fungal hyphae.

Soil rhizosphere microorganisms are a key link between the soil and plant nutrient supplies. Microorganisms are concentrated around the roots, transforming the organic matter released during plant growth into inorganic matter and providing effective nutrition for plants (Xu et al., 2008). Studies have shown that the major groups of soil rhizosphere fungi are Penicillium, Trichoderma, Aspergillus, Pythium, Gliocladium and Fusarium, but their specific species vary widely (Zhang et al., 2010). Nitrogen application also significantly changes the community structure of soil fungi (Zhang et al., 2018). Chanyarat et al. (2015) have shown that nitrogen application strongly changed the population structure of soil fungi, with the increase of nitrogen fertilizer having potential negative effects on the soil carbon and nitrogen cycle; excessive application of nitrogen fertilizer also increased the proportion of pathogenic fungal species. The results of Zhou et al. (2016) and Bi et al. (2010) also showed that the long-term application of nitrogen fertilizer not only reduced the diversity of soil fungi but also changed the fungal community composition. However, the change in the soil rhizosphere fungal community in different types of soil with the same fertilization application levels has not been investigated sufficiently.

Reasonable nitrogen fertilizer application is a concern of an increasing number of agricultural producers. Lishu, Jilin Province, China is the national key commodity grain production base, and sustainable cultivation of corn is crucial in this region, the contribution of Jilin province to Chinese total production is about 15% (Lin et al., 2018). In this work, we conducted a field trial of maize cultivation for 9 consecutive years. We investigated the changes in the composition and diversity of the maize rhizosphere fungal community under the same climatic conditions in three soil types under different long-term nitrogen application levels based on sequence variation of ITS rDNA using Illumina MiSeq sequencing. The results will provide a theoretical basis for rational N application in different types of soil.

Materials and methods

Collection of soil samples and field experiments

Soil samples were collected from Lishu County, Siping city, Jilin Province, Northeast China (123°45′–124°53′E, 43°02′–43°46′N). The area has a temperate

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continental monsoon climate, with four distinct seasons. The average annual rainfall is 594.8 mm and the average temperature is 5.6 °C. The main farming method this region is continuous cultivation of corn. There are three types of soil in the area: sandy soil (S) in Fujia Street village, meadow black soil (M) in Sankeshu village and alluvial soil (A) in Wangjiaqiao village.

There were five fertilization treatments in each soil: (1) N0 (no fertilization), (2) N168 (N, 168 $kg \cdot ha^{-1}$), (3) N240 (N, 240 $kg \cdot ha^{-1}$), (4) N270 (N, 270 $kg \cdot ha^{-1}$), and (5) N313 (N, 313 $kg \cdot ha^{-1}$). N fertilizer (urea) was applied to soil twice as basal fertilizer and as top dressing. Phosphate (P_2O_5) and potassium (K_2O) fertilizers were applied as basal fertilizer at rates of 100 $kg \cdot ha^{-1}$ and 120 $kg \cdot ha^{-1}$, respectively. Each treatment was repeated three times, with random permutation. All plots were planted with the Xianyu 335 corn variety. It was sown in April of each year, and all corn residues were removed from the plot after harvesting in October.

The soil samples were collected after the harvest period. To investigate the response of fungal communities for different soils, each plot was sampled at 5 points, and the samples were mixed into one sample, for a total of 15 test samples; Another15 test samples were obtained to investigate the effect of N application level on soil fungi (each treatment was took 3 samples). The maize rhizosphere soil were collected in polyethylene bags and then shaken vigorously by hand for 10 min until the non-adhering soil fell off. All samples were conserved on ice and taken back to the laboratory. Aliquots of 5 to 10 g of soils were directly shock frozen in liquid nitrogen after sampling and stored at −80 °C until DNA extraction. The remaining soil samples were conserved at 4 °C for analysis of the soil physicochemical properties after 2 weeks. Before analysis, the soil samples were air dried and sieved using a 2 mm mesh. Soil pH, moisture content (MC), organic carbon content (SOC), total nitrogen (TN), available phosphorus (A-P), available potassium (A-K) were measured by Soil agrochemical analysis. Ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were measured by a continuous flow analyser (Skalar San++, Netherlands) (Cui et al., 2018).

Extraction and detection of soil nucleic acids

Total genomic DNA was isolated from 0.50 g of soil samples using the PowerSoil® DNA Isolation kit (MoBioLaboratories, Inc., Solana, CA, USA) according to the manufacturer's instructions, with some modifications (the centrifugation time was extended to 15 min in step 5). Extraction and purification of soil nucleic acids was performed by the TIAN quick Midi purification kit (TIANGEN). Next-generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (Suzhou, China). DNA samples were quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). An amount of 50-100 ng DNA was used to generate amplicons using a panel of primers designed by GENEWIZ (GENEWIZ, Inc., South Plainfield, NJ, USA). Multiple oligonucleotide primers were designed to anneal to the relatively conserved sequences spanning fungal ITS regions (the forward primer containing the sequence "ACCTGCGGARGGAT"; the reverse primer containing the sequence "GAGATCCRTTGYTRAA"). In addition to the ITS target-specific sequences, the primers also contained adaptor sequences allowing the uniform amplification of the library, which had high complexity and was ready for downstream NGS sequencing on the Illumina Miseq platform. DNA libraries were validated by an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA) and quantified by Qubit 2.0 fluorometer. DNA libraries were multiplexed and loaded on an Illumina

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MiSeq instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 300/250 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) embedded in the MiSeq instrument. To ensure the purity of the measured DNA, we used agarose electrophoresis and determination of the extracted soil DNA and determined the absorbance of the soil DNA dilution at 230, 260 and 280 nm using an ultraviolet photometer (UV5 Nano, Switzerland).

Statistical methods

First of all, the original data were performed and filtered with low quality (sequence length < 200 bp, no ambiguous bases, mean quality score ≥ 20). Then, clustering analysis was carried out, each cluster was called an operational taxonomic units (OTU). Alpha diversity indices were calculated in QIIME from rarefied samples using the Chao1 richness index, the Shannon diversity index, Simpson diversity and Goods coverage (Huang et al., 2019). An unweighted pair group method with arithmetic mean (UPGMA) tree was constructed from the beta diversity distance matrix (Deng et al., 2007). We also performed a redundancy analysis (RDA) using the to analyse the relationships between the environment, the samples and the top 30 most abundant fungal species (Zhang et al., 2018). To determine the effects of nitrogen fertilization on soil properties and fungal characteristics, statistical software (SPSS 21) was used to perform a one-way ANOVA and LSD tests.

Results

The physical and chemical properties of the maize rhizosphere

The physical and chemical properties of the three types of soil under different N application levels are shown in *Table 1*. The physical and chemical properties: Soil organic carbon (SOC), total nitrogen (TN), total nitrogen (TP), total potassium (TK), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), available phosphorus (AP) and available potassium (AK) in meadow black soil were higher than that in the other two soil types, and pH was highest in alluvial soil; The physical and chemical properties were changed significantly with N application levels: when N application level arrived at 312 kg·ha^{-1}, the pH decreased 6.3%, the contents of NH_4^+-N and NO_3^--N increased 1.7 and 5.62 mg·kg^{-1}. But the total nutrients (SOC, TN and TP) had no significant changes with the increasing N application levels (P > 0.05) (*Table A1*).

Table 1. *Soil chemical properties of different types of soil*

	pH	MC	SOC	TN	TP	TK	NH_4^+-N	NO_3^--N	AP	AK
S	6.06±0.28a	7.56±0.61c	12.56±0.29c	0.95±0.07c	6.05±0.65c	4.81±0.06b	2.57±0.84b	1.17±0.55b	90.45±31.54b	126.27±33.76b
M	5.93±0.31ab	18.96±1.07a	25.91±0.62a	1.45±0.07a	9.28±0.47a	5.35±0.16a	4.12±0.58a	5.48±0.64a	160.60±34.46a	196.67±47.63a
A	5.58±0.18b	13.24±0.65b	11.08±0.78b	1.10±0.10b	7.95±0.76b	5.11±0.22a	2.82±0.11b	2.04±0.08ab	141.22±32.86a	120.93±30.26b
N0	5.83±0.13a	13.72±1.57a	12.71±0.84a	1.11±0.38a	5.20±0.49a	2.78±0.35b	2.78±0.35b	0.67±0.42b	149.00±8.43a	112.65±23.28b
N168	5.72±0.07ab	13.80±1.00a	12.90±0.61a	1.20±0.09a	5.45±0.43a	2.27±0.13b	2.27±0.13b	0.94±0.68b	119.00±11.61b	194.88±15.87a
N240	5.48±0.09ab	12.46±2.42a	12.41±0.29a	1.16±0.15a	4.92±0.32a	2.48±0.20b	2.48±0.20b	1.03±0.32b	113.67±7.51b	124.56±19.06b
N270	5.59±0.35ab	12.62±1.41a	12.14±1.75a	1.07±0.09a	4.98±0.19a	2.00±0.94b	2.00±0.94b	1.19±0.44b	106.67±3.21b	124.56±27.42b
N312	5.46±0.04b	13.60±3.01a	12.62±1.59a	0.95±0.28a	4.98±0.19a	4.58±1.72a	4.58±1.72a	6.35±4.67a	116.33±19.55b	149.47±36.96ab

The lower-case letters 'a' and 'b' indicate significant difference (P < 0.05) among different samples for each treatment. "S" indicates sandy soil, "M" indicates meadow black soil and "A" indicates alluvial soil. MC is the moisture content of soil; SOC is Soil organic carbon content; TN is soil total nitrogen; TP is soil total nitrogen; TK is soil total potassium; NH_4^+-N is ammonium nitrogen; NO_3^--N is nitrate nitrogen; AP is available phosphorus; AK is available potassium

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The fungal community diversity

A total of 1.5 million sequences were obtained from the ITS gene sequencing, with a total gene base number of approximately 10^8 bp. When grouped at the 97% similarity level, there were 630 OTUs for all of the soils. The number of OTUs in meadow black soil was higher than that in the other two soils. As the N application levels increased, the number of OTUs first increased then decreased, the highest under N168 (*Table 2*), and we found pH, SOC, NH_4^+-N, NO_3^--N, TP, TK and AK had negative effect on the number of OTU (*Table A2*). The findings suggested that fungi were abundant in high quality soil. The fungal community alpha diversity in the three types of soil was different: the average Chao 1, Shannon and Simpson indices were highest in meadow black soil, and that was lowest in sandy soil ($p < 0.05$). The fungal community alpha diversity varied with different nitrogen applications: as the N application levels increased, the average Chao 1 index increased, but remained were lower than that under N0. The Shannon and Simpson index had no obvious change pattern under nitrogen application ($p > 0.05$) (*Table 2*). In order to discover which factors affected fungal community diversity, we analysed the correlations between alpha diversity indices and soil physical-chemical properties and N application levels. The correlation between fungal alpha diversity and soil physical-chemical properties was significant, especially SOC had significant effect on Chao 1 and Shannon index, but the correlation between fungal alpha diversity and N application level was not significant (*Table A2*). These findings indicated that the alpha diversity of fungal communities in this study was mainly influenced by the type of soil.

Table 2. *The alpha diversity and OTU richness for N application levels in three types of soil*

	OUT (×10^4)	**chao1**	**Shannon**	**Simpson**
A	10.64 ± 0.76a	436.617 ± 19.631b	5.319 ± 0.259ab	0.933 ± 0.015ab
M	9.18 ± 0.46a	489.906 ± 26.071a	5.814 ± 0.453a	0.957 ± 0.018a
S	9.77 ± 0.15a	452.877 ± 22.968b	5.111 ± 0.499b	0.914 ± 0.032b
N0	10.39 ± 0.19b	476.653 ± 18.633a	5.421 ± 0.384a	0.938 ± 0.017a
N168	11.87 ± 0.03a	429.807 ± 26.164b	5.234 ± 0.728a	0.922 ± 0.037a
N240	9.48 ± 0.42c	433.195 ± 28.116ab	5.38 ± 0.370a	0.931 ± 0.027a
N270	9.18 ± 0.51c	453.621 ± 18.402ab	5.661 ± 0.388a	0.952 ± 0.018a
N312	7.84 ± 0.08d	461.726 ± 22.605ab	5.376 ± 0.766a	0.929 ± 0.046a

The fungal community structure

We obtained 5 phylum groups and an unclassified group by comparing with the UNITE database (*Fig. 1*). The dominant phylum in the three types of soil was the same. Among the identified groups, Ascomycota was the most dominant phylum (53.33-64.60%), followed by Basidiomycota (17.51-34.42%). The relative abundances of Ascomycota, Chytridiomycota and the unclassied in sandy soil were lower than those in the other two soils, but the abundances of Basidiomycota and Glomeromycota were the highest in sandy soil. The relative abundance of Zygomycota in black meadow soil was 2.95% higher than that in the sandy soil. With the N application level increased, the relative abundance of Ascomycota decreased, but Basidiomycota increased significantly. Moreover, Glomeromycota abundances were negatively correlated with the N application level (*Table A3*).

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We also identified fungi by genus to analyse the response of fungi to N application in three types of soil. The results showed that the top five most-changed genera level in three types of soil at the different N application levels. *Cryptococcus, Fusarium, Holtermanniella, Thielaviopsis and Trichoderma* were the most affected by the different types of soil (*Fig. 2a*). However, *Alternaria, Geomyces, Gibberella, Rhizopus and Waitea* changed significantly with the N application level (*Fig. 2b*). And *Alternaria, Rhizopus and Waitea* belonged to fungal pathogens, it suggested that excessed N application could increase fungal pathogens.

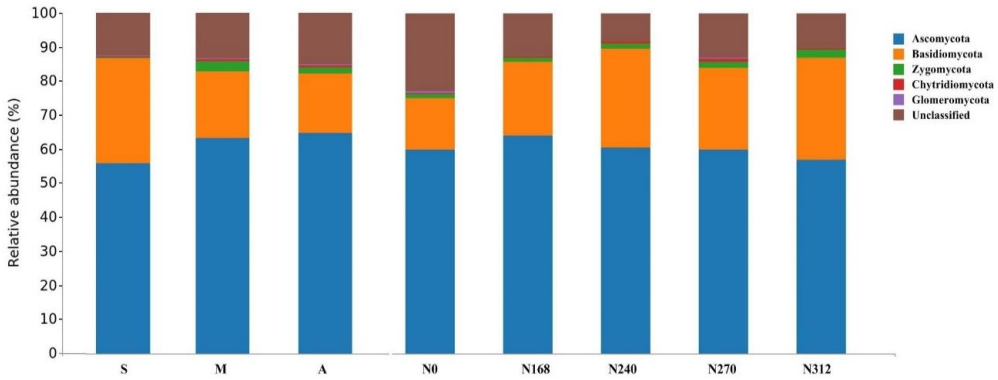

Figure 1. *Relative abundance at the fungus phylum level of N application levels in three types of soil*

An UPGMA tree was used to calculate and analyse Beta diversity. The results showed that all samples were clustered into two main groups. Samples from the sandy soil and black meadow soil clustered into one clade, and samples from alluvial soil clustered in another clade. Samples in the sandy soil and black meadow soil also clustered into two clades; one clade was the N0 treatment, and the other treatments were in another clade (*Fig. 3*).

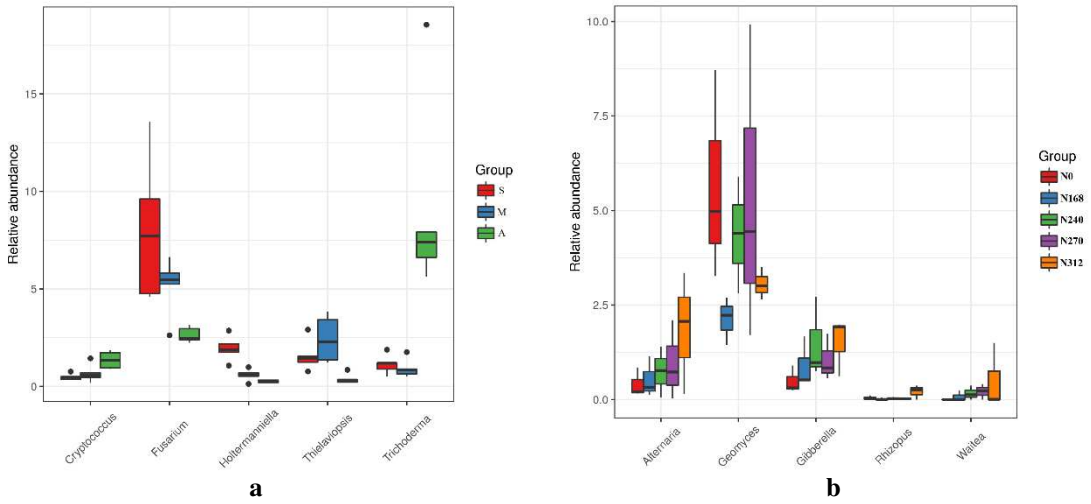

Figure 2. *The top 5 changed relative abundance of fungi in genus level. (a) Fungi in different types of soil; (b) fungi in different N applications*

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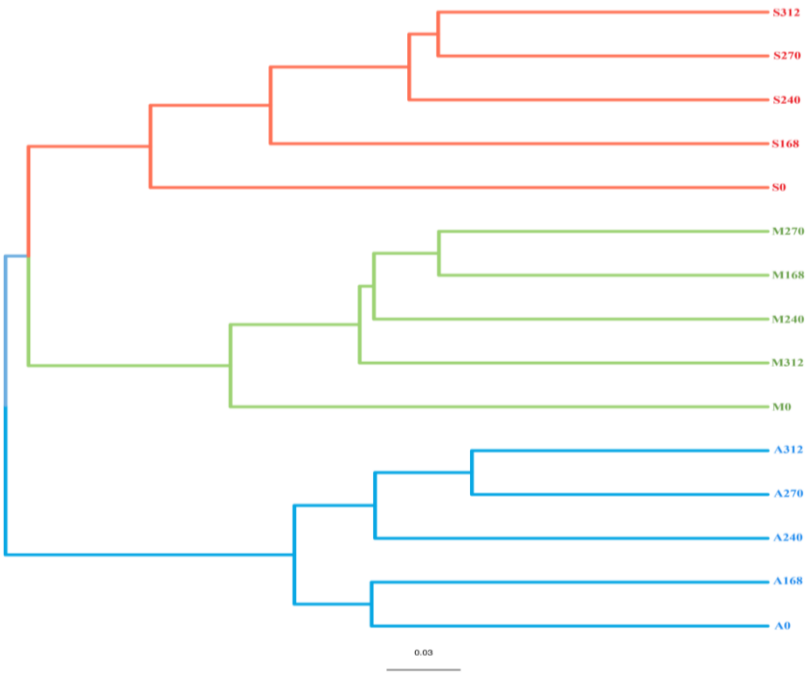

Figure 3. *OPGAM_Tree analyze of soil fungal community under different N application levels in three types of soil. Red indicates sandy soil, green indicates meadow black soil and bule indicates alluvial soil*

To further investigate the effects of N application levels on fungal communities in different types of soil, the interdependence between the ITS transcript abundance of individual phylotypes and each of the environmental factors was investigated by redundancy analysis. Among the 10 environmental variables tested, all variables showed significant correlation with fungal community composition. We found that *Humicola, Mortierella* and *Leptosphaeria* were affected by most of the environmental factors, and they were all positively correlated with environmental factors. pH was significantly positively correlated with *Fusicolla, Entrophosora, Verticillium,* and *Preussia* but was negatively correlated with *Trichocladium, Arthrinium, Penicillium* and *Cryptococcus*. SOC was positively significantly correlated with *Leptosphaeria, Thielaviopsis, Microdochium, Verticillium* and *Preussia* and negatively correlated with *Trichoderma, Cordyceps* and *Arthrinium*. TK was significantly correlated with 7 fungal community compositions. TN was positively correlated with *Humicola, Mortierella, Lestosphaeria, Lecanicillium* and *Chaetomium*. And we found that pH, SOC, TN, MC and TP were the main influences on the fungal community (*Fig. 4*).

Discussion

Soil properties

In agro-ecosystems, appropriate nitrogen input can provide the required nutrients for crop growth, but excessive application of nitrogen may have a negative impact on the soil environment. In this study, the physical and chemical properties in different types of soil were different, which was same as Jirků et al. (2013), as shown in *Table 1*. Wang et al. (2017) suggested that meadow black soil contained higher soil organic matter

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content and SOC, and was more fertile. In addition, the soil responses to nitrogen application were different. The content of AP decreased significantly with increasing levels of nitrogen application in the black meadow soil, but AP content increased in the sandy soil and the alluvial soil. In addition, the application of high nitrogen can lead to the accumulation of $NH4^+$-N and $NO3^-$-N in the soil. N application had no significant correlation with total nutrients ($P > 0.05$) (Yu et al., 2016), but it was significantly correlated with available nutrients ($P < 0.05$) (*Table A1*). Although the input of nitrogen did not increase the content of total nutrients in the soil, the accumulation of different forms of N promotes N cycling in the soil.

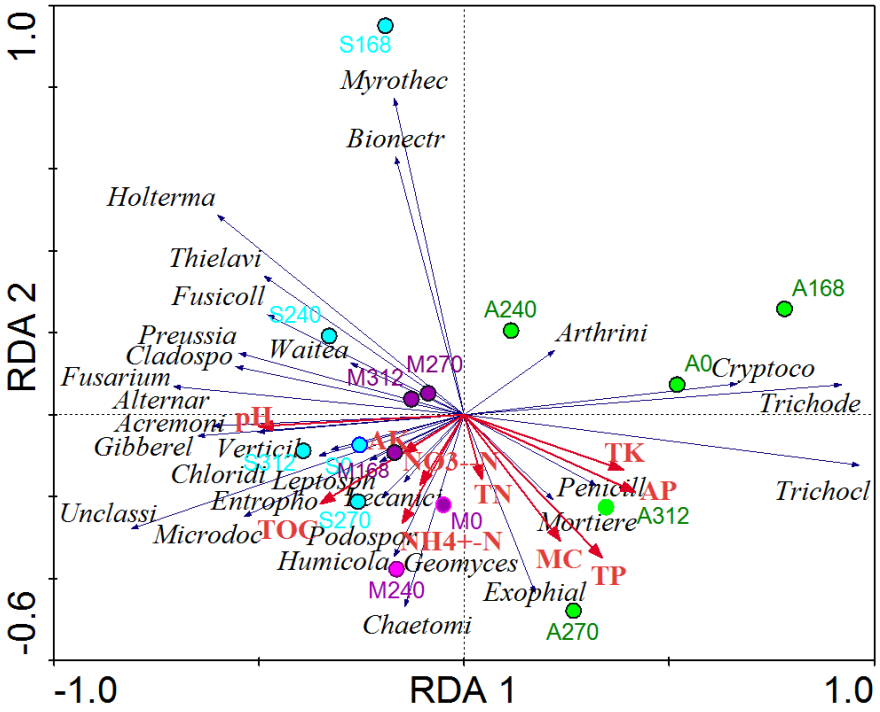

Figure 4. *Triplots based on redundancy analysis (RDA) of 16S rDNA finger print patterns, showing the contribution of environmental parameters to variability. Arrows indicate environmental factors and their relative effects on fungal community structure*

The pH decreased significantly with increasing nitrogen application in the three types of soil. The continuous application of chemical fertilizers causes incomplete circulation of nitrogen in the soil, leading to soil acidification (Kamaa et al., 2011; Xia et al., 2015; Sun et al., 2015). The pH changes in the alluvial soil were relatively smaller than those in the sandy soil and the meadow black soil. The pH of the alluvial soil was the lowest when the nitrogen application level was 312 kg·ha^{-1}, but it only decreased 0.37 compared to that in the control, whereas pH decreased by 0.7 and 0.92 in the sandy soil and black meadow soil, respectively. This may be because the buffer capacity of alluvial soil is relatively higher than that of sandy soil and meadow black soil (Chen et al., 2005).

Rhizosphere soil fungal diversity

Currently, the use of ITS sequencing technology to study the diversity and structure of fungal communities in the soil environment is becoming more common. Some

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researchers believe that fungal diversity is closely related to the soil ecological environment, which can lead to differences in the species and quantity of fungi in rhizosphere soil (Corey et al., 2008). Agricultural practices, such as tillage, fertilization, and machinery, which are used for crop management, have modified the physical and chemical properties of soils (Bissett et al., 2011, Suleiman et al., 2013) and consequently altered the soil fungal community diversity. In this paper, the diversity of fungi was relatively rich, and the diversity index was different in different soils (Jun et al., 2008). The fungal diversity in black meadow soil was higher than that in the other two soils, possibly because there was rich soil organic carbon in the black meadow soil, and soil organic carbon can provide nutrients for fungi (Jiang et al., 2018). The Chao 1 index under no nitrogen was the highest, and with the N application level increased, the Shannon and Simpson index had no significant changed, which was the opposite of the results of Qin et al. (2015) but the same as the results of Chanyarat et al. (2015). This may be because the fungal communities in different soils are different, and fungal community diversity changed significantly with the soil properties (*Table A3*). Liu et al. (2012a) found chemical fertilization could significantly alter the community diversity, and our results showed N application had negative relation with OTUs and fungal community diversity. This may be because excessive N application could increase the content of nitrate nitrogen, then decreased the pH and soil pH had significant negative relation with fungal diversity (Lamabam et al., 2012).

Rhizosphere soil fungal structure

The soil microbial community structure refers to the abundances and relative proportions of major microorganisms in soil (Bissett et al. 2011). In addition, the relative abundances of dominant fungal phyla in the rhizosphere soil change with nitrogen application (Yan et al., 2018). As shown in previous work, Ascomycota and Basidiomycota are dominant microorganisms that can frequently be found in maize soils (Yin et al., 2018). In this study, Ascomycota and Basidiomycota were the dominant fungal phyla among the three types of soils (*Fig. 1*), but the relative abundances of different samples from different soils were different. The relative abundance of Ascomycota in the sandy soil was lower than that in the other two soils. However, the relative abundance of Basidiomycota in the sandy soil was the highest of the three types of soil. This may be because of the different soil physical-chemical properties and nutrient contents in the three types of soil (Ruan et al., 2009). Specifically, Basidiomycota and Ascomycota could decompose animal and plant residues, cellulose and hemicellulose, etc. and provide nutrients for crops. We discovered Basidiomycota had a significant positive relationship with nitrogen application. Increasing levels of nitrogen addition favoured Basidiomycota, which may be ascribed to increases in low-lignin, high-cellulose substrate availability under elevated N conditions (Wang et al., 2015). However, in black meadow soil, the relative abundance of Ascomycota increased under N168 but decreased with excess N. This indicated that the dominant fungal community response to N application in different soils is different. The unclassified fungal phyla decreased with nitrogen addition, indicating that nitrogen application has a positive effect on known fungal communities. In addition, we found that the relative abundance of Chytridiomycota and Glomeromycota which showed linear increases with CO_2 (Procter et al., 2014) altered significantly with N application. Glomeromycota abundance decreased with increasing N application levels (Liu et al., 2012b). But Chytridiomycota relative abundance was

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higher than Glomeromycota, and the relative abundance of Chytridiomycota increased with N application. We also found that abundances of *Cryptococcus* and *Holtermanniella*, belonging to the Basidiomycota phyla, and *Fusarium, Thielaviopsis* and *Trichoderma*, belonging to the Ascomycota phyla, were significantly different among three types of soil. Moreover, *Alternaria*, (P_Ascomycota), *Rhizopus* (P_Zygomycota) and *Waitea* (P_Basidiomycota) showed significant positive responses to nitrogen. This suggested N application could increase the transmission of fungal pathogens. In these most-changed fungi, *Fusarium* is an example of a fungal genus in the class Sordariomycetes and is known to function in denitrification. Sordariomycetes was the only fungal class that responded to N addition (Mueller et al., 2015). In this study, *Fusarium* had a positive response to N application levels, and it was highest in the sandy soil (*Fig. 3*), which is the opposite of the results found by Lauber et al. (2008) and Mueller et al. (2015). This may be because *Fusarium* has different responses to N addition in different soils.

Soil fungal community structure is an important index for measuring soil quality and maintaining soil fertility (Swer et al., 2011). Moreover, the fungal community notably changes in different types of soil (Zhang et al., 2013). In this study, the Beta diversity of soil fungal colonies across different soils under different levels of nitrogen using an UPGMA tree showed that all samples clustered into two large groups, i.e., the sites of the sandy soil and black meadow soil were clustered into one clade, while those of alluvial soil were clustered into the other clade. In these two large groups, the samples clustered into two clades: one clade included the N0 samples, and the other included the samples with N application. However, the samples of N0 and N168 were closer to each other than to the others in the alluvial soil. This structure indicates that the N fertilizer effects on fungal community structure in different types of soils were different: the fungal community rapidly increased with the level of N fertilizer in sandy soil, but the fungal community did not increase significantly with N fertilizer until the level was 168 $kg \cdot ha^{-1}$ (Girvan et al., 2004). As the N application levels increased, the soil physical and chemical properties changed, and the soil fungi had a significant relationship with the soil environment (*Fig. 4*). Huang et al. (2017) showed that low levels of bio-organic fertilizer had little effect on soil microorganisms, but high levels of bio-organic fertilizer could change the soil microbial community.

To determine the effects of soil physical-chemical properties on the fungal community, we used a heatmap analysis of the relative abundance of 30 main fungal taxa (*Fig. 4*). The soil physical-chemical properties had a significant effect on the fungal community (Ana et al., 2010). However, Carles et al. (2016) considered that drying procedures had no effect on fungal community composition or on fungal diversity. In this paper, soil MC had a positive effect on Holtermanniella and a negative effect on Chaetomium, Leptosphaeria, Mortierella and Humicola, possibly because the fungal community was collected from different soils or in different seasons and the response of the fungal community to different soils varies (Chemidlin et al., 2014). In this paper, pH, SOC, MC (Ke et al., 2016), TN and TP had a significant influence on the fungal community. Although the soil physical-chemical properties did not strongly change with the N application level, they were significantly different in different types of soil. Therefore, we concluded that the most important factor for the fungal community was the soil type, followed by the N application level. As the level of nitrogen application increased, the soil active nutrients were altered, and the applied N could improve nutrient availability for soil fungi.

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Conclusions

Our study revealed that the responses of fungal community to long-term N application. The diversity of soil fungal communities was higher in black meadow soil than in sandy soil and alluvial soil due to the higher soil nutrition in black meadow soil. Our results also confirmed that N application level had no significant effect on fungal diversity, but had significantly correlated with soil physical-chemical properties. Then pH, MC, TN and TP could alter soil fungal community diversity and structure. From the point of view of soil fungal structure, it seemed that increasing N application level could increase CO_2 emissions and transmission of fungal pathogens. Ascomycota abundance was highest in S270, M168 and A240 respectively. Here, we suggest that the amount of fertilizer application should be based on different types of soil, and the ordination of N application should be sandy soil > alluvial soil > meadow black soil. In this way, it could not only reduce the application amount of nitrogen fertilizer, while reducing environmental pollution, but also could optimize the structure of fungal community.

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APPENDIX

Table A1. *Correlation analysis between soil physical-chemical properties and N application levels*

	pH	MC	SOC	TN	NH_4^+-N	NO_3^--N	TP	AP	TK	AK
R	**-0.98****	**-0.98****	0.386	0.287	**0.893***	**0.89***	-0.11	0.07	-0.8	-0.17
P-value	**0.00**	**0.00**	0.52	0.64	**0.04**	**0.04**	0.86	0.91	0.10	0.78

MC is the moisture content of soil; SOC is Soil organic carbon content; TN is soil total nitrogen; TP is soil total nitrogen; TK is soil total potassium; NH_4^+-N is ammonium nitrogen; NO_3^--N is nitrate nitrogen; AP is available phosphorus; AK is available potassium. **indicates $p < 0.01$, * indicates $p < 0.05$

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Table A2. *Correlation analysis between fungal community and soil physcial-chemical properties*

	chao1		Shannon		Simpson		OTU	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**
pH	0.331	0.228	-0.024	0.932	-0.104	0.711	-0.620*	0.014
MC	0.469	0.078	0.523*	0.046	0.227	0.415	-.0602*	0.018
SOC	0.707**	0.030	0.587*	0.022	0.025	0.928	-0.672**	0.006
TN	0.562*	0.290	0.559*	0.030	-0.310	0.261	-0.337	0.219
$NH4^+$-N	0.498	0.059	0.307	0.265	-0.409	0.130	-0.644**	0.010
$NO3^-$-N	0.485	0.067	0.617*	0.014	-0.057	0.841	-0.720**	0.002
TP	0.473	0.075	0.638*	0.010	-0.101	0.720	-0.608*	0.016
AP	0.277	0.318	0.478	0.072	0.056	0.842	-0.398	0.142
TK	0.392	0.149	0.307	0.266	0.556*	0.031	-0.530*	0.042
AK	0.656	0.008	0.470	0.077	-0.019	0.946	-0.936**	0.000
N	-0.020	0.944	-0.116	0.681	0.060	0.830	-0.016	0.955

Table A3. *Correlation analysis between fungal phylum and N application levels*

	Ascomycota	**Basidiomycota**	**Unclassified**	**Zygomycota**	**Chytridiomycota**	**Glomeromycota**
R	-0.55	**0.89**	**-0.97**	-0.81	-0.66	**-0.93**
P-value	0.34	0.05	0.01	0.10	0.22	0.02

Table A4. *The location of the sampling sites*

Soil types	**Sampling location**	**Latitude and longitude**	
Sandy soil	Fujiajie country	43° 21′ N	124° 05′ E
Meadow black soil	Sankeshu country	43° 20′ N	124° 00′ E
Alluvial soil	Wangjiaqiao country	43° 15′ N	124° 29′ E

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SAFETY OF INSECTICIDES TO HONEY BEES TARGETED FOR THE MANAGEMENT OF *HELICOVERPA ARMIGERA* IN PIGEON PEA

KAMBREKAR, D. N.[1] – JAHAGIRDAR, S.[2]

[1]*Department of Agricultural Entomology, University of Agricultural Sciences, Dharwad 580005, Karnataka, India*

[2]*Department of Plant Pathology, University of Agricultural Sciences, Dharwad 580005, Karnataka, India*

**Corresponding author*
e-mail: kambrekardn@gmail.com

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Abstract. The present study aimed to investigate the repellent proprieties of recommended insecticides targeted at the management of pigeon pea pod borer, *Helicoverpa armigera* along with the recuperation of three species of honey bees under field condition during 2018-19 and 2019-20 at Vijayapur and Bagalkot districts of Karnataka-India. The repellency studies of insecticides concerning honey bees in both locations and during both the years indicated that the recovery percentage of honey bees (*Apis florea, Apis cerana* and *Apis dorsata*) for the treated field had the fastest rate in the plots treated with Neem Seed Kernel Extract followed by chlorantraniliprole compared to other insecticides. Further, normal activity (100% recovery) of the bees was realized in the treatment with Neem seed kernel extract and chlorantraniliprole which took only 2 and 3 days as compared to more than 7 days for other insecticides. Among the bee species studied, *A. dorsata* resumed its normal activity sooner than *A. cerana* and *A. florea. A. florea* was the most sensitive species to insecticides in pigeon pea. The results indicate the faster recovery of bees which has augmented pollination services and has a greater relevance in enhancing the yield in pigeon pea, whenever the insecticides with less repellent activity were used in the IPM programmes.
Keywords: *chlorantraniliprole, Helicoverpa armigera, neem seed kernel extract, pollination*

Introduction

Pigeon pea *(Cajanus cajan* (L.) Millsp.) is cultivated in tropical and sub-tropical areas and is an important legume crop of Asia (especially, the Indian subcontinent), Latin America and Eastern and Southern Africa. Globally, it is grown on around 5 million hectares (m ha) in about 82 countries of the world. Pigeon pea has a significant place in Indian farming and India contributes 90% of the world pigeon pea production. It is the second most important pulse crop next to chickpea, covering an area of around 4.42 m ha (occupying about 14.5% of area under pulses) and production of 2.86 mt (contributing to 16% of total pulse production) and productivity of about 707 kg/ha. It is staple diet throughout the country that is mainly consumed as dry split daal besides several other uses of various parts of pigeon pea plant. It is an excellent source of protein (20-22%), supplementing energy rich cereal diets in a mainly vegetarian population. In addition to food, it is used as fodder, feed, and fuel and has functional utility for making baskets, huts, fences, etc.

Pollinators play an important role in providing key component of ecosystem services in the form of crop pollination that is vital to maintain the plant communities and to enhance the quality and quantity of agricultural produce. It has been estimated that one

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third of the food eaten by humans, either directly or indirectly, comes from honey bee pollination (Free, 1993). In addition, the proportions of agricultural crops that depend on honey bee are increasing because of their versatility, low cost, and the ease with which they are moved and managed. Multiple pressures threaten pollinator populations and the pollination services they provide, including the negative effects of insecticides. Bees provide pollination services to various food crops as well as wild plants (Delaplane and Mayer, 2010). Recently, decline in various pollinators have been reported worldwide (Potts et al., 2010; Cameron et al., 2011). Although many environmental and anthropogenic factors remain under investigation for their role in annual honey bee colony losses, pesticides is a major factor (Smith et al., 2014).

Helicoverpa armigera (Hubner) is most destructive pests of field crops worldwide. Its wide dissemination high mobility, survival rate under adverse conditions, capacity to complete several generations in a year (polyvoltine), ability to develop resistance against insecticides, its polyphagy, its ability to undergo facultative diapauses and migration has made its management very difficult (Kumar et al., 2012a).

Pigeon pea is being ravaged by several insect pests, of which the damage caused by the pod borer, *Helicoverpa armigera* (Hubner) is immensely attributed to greater yield losses and increased cost on crop protection. The severity of pod borer in pigeon pea coincides with the flowering and pod formation of the crop, the activity of honey bees is also characterized as a major contributing factor for yield.

The study of pesticide repellent activity on the pollinators is vital because of the need to manage the insect pest occurring concurrently. The effects of insecticide on honey bees includes direct mortality, sublethal effects, repellent effects and toxicity of the residues present on the floral parts and nectar of the crop plant (Desneux et al., 2007). The repellent effects of insecticides on honey bees have already been reported by many workers in the past on different crops (Thompson and Wilkins, 2003 and Abrol and Kumar, 2009).

In pigeon pea, flowers are yellow with red to reddish-brown. The flowers are self-compatible and usually self-pollinated. However, there is a good amount of cross pollination which occurs with insect visitations (Saxena et al., 1990). In redgram, honey bees are the major pollination contributors and the peak foraging activity of honey bees in pigeon pea is between 10.00 to 14.00 h and the major pollinators are *Apis florea, Apis cerana* and *Apis dorsata* (Kambrekar et al., 2019). Since, the foraging is during 10.00 to 14.00 h, there is a greater relevance of study of repellent properties of recommended insecticides on honey bees.

During the course of application of insecticides, honey bee directly come in contact with the insecticides or the insecticide treated floral parts which affects behaviors such as communication dances, return flights, orientation, and foraging efficacy during visits to flower (Vandame et al., 1995). Pesticides also known to reduce the ability of honey bees in gathering food from plant and also have lethal effects on the bees. There are two insecticides commonly used by the farmers namely neonicotinoids and organo phosphorous which could affect bees' brains. Studies also indicate that bees fed on neonicotinoid contaminated pollen and nectar produces fewer offspring. On the other hand, certain pesticides can destroy cells in the gut, brain, other tissues, thus affecting the bee's physiology and behavior. Pesticides have been reported to affect the reproductive potential of the bees by reducing sperm viability in drones that causes poor mating and destruction of ovary activation in the developing queen (Tosi et al., 2017).

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Both in response to threats to honeybees and in recognition of the potential benefits of augmenting honey bees, methods are being developed to conserve native and domesticated bee populations. One such strategy involves spraying of insecticides after or before the peak foraging activity of the pollinators including managing agricultural field edges to increase the diversity of floral provisioning resources (Winfree et al., 2008; Egan and Mortensen, 2012) and the abundance of specific floral hosts (Isaacs et al., 2009).

It has been analyzed that decline of honeybee population is due to insecticides like organochlorine, carbamate, organophosphorus and pyrethroid. The damage to honey bee colony by application of pesticides not only depends by toxicity of chemical substances, number and methods of insecticides application, time of application, weather, but also by type of nectar, type of food flower collected, season of damage, number of honeybees in colony and also the type of insecticides used to control the insect pests co-exists.

The purpose of the present investigation was to assess the detrimental effects of insecticides targeted for the management of pod borer in pigeon pea on honey bees under field condition. For this purpose, the repellent activity of the insecticides on honey bees was assessed after being applied on the crop and the activity of the bees was regularly monitored and the recovery of the bees was studied in pigeon pea ecosystem.

Materials and methods

Study area

Investigation on repellent activity of insecticides on pollinators of pigeon pea was carried out at two locations *viz.*, Vijayapur and Bagalkot districts of Karnataka (India) during *kharif* 2018 and 2019. Karnataka is the eighth largest state in India with an area of 190 lakh ha. It is situated between 11.5° and 19.0° N latitude and between 74° and 78° E longitude in the southern plateau. The State receives an average annual rainfall of about 1139 mm both from southwest and north-east monsoons. Vijyapur district is situated well in the interior of the Deccan Peninsula and lies between north latitude 15° 20’ and 17° 28’. The average annual rainfall of the district is 668.2 mm, the temperature ranges from 14.8 to 43 °C. The climate of the district is generally dry. Bagalkot is located in Northern Dry Zone (Zone-3) of Karnataka. The centre is located at 75° 42’ East longitude and 16° 10, North latitude with an altitude of 542.00 m above Mean Sea Level (MSL). The average annual temperature is 25.8 °C in Bagalkot. The rainfall here averages 683 mm. The crop was sown at 90 cm of row spacing and 30 cm of plant to plant spacing. The crop flowering starts at 70 days after sowing. The first spray was given at 75 days after sowing followed by next two sprays at an interval of 10 days apart. The crops flowers for 30-35 days with staggered flowering.

Insecticides for the management of pod borer, Helicoverpa armigera in pigeon pea

The spraying of insecticides was done, commensuration to the incidence of pod borer. In each treatment 0.5 ac land was used to know the impact of insecticides on honey bees. Spraying of recommended insecticides was done between 14.00 to 18.00 h of the day to avoid the peak foraging activity of the honey bees. The spraying was carried out with the help of knapsack sprayer with total volume of spray being 500 L/ha.

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Sl. No.	Insecticide	Group	Mode of action	Dose (ml/g/l)
1	Thiodicarb 75 WP	Carbamate	Inhibit acetylcholine esterase	1.00
2	NSKE (%)	Botanical	Feeding deterrent	5.00
3	Chlorantraniliprole 18.5 SC	Anthranilic diamide	Ryanodine receptor activator	0.15
4	Indoxacarb 14.5 SC	Oxadiazine	Blocks the neuronal sodium channels	0.30
5	Emamectin benzoate 5% SG	Abamectin	GABA - and glutamate-gated chloride channel agonist	0.20
6	Flubendiamide 480 SC	Phthalic acid diamide	Ryanodine receptor activator	0.075

Repellent property of the insecticides

The observation on pollinator visitation was initiated during 10% flowering till its complete cessation. Observations were made for different species of honey bees visiting the field during flowering at regular interval of time for 5 min in a square meter area from five spots. The observation on bee visitation was made before the initiation of spray and after the spray of insecticides at one to seven days. Totally three sprays were taken up at ten days interval. The bee visitation recorded after the spray of each insecticide at different intervals was further used to calculate the per cent recovery of the honey bees in comparison with the bee visitation recorded before the spray.

Statistical analysis

The number of bee visits at different intervals after each spray was assessed and the per cent recovery of bees in the sprayed plots were done in correspondence to the pre count of honey bees before spraying. The transformation values were subjected to ANOVA (Analysis of Variance) (Panse and Sukhatme, 1954) and DMRT (Duncan's Multiple Range Test) by using SAS 9.1 (Statistical Analysis Software) programme.

Results

The pooled results on the investigation on the effect of insecticides on the foraging activity of different honey bee species as influenced by their visitation after the spraying is herewith presented in *Tables 1-3*. The average data of two years and two locations is presented.

Apis florea

Before the spray, the population of *A. florea* in different plots ranged between 58.50 to 62.50 bees per square meter area during 5 min. The effect of different insecticides on the foraging activity of *Apis florea* after the spraying revealed statistical difference among the treatments in the restoration of *A. florea*. Bee restoration varied between 7.93 to 81.08% on the 1st day after spraying. Neem Seed Kernel Extract (hereafter NSKE) @ 5% recorded highest restoration bees to the extent of 81.08% indicating its safety and less repellent activity against *A. florea*. Chlorantraniliprole has recorded 42.61% recovery of *A. florea* which is the next safe insecticides to *A. florea.*

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Response of *A. florea* to different insecticides over intervals of post treatment indicated that among the insecticides, normal bee activity (100% restoration) was realized in the plots treated with NSKE (@ 5%) on the 3rd day after the spraying followed by chlorantraniliprole on the 4th day after the spraying. However, in untreated check, normal activity of bee was realized throughout the internals (*Table 1*).

Table 1. *Impact of different insecticides on the activity of Apis florea Fabricius on pigeon pea*

Sl. No	Treatments	Dose (ml/g/l)	Bees/m²/5m	Per cent recovery of bees after spraying						
			DBS	1DAS	2DAS	3DAS	4DAS	5DAS	6DAS	7DAS
1	Thiodicarb 75 WP	1.00	62.50	08.01 (16.43)	16.54 (23.99)	23.30 (28.85)	51.5 (45.89)	70.93 (57.35)	92.41 (73.98)	100.00 (90.00)
2	NSKE (%)	5.00	60.50	81.08 (64.19)	95.50 (77.72)	100.00 (90.00)	100.00 (90.00)	98.92 (84.00)	98.92 (84.00)	99.47 (85.79)
3	Chlorantraniliprole 18.5 SC	0.15	59.50	42.61 (40.73)	70.34 (56.98)	87.43 (69.21)	100.00 (90.00)	100.00 (90.00)	98.92 (84.00)	99.12 (84.58)
4	Indoxacarb 14.5 SC	0.30	60.50	08.27 (16.71)	23.32 (28.86)	32.69 (34.86)	50.10 (45.04)	72.14 (58.12)	88.84 (70.46)	91.98 (73.52)
5	Emamectin benzoate 5% SG	0.20	59.50	14.53 (22.40)	28.02 (31.95)	76.32 (60.86)	89.54 (71.10)	89.89 (71.43)	87.92 (69.63)	91.29 (72.81)
6	Flubendiamide 480 SC	0.075	58.50	07.93 (16.35)	25.28 (30.17)	43.67 (41.35)	75.34 (60.20)	92.68 (74.27)	89.81 (71.36)	75.92 (60.59)
7	Untreated check	-	60.50	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	99.28 (85.10)	99.47 (85.79)	98.67 (83.34)
	S.Em±	-	-	2.77	3.09	3.28	2.39	3.26	3.60	4.24
	CD (P = 0.05)	-	NS	8.55	9.39	10.07	7.37	10.06	10.94	12.91
	CV	-	-	10.56	12.43	10.96	10.85	10.72	9.58	10.72

DBS: Day before spraying, DAS: Days after spraying. Figures in the parenthesis are arcsine transformed values. Bees/m²/5m: Number of bees per square meter area per 5 min

Emamectin benzoate recorded slow and steady visitation by bees after the spraying wherein at the 7th day after spraying 91.29% recovery was recorded indicting the persistent repellency over a long period of time. Similarly, thiodicarb and indoxacarb recorded lowest recovery of the bees up to 3rd day after the spray and the recovery gradually increased towards 7th day and allowed almost normal bee activity on the 7th day indicating their acute repellency up to 3rd day of the spraying.

Apis cerana indica

The normal foraging activity of *A. ceran* before spraying was 48.50 to 52.50 bees per square meter area during 5 min. The repellent effect of different insecticides on the recovery of *Apis cerana* after the spraying indicate Neem Seed Kernel Extract with higher bee activity (77.76%). Emamectin benzoate and chlorantraniliprole have recorded 50.14 and 41.61% recovery of *A. cerana* and are the next safe insecticides to *A. cerana.* Indoxacarb was the most toxic insecticide where only 2.39% and 7.24% bees returned back to the treated plot on 1st and 2nd day after the spray. The normal activity of the bees was assumed on 3rd, 4th and 5th day after spray in the plots sprayed with NSKE, chlorantraniliprole and flubendiamide respectively (*Table 2*). This indicates the diverse response of *A. cerana indica* to the insecticides used for the management of the pod borer in pigeon pea.

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Table 2. *Impact of different insecticides on the activity of Apis cerena Fabricius on pigeon pea*

Sl. No	Treatments	Dose (ml/g/l)	Bees/m²/5m	Per cent recovery of bees after spraying						
			DBS	1DAS	2DAS	3DAS	4DAS	5DAS	6DAS	7DAS
1	Thiodicarb 75 WP	1.00	52.00	10.81 (19.19)	21.67 (27.73)	56.01 (48.43)	76.06 (60.68)	91.84 (73.46)	96.27 (78.91)	87.14 (68.95)
2	NSKE (%)	5.00	49.50	77.76 (61.84)	91.06 (72.57)	98.78 (83.62)	100.00 (90.00)	97.57 (81.09)	100.00 (90.00)	100.00 (90.00)
3	Chlorantraniliprole 18.5 SC	0.15	50.50	41.61 (40.15)	87.33 (69.12)	95.27 (77.41)	100.00 (90.00)	97.22 (80.37)	94.78 (76.83)	100.00 (90.00)
4	Indoxacarb 14.5 SC	0.30	52.00	02.39 (8.89)	7.24 (15.60)	38.52 (38.35)	53.77 (47.14)	85.33 (67.45)	100.00 (90.00)	89.76 (71.37)
5	Emamectin benzoate 5% SG	0.20	52.50	50.14 (45.06)	70.98 (57.38)	79.02 (62.71)	85.33 (67.45)	80.37 (63.72)	73.80 (59.21)	49.87 (44.89)
6	Flubendiamide 480 SC	0.075	49.00	10.76 (19.14)	35.34 (36.46)	58.51 (49.88)	96.32 (78.91)	100.00 (90.00)	86.32 (68.28)	96.42 (79.06)
7	Untreated check	-	48.50	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	98.26 (82.51)	96.69 (79.53)	99.43 (85.67)
	S.Em±	-	-	3.27	3.66	3.51	3.39	3.26	3.60	4.24
	CD (P = 0.05)	-	NS	10.09	11.29	10.82	10.45	10.06	10.94	12.91
	CV	-	-	11.93	11.54	9.60	10.14	10.72	9.58	10.72

DBS: Day before spraying DAS: Days after spraying. Figures in the parenthesis are arcsine transformed values. Bees/m²/5m: Number of bees per square meter area per 5 min

Apis dorsata

Before spraying, the total number of *A. dorsata* bees in all the experimental plots ranged from 21.00 to 23.00 bees per square meter area for 5 min and was significantly less compared to *A. cerana* and *A. florea* indicating the less contribution by *A. dorsata* in pigeon pea pollination. However, the response of *Apis dorsata* to different insecticides in pigeon pea revealed variation compared to the other two species of honey bees. NSKE and chlorantraniliprole have recorded 100% recovery of the bees on the very second day of the spray indicating the quick resumption of *A. dorsata.* Further*, A. dorsata* responded differently to indoxacarb, flubendiamide and Thiodicarb wherein there was a gradual increase in the foraging activity of bees over days after spraying. Interestingly emamectin benzoate encouraged bees better up to 4 days of spray and thereafter there was a decline in the foraging as indicated by reduced recovery of bees on towards 6 and 7 days after spray with 57.78 and 59.70% respectively (*Table 3*).

The mean of all the intervals is calculated and there was a clear indication that NSKE and chlorantraniliprole encouraged all the three species of honey bees to come back to the farm. NSKE recorded an average of 96.34 and 88.52% recovery irrespective of the interval and the honey bee species. As per the present investigation, indoxacarb and thiodicarb were the insecticides with more repellent activity to the bees. The categorization of insecticides with the increased repellent activity to *A. florea* is NSKE > chlorantraniliprole > emamectin benzoate > flubendiamide > indoxacarb > thiodicarb, whereas, against *A. cerana* the repellent activity is in the hierarchy of NSKE > chlorantraniliprole > emamectin benzoate > flubendiamide > thiodicarb > indoxacarb. Further, against *A. dorsata* the repellent activity of the insecticides in the increasing order is NSKE > chlorantraniliprole > flubendiamide > emamectin benzoate > thiodicarb > indoxacarb (*Table 4*; *Fig. 1*).

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Table 3. *Impact of different insecticides on the activity of Apis dorsata Fabricius on pigeon pea*

Sl. No	Treatments	Dose (ml/g/l)	Bees/m²/5 m	Per cent recovery of bees after spraying						
			DBS	1DAS	2DAS	3DAS	4DAS	5DAS	6DAS	7DAS
1	Thiodicarb 75 WP	1.00	21.50	17.09 (24.41)	37.87 (37.96)	68.11 (55.60)	87.96 (69.67)	91.90 (73.44)	90.85 (72.36)	86.78 (68.65)
2	NSKE (%)	5.00	22.00	87.51 (69.28)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	97.99 (81.82)	98.74 (83.52)	100.00 (90.00)
3	Chlorantraniliprole 18.5 SC	0.15	22.50	54.47 (47.55)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	98.13 (82.11)	100.00 (90.00)	91.31 (72.83)
4	Indoxacarb 14.5 SC	0.30	23.00	6.37 (14.61)	26.31 (30.85)	52.74 (46.55)	85.50 (67.59)	95.40 (77.58)	88.49 (70.14)	100.00 (90.00)
5	Emamectin benzoate 5% SG	0.20	21.00	34.60 (36.02)	80.15 (63.52)	96.79 (79.65)	100.00 (90.00)	74.68 (59.76)	57.78 (49.46)	59.70 (50.57)
6	Flubendiamide 480 SC	0.075	23.00	35.76 (36.71)	61.01 (51.34)	82.49 (65.24)	96.31 (78.89)	100.00 (90.00)	96.23 (78.77)	100.00 (90.00)
7	Untreated check	-	22.00	99.62 (86.43)	96.50 (79.19)	100.00 (90.00)	100.00 (90.00)	92.75 (74.35)	99.62 (86.43)	77.71 (61.80)
	S.Em±	-		3.32	3.69	3.47	3.42	3.90	3.51	3.42
	CD (P = 0.05)	-		10.09	11.38	11.01	10.40	11.70	10.68	10.54
	CV	-		9.18	9.74	10.16	9.81	12.61	10.66	11.58

DBS: Day before spraying DAS: Days after spraying. Figures in the parenthesis are arcsine transformed values. Bees/m²/5m: Number of bees per square meter area per 5 min

Table 4. *Impact of different insecticides on the activity of different species of honey bees in pigeon pea (Mean of all the intervals)*

Sl. No	Treatments	Dose (ml/g/l)	Average per cent recovery of different species of bees after spraying			Average
			A. florea	*A. cerana*	*A. dorsata*	
1	Thiodicarb 75 WP	1.00	51.82	61.127	68.65	60.53
2	NSKE (%)	5.00	96.27	94.987	97.75	96.34
3	Chlorantraniliprole 18.5 SC	0.15	85.49	88.893	91.18	88.52
4	Indoxacarb 14.5 SC	0.30	52.48	50.697	64.97	56.05
5	Emamectin benzoate 5% SG	0.20	68.22	79.224	71.96	73.13
6	Flubendiamide 480 SC	0.075	58.66	65.620	80.48	68.25
7	Untreated check	-	99.63	99.631	95.17	98.14

It is evident from the results that NSKE and chlorantraniliprole were safe to all the three species of honey bees with less repellent activity which is envisaged by the quick recovery of bee species. Indoxacarb was with a high repellent activity against *A. cerana* and *A. dorsata* whereas, thiodicarb is more toxic to *A. florea*. Emamectin benzoate is more toxic to *A. dorsata* compared *to A. florea* and *A. cerana indica*. Further, flubendiamide is safer to *A. dorsata* but toxic to *A. florae* and *A. cerana indica*.

Response of honey bee species to different insecticides

Irrespective of the interval and the insecticides tested, *A. dorsata* (81.45%) recovered very fast to the sprayed field compared to *A. cerana* (77.17% recovery) and *A. florea* (73.22% recovery). *A. florea* is the most sensitive species of honey bees in pigeon pea to insecticides (*Fig. 2*).

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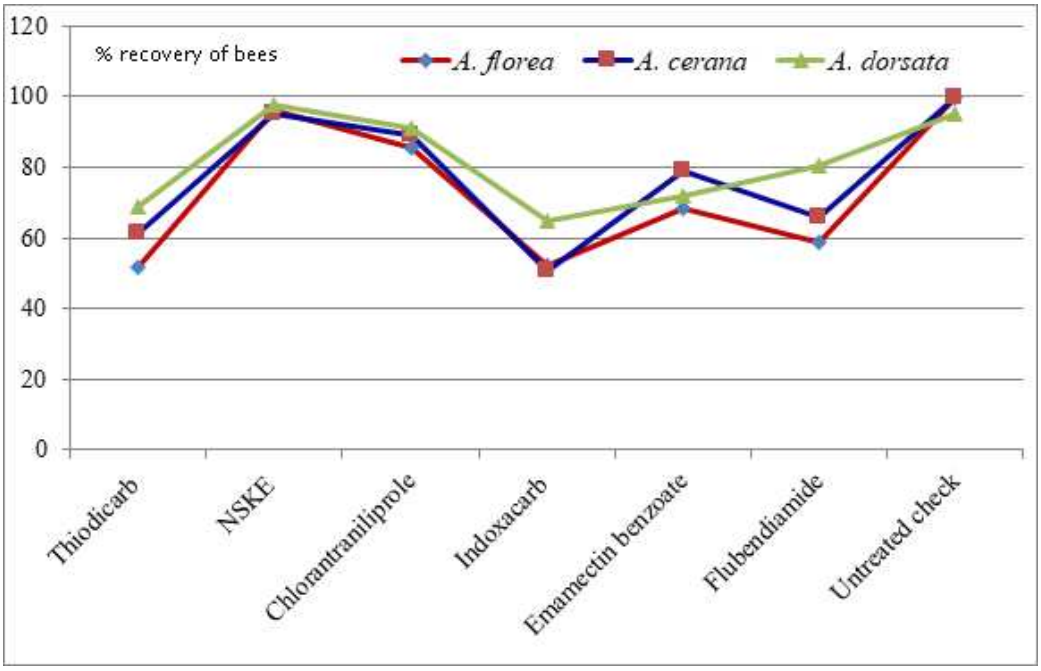

Figure 1. *Influence of different insecticides on three species of honey bees in pigeon pea (mean of all intervals)*

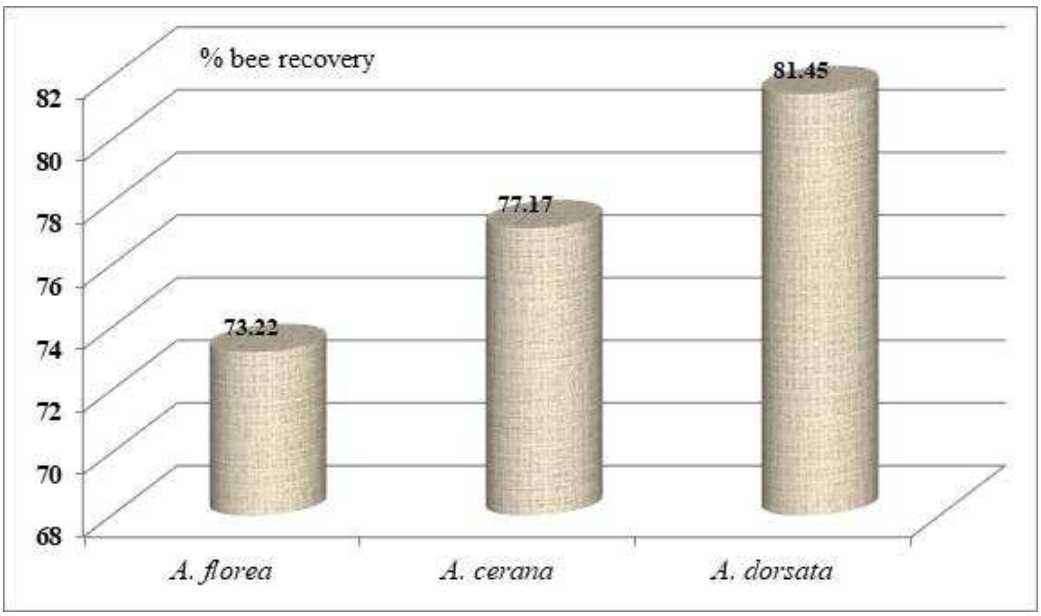

Figure 2. *Recovery of different species of honey bees in pigeon pea (mean of all intervals and insecticides)*

Repellent activity of insecticides to honey bees

The analysis was also made to understand the impact of insecticides to honey bee irrespective of the species studied. It is indicative that the neem-based insecticide has recorded almost more than 80% recovery on the very next day of the spray and attained almost 100% on the second to third day of spraying and remained safer to all the species

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throughout the week. Chlorantraniliprole has encouraged more than 40% of the bees and took almost 3 to 4 days to assume the normal activity and thereafter it remained as safe as that of NSKE. Emamectin benzoate and flubendiamide took 4 days to reach the maximum recovery of 90%. Thereafter, the safety of emamectin benzoate declined steadily and reached less than 80% during 6 and 7 days of the spray. Whereas, in case of flubendiamide, the recovery of bees increased gradually on 6th day and thereafter declined gradually toward 7th day and remained on par with emamectin benzoate at 7th day of the spray (*Fig. 3*). The increase in the repellent activity of emamectin benzoate after the 4th day is a fact to be investigated keeping its mode of action and release of secondary metabolites on the plan surface which are more toxic than its original ingredient.

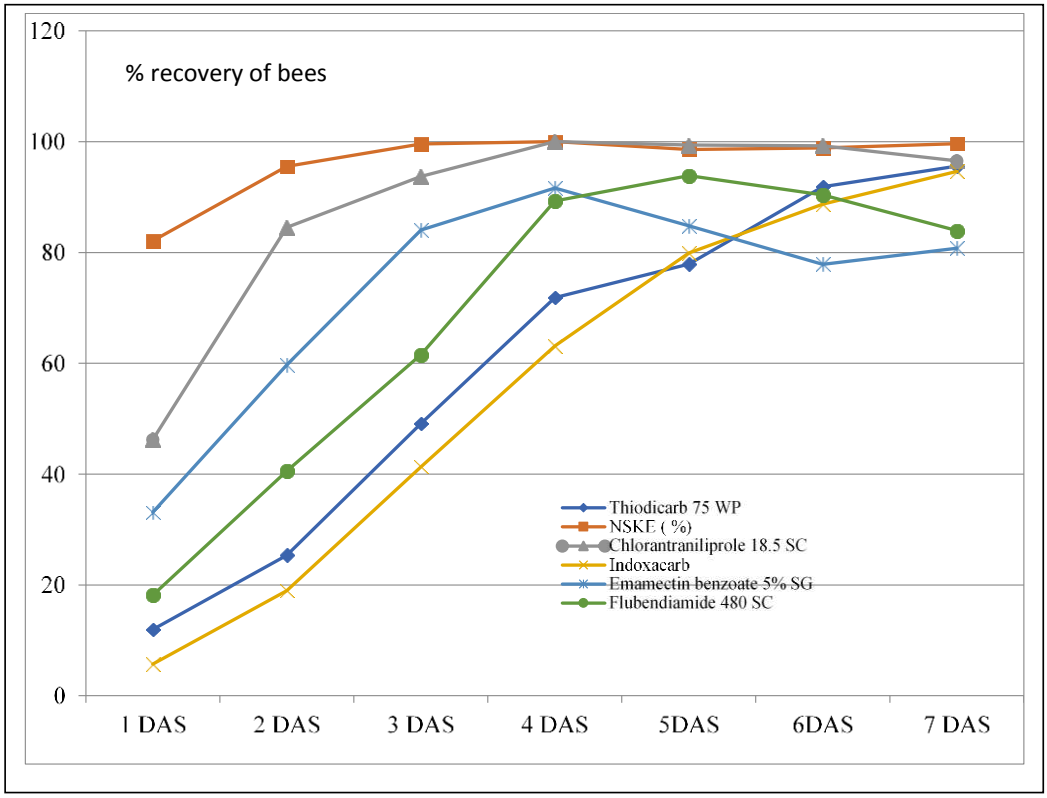

Figure 3. *Repellent activity of insecticides to honey bees (mean of three species)*

The recovery in case of thiodicarb and indoxacarb is consistent and it gradually increasing towards 5th day of the spray and thereafter there was a varied response. The 2-3 day time to assume the normal foraging activity in NSKE and chlorantraniliprole has a greater relevance in augmenting the bee activity in the treated area where they can contribute immensely towards pollination during the peak flowering period of pigeon pea.

Discussion

Among the bad effects of insecticides on beneficial insects, sublethal effects imparted by the insecticides have greater significance and thereby gaining more attention in the present-day agriculture (Desneux et al., 2007). Both lethal and sublethal

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effects should be taken into consideration during risk assessments of pesticides on the ecosystem services rendered by the pollinators under filed condition. Although it was documented that neonicotinoid insecticides have contributed to honey bee losses, little has been known about the impact of other group of insecticides on pollinator services under field condition. This study is the first to show the repellent properties of insecticides under field conditions that directly affect the foraging activity of honey bees in pigeon pea ecosystem.

The results recorded on the impact of different insecticides used in the management of *H. armigera* on pollinators of pigeon pea indicated that except untreated control, there was marked reduction in the foraging activity of bees. In our present findings, NSKE and chlorantraniliprole imparted less repellent effect and achieved maximum bee recovery at a faster rate within 2 to 3 days after the spray. There was a sustained repellent effect in case of thiodicarb, indoxacarb and flubendiamide. Kumar et al. (2013) found that neem extracts significantly ($P < 0.0001$) reduce the *Helicoverpa armigera* larval population and adult emergence, some adult abnormalities were also recorded from 2.5% NSE, 2.5%NLE and 10%NLE. The results of the present finding on safety of azadirachtin showing fast recovery of bees is in conformity with the findings of Egan and Mortensen (2012) and Kumar et al. (2010), who reported azadirachtin did not deter the honey bees in the field. Similarly, least repellence of neem products was observed by Umrao et al. (2012) and Kumar and Singh (2012), which endorse the results of the present investigations.

In the present findings, chlorantraniliprole treated plots regained the normal bee population within short period which is in accordance with the findings of Jonathan et al. (2013) who observed neither bumble bees nor honey bees avoided foraging on treated white clover in open plots with chlorantraniliprole. Chlorantraniliprole is primarily active on chewing insect pests by ingestion and by contact, showing good larvicidal activity. The remarkably low toxicity combined with low use rates provides large margins of safety for consumers, beneficial insects and with minimal impact on pollinators and beneficial insects (Bassi et al., 2009).

As evidenced in the study, the normal bee activity was on the 2ndafter spray in case of *Apis dorsata* and *Apis cerana*. Chlorantraniliprole has an excellent profile of safety to beneficial arthropods, pollinators and non-target organisms such as earthworms and soil microorganisms. The product effects on honeybees have been studied extensively, demonstrating low intrinsic toxicity and no negative effects were observed under semi-field conditions on foraging honey bees in numerous tunnel tests (Dinter et al., 2008). Further, he also concluded that chlorantraniliprole in addition to its excellent performance in IPM programmes, conserves pollinating honey bees and bumble bees on flowering Phacelia or wheat. In the present findings, emamectin benzoate also showed good recovery of honey bees for 4 days. The inferences of the present results are in line with the findings of Amechi et al. (1997) who reported better colonization of *Apis mellifera* (L.) and *Diglyphusisaea* (Walker) on the emamectin benzoate treated crops within relatively short intervals (≤24 h) after applications.

As witnessed in the findings, the population of honey bees showed more variation in their repellent activity against different insecticides. If the spray is scheduled with safe and effective insecticide during peak foraging, that will cause less direct impact on beneficial insects mainly pollinators. Hence, scheduling spraying with the insecticide with low repellent activity has immense significance in augmenting the ecosystem services by pollinators for better yields which in turn conserve the insect pollinators and biodiversity.

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Thus, we propose that significant attention should also be paid on direct exposure of repellent insecticides on honey bees. In conclusion, although there is a variation in the impact of insecticides on honey bees, this study suggested that the insecticides like the NSKE and chlorantraniliprole can restore the honey bees at faster rate and bring the bees back to the farm for effective pollination.

Conclusion

The present investigation indicates the importance of repellent properties of insecticides concerning honey bees in pigeon pea which elucidates the fastest recovery percentage of honey bees (*Apis florea, Apis cerana* and *Apis dorsata*) in the plots treated with Neem Seed Kernel Extract followed by chlorantraniliprole compared to other insecticides. Further, among the bee species studied, *A. dorsata* resumed its normal activity sooner than *A. cerana* and *A. florea. A. florea* was the most sensitive species to insecticides.

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EFFECT OF LOW DIETARY PROTEIN SOURCES ON THE INTESTINAL MICROBIOTA OF FINISHING PIGS

SUN, K. C.[1,2,3] – CAO, K. F.[1,2,3] – ZHAO, L.[1,2,3] – SU, S.[1,2,3] – GENG, T. T.[1,2,3] – HE, F.[1,2,3] – BAI, X. L.[1,2,3] – ZHANG, H. H.[1,2,3] – GUO, H. L.[1,2,3] – SUN, H.[1,2,3*]

[1]*College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, P. R. China*

[2]*Key Laboratory of Animal Production, Product Quality and Security, Ministry of Education, Jilin Agricultural University, Changchun 130118, P. R. China*

[3]*Jilin Provincial Key Laboratory of Animal Nutrition and Feed Science, Jilin Agricultural University, Changchun 130118, P. R. China*

[*]*Corresponding author*
e-mail: hui1688@163.com; phone: +86-189-4392-1381

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Abstract. The objective of this study was to evaluate the effect of low dietary protein sources on the gut microbiota in finishing pigs. Thirty-six Duroc × Landrace × Large White finishing barrows were randomly allocated to four equal groups. Soybean meal (SBM) as a protein source is a control group and had a general protein level of 15% (SBM15). The pigs fed soybean meal (SBM11), soybean-cottonseed-corn germ meal (SCCM11), and cottonseed-corn germ meal (CCM11) were offered low protein (11%) diets. The results showed that the ileum and feces microbiota of the SCCM11 group were significantly increased compared to the SBM15 group (P<0.05). At the phylum level, the relative abundance of *Proteobacteria* phyla was significantly increased in the ileum microbiota of the SCCM11 group compared to the SBM15 group (P<0.05). At the genus level, the abundance of *Lactobacillus* and *Bifidobacterium* showed a significant increase in the ileum microbiota of the SCCM11 group (P<0.05). The abundance of *Ruminococcaceae, Phascolarctobacterium* was significantly increased in the feces microbiota of the SCCM11 group than the other three groups (P<0.05). These results indicated that the SCCM11 group with added crystalline amino acid may modulate intestinal microbiota.
Keywords: *soybean-cottonseed-corn germ meal, ileum microorganisms, fecal microorganisms, intestinal health, 16SrRNA*

Introduction

Intestinal health is associated with the growth of pigs (Zhou et al., 2015). Commonly, intestinal microbiota plays an important role in intestinal healthy (Brestoff and Artis, 2013). Because of the impact on physiological, nutrition, and immunological process, the intestinal microbiota is a uniquely diverse ecosystem that influences many aspects of its host (McDonald et al., 2018).

Wellock et al. (2006) demonstrated that a decrease in dietary protein intake results in higher fecal and colonic lactobacilli to the coliform ratio in pigs. Additionally, Rist et al. (2014) reported that variations in dietary protein supply may beneficially affect microbiota composition in the intestinal tract of pigs. Cottonseed meal (CSM) and corn germ meal (CGM) have been used in the non-ruminant animal diets and shown to achieve good results, such as improve bacterial diversity (González-Vega and Stein, 2012). From previous studies, we can see that suitable protein sources and low protein (LP) levels can be used in finishing pigs' diets and influence the composition and function of intestinal microbiota.

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Diet composition has been identified as one of the important factors that influence the composition of intestinal microbiota (Liao et al., 2017). Amino acid is an important part of the diet that can support the growth of bacteria and host (Morales et al., 2012). These microbes also can utilize amino acids to offer nutrition for hosts and are beneficial for the balance of intestine microecological and guaranteed the health and development of intestine (Dersjant-Li et al., 2019).

This study aims to determine LP diets with different protein sources including Soybean meal (SBM), CSM, CGM in feeding finishing pigs as well as to estimate the intestinal microbiota. These were assessed through high-throughput sequencing-based on Illumina Miseq to define the diversity, abundance, and composition of ileum and feces microbiota in finishing pigs.

Material and methods

Animals, housing

Thirty-six (Duroc× Landrace× Large White) finishing barrow pigs (Ji Feng Company, Gongzhuling, China), according to initial body weight (BW, 58.65±3.71 kg) were randomly allocated to four groups using a randomized block design and the sex was considered as a random effect. Each group consisted of three replicates (pens) and three pigs each. Every pig was in a single cage (150 cm in length × 70 cm in width), the pre-feeding period was 7 days, formal feeding period was 28 days. All pigs were conducted at Farm of Jilin Agriculture University, Changchun city, China. The pigs were kept individually throughout the experimental period. All animals had unlimited access to feed and water throughout the experimental period. The average of the ambient temperature in the barn was 25°C. The treatment, housing, husbandry, and slaughtering conditions conformed to the Guide for the Care and Use of Laboratory Animal (2012).

Diets

The experimental diets referred to the feeding standard of finishing pigs (NRC, 2012, 50-80 kg) and were formulated by the net energy system. SBM as a protein source is a control group and had a general protein level of 15% (SBM15). The pigs in the SBM11, soybean-cottonseed-corn germ meal (SCCM11), and cottonseed-corn germ meal (CCM11) groups as treatment groups were offered LP level (11%) diets (*Table 1*). All experimental diets were meet the requirement of standardized ileum digestibility (SID) of amino acid in finishing pigs and balanced with some crystalline amino acid (NRC, 2012).

Sample

On the day 29th, all the pigs were fasted for 12 h before slaughter and slaughtered at the slaughterhouse in Jilin Agriculture University via electrical stunning followed by exsanguination. The pigs were opened up immediately. The ileum content and feces in the rectum were collected into tubes (freezing, approximately 5 cm in length × 1 cm in width, huayi biological Company, China). Samples kept in liquid nitrogen were immediately and at −80°C until further analysis.

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Table 1. *Composition and nutrient analysis of experimental diets*

Diets	Normal protein (15%CP)	Low protein (11% CP)		
	SBM15②	SBM11②	SCCM11②	CCM11②
Maize (8.7% CP)	71.50	77.50	77.00	76.60
Soybean meal (44.0% CP)	18.00	6.00	3.00	—
Cottonseed meal (40.6% CP)	—	—	2.45	4.90
Corn germ meal (20.8% CP)	—	—	1.76	3.52
Rice hull powder	1.29	2.82	2.22	1.63
Soybean oil	4.05	5.50	5.50	5.50
Lysine (98%)	0.25	0.59	0.65	0.70
Methionine (98%)	0.36	0.43	0.43	0.43
Threonine (98%)	0.17	0.34	0.36	0.38
Typtophan (98%)	0.06	0.11	0.11	0.11
Isoleucine (98%)	—	0.24	0.27	0.30
Leucine (98%)	0.06	0.26	0.29	0.32
Valine (98%)	0.09	0.27	0.27	0.27
Phenylalanine (98%)	0.05	0.25	0.25	0.25
Histidine (80.1%)	0.04	0.14	0.15	0.14
Dicalcium phosphate	0.72	0.86	0.81	0.78
Limestone	0.94	0.96	1.00	1.02
Salt	0.30	0.30	0.30	0.30
Zeolite	1.12	2.43	2.18	1.85
Vitamin and mineral premix①	1.00	1.00	1.00	1.00
Nutrition composition				
Net energy (kcal/kg)	2475.42	2475.81	2474.61	2475.48
Crude protein	14.93	10.97	11.04	11.08
Lys	0.91	0.91	0.91	0.91
Met+Cys	0.51	0.51	0.51	0.51
Thr	0.56	0.55	0.56	0.56
Trp	0.17	0.17	0.16	0.16
Ile	0.47	0.47	0.47	0.48
Leu	0.94	0.91	0.91	0.91
Phe	0.59	0.59	0.59	0.59
Val	0.54	0.55	0.54	0.54
His	0.31	0.31	0.32	0.31
Ca	0.59	0.59	0.59	0.59

Note:
①Premix per kg diet provided: cobalt 1 mg; copper 150 mg; iron 150 mg; manganese 80 mg; zinc 120 mg; iodine 0.3 mg; selenium 0.3 mg; nicotinic acid 10 mg; calcium pantothenate 5 mg; folic acid 0.4 mg; biotin 0.05 mg; retinal 38,000,000 IU; cholecalciferol 8,000,000 IU; alpha-tocopherol 90,000 IU; menadione 1 mg; thiamin mononitrate 1 mg; riboflavin 2 mg; pyridoxine hydrochloride 1.2 mg; cyanocobalamin 0.01 mg; antioxidant 0.02 mg.
②SBM15 represents soybean meal (SBM) and crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%

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High- throughput sequencing

Total sample DNA was extracted with a QIAamp® DNA Stool Mini Kit (QIAGEN Company, USA) (Sbardella et al., 2016). The concentration of the extracted DNA was determined using 1% agarose gel electrophoresis (Ren et al., 2013). The bacterial 16SrRNA gene was amplified with a set of primers targeting the V3-V4 region and the primers were 338F (5′- ACTCCTACGGGAGGCAGCAG -3′) and 806R (5′- GGACTACHVGGGTWTCTAAT -3′). The PCRs were carried out in triplicate using 20 μL reactions with 4 μL of 5×FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of 5 μmol l-1 each primer, 0.4 μL of FastPfu polymerase, 10 ng of DNA, and added ddH2O to 20 μL, according to the manufacturer's instructions. The PCR amplification was conducted according to described by Metzler-Zebeli et al. (2009). Initial denaturation at 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, with a final elongation step at 72°C for 10 min. The concentrations of each PCR product were checked by agarose gel electrophoresis (2% agarose) and SanPrep Column DNA Gel Extraction Kit (Biotech, Sangon Company, China) to ensure correct primer specific products (Agyekum et al., 2016). Quantity and quality of purified PCR amplification products were determined using QuantiFluor® Single-Tube Fluorometers (Promega Company, USA) (Castillo et al., 2006). High-throughput sequencing was performed on Illumina Miseq (PE300) sequencing platform at Majorbio Bio-Pharm Technology Co., Ltd. (Meiji Company, China).

Bioinformatics analysis

All of the sequences were analyzed and filtered according to the barcode and primer sequences using Trimmomatic software. Raw reads were processed to remove low-quality sequences if they were shorter than 300 bp or longer than the expected PCR product size. Operational taxonomic units (OTUs) were chosen, which were defined by 97% of similarity, using the Usearch (version 7.1). According to Ribosomal Database Project (RDP) classifiers against the Silva 16SrRNA database (Quast et al., 2013) assigned taxonomically and calculation abundance, make rarefaction curves of microbe structure, the figure of Species abundance and principal components analysis (PCA) (Qiong et al., 2007). Rarefaction curves, Shannon index, abundance-based coverage estimator (ACE) index, and Chao1 richness were determined using Mothur (version v.1.30.1) (Schloss et al., 2011). Beta diversity was evaluated with the Unifrac metric. Weighted Unifrac distance matrices were used to compare the hierarchical relationships among the samples (Lozupone et al., 2011).

Statistical analysis

All experimental data were initially subjected to analysis of variance (ANOVA) with General Linear Model (GLM) procedures of SPSS software (19.0, SPSS Inc, Chicago, IL, U.S.) using a randomized block design. Growth performance was analyzed with the pen as the experimental unit (n=9); Microbiota diversity, abundance, and composition of ileum and feces were analyzed with the pen as the experiment unit (n=6). When the interactions were significant (P<0.05) for most of the parameters, data were then analyzed using one-way ANOVA among the groups within a diet. When F-test was significant (P<0.05), the least significant difference (LSD) test was utilized to compare significant differences (P<0.05) among the groups.

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Results

Effect of variations in dietary protein levels with different protein sources on the diversity of ileum and feces bacterial

The diversity of bacterial based on OTUs and estimated OTUs from ACE, Chao 1, and Shannon indices. It reflects the bacterial diversity in the sample. A total of 658,744 valid reads and 660 OTUs were obtained from 48 samples through Illumina Miseq analysis after screening them with strict criteria. The diversity estimates of the pig ileum and feces were shown in *Table 2*. For the ileum samples, the SBM11 group richness the number of OTUs and estimated OTUs from Shannon compared with the SBM15 group (P<0.05). The number of OTUs and estimated OTUs from the ACE, Chao 1 indices showed that the SCCM11 and CCM11 groups were much higher than the SBM15 and SBM11 group (P<0.05). The number of OTUs, Chao 1, and ACE indices of the SCCM11 group was higher than the other three groups (P>0.05). For the feces samples, the number of OTUs of the SBM11 and SCCM11 groups was significantly higher than the SBM15 group (P<0.05). The number of OTUs and Chao 1 indices showed that the SCCM11 group was significantly higher than the groups of SBM15 and SBM11 (P<0.05).

Table 2. *Effect of low protein diet and different protein sources on diversity estimation of the 16S rRNA gene libraries from ileum and feces microbiota in finishing pigs (n=6)*

Items	Group	OTU	ACE	Chao1	Shannon
Ileum	SBM15	42.333±10.504^{c}	53.662±5.670^{c}	53.323±17.287^{b}	1.353±0.295^{b}
	SBM11	60.667±6.028^{b}	66.210±3.045^{c}	66.333±15.939^{b}	2.037±0.057^{a}
	SCCM11	85.067±12.133^{a}	206.067±35.388^{a}	157.250±23.750^{a}	1.960±0.433^{ab}
	CCM11	83.7500±7.2500^{a}	127.517±23.000^{b}	128.520±25.517^{a}	1.820±0.080^{b}
	P value	0.050	0.049	0.045	0.053
Feces	SBM15	382.333±29.501^{b}	459.000±37.363	365.000±43.313^{b}	4.447±0.119
	SBM11	318.0000±22.338^{c}	475.000±12.124	481.667±20.033^{b}	4.280±0.131
	SCCM	459.667±25.686^{a}	489.000±10.149	496.667±18.963^{a}	4.560±0.125
	CCM	419.000±14.933^{ab}	462.333±21.008	484.000±36.056^{a}	4.347±0.318
	P value	0.047	0.414	0.042	0.368

Different letters in the same column identify significant differences at 0.05 level among parameters. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and the CP level is 11%

The analysis of bacterial community structure is mainly based on principal component analysis (PCA). It is based on the distance to describe the relationship between samples. The pig ileum and feces were divided into two groups based on PCA (*Fig. 1*) and the unweighted pair group method with arithmetic mean (UPGMA) from the Unifrac and Mothur analyses (*Fig. 2*). Pairwise comparisons revealed that the bacterial community structure of ileum samples in SBM15, SBM11, SCCM11, and CCM11 groups showed a high level of distance to feces samples for SBM15, SBM11, SCCM11, and CCM11 groups. Additionally, rarefaction curves from the ileum and feces microbiome appear to approach a horizontal asymptote, indicating that the current sequencing effort saturates diversity (*Fig. 3*). However, the distance of bacterial community structures in ileum or feces was very short among the four groups.

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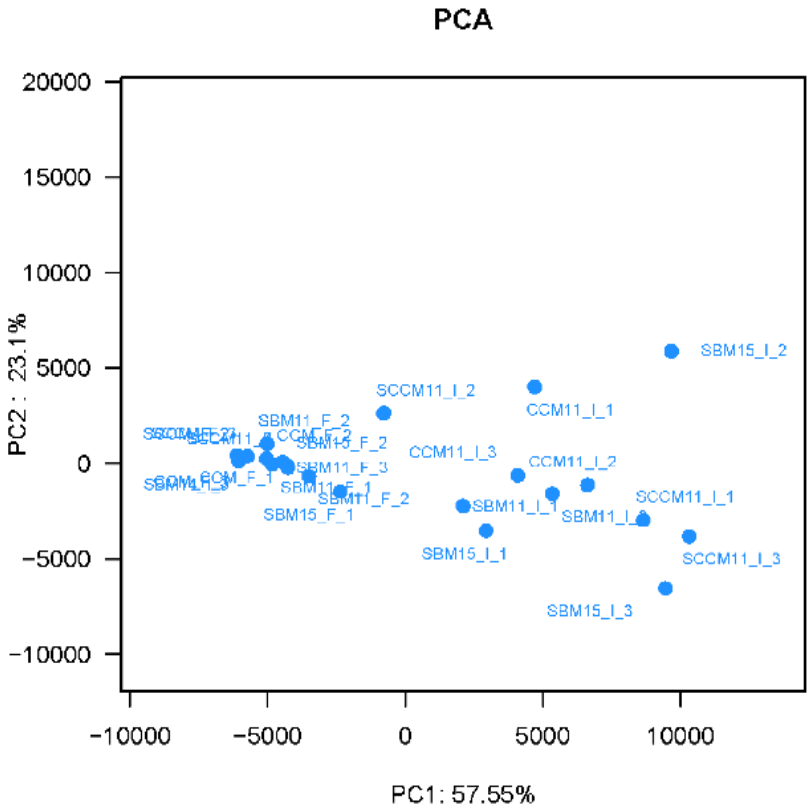

Figure 1. *The score plot of principal component analysis for different bacterial communities using weighted Unifaces distance. This analysis is based on principal component analysis (PCA) and the distance describes the relation between samples. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%. The "I" represent ileum; "F" represents feces. The number, in the end, is pig number (one to three pigs). To make the figure clear, I did not put all pig number (one to six pigs)*

Effects of variations in dietary protein levels with different protein sources on the abundance and composition of ileum and feces microbiota for phylum and genus level

In order to evaluate the abundance and composition of ileum and feces microbiota, a phylum and genus level was a calculation to define the microbiota of ileum and faces. At the phylum level, the pig ileum microbiomes of four groups were dominated by the *Firmicutes* phyla (*Fig. 4*). The result showed that three LP diets significantly increased the relative abundance of *Proteobacteria* phyla ($P<0.05$). The pig fecal microbiomes among the dietary treatments had no effect on the bacterial community composition. *Firmicutes, Bacteroidetes, Proteobacteria,* and *Spirochaetae* amounting to beyond 99% of the total reads.

At the genus level, the most predominant genus in SBM15, SBM11, SCCM11, and CCM11 were the *Clostridium sensu stricto, Terrisporobacter, Peptostreptococcaceae,* and *Turicibacter* genus in the ileum (*Fig. 5*). The SBM15 diet significantly decreased the abundance of *Actinobacillus* (0.501%), *Lactobacillus* (0.017%), and *Bifidobacterium* (0%) ($P<0.05$), but increased the abundance of *Streptococcus* (2.895%)

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comparing to the three LP diets groups (P<0.05). The SCCM11 group diet significantly increases the abundance of *Lactobacillus* (5.214%) and *Bifidobacterium* (3.923%) comparing to the other three groups (P<0.05) (*Table 3*). In the feces, four diet groups library included sequences most similar to *Clostridium sensu stricto, Terrisporobacter*, *Peptostreptococcaceae, Rikenellaceae, Treponema, Christensenellaceae, Lachnospiracea* and *Prevotella* (*Fig. 5*). The SBM15 group significantly increase the abundance of *Streptococcus* (7.785%) and *Anaerovibrio* (1.394%) genus (P<0.05) and a lower relative abundance of *Prevotellaceae* UCG-001 (0.243%) (P<0.05) than the three LP groups. Nevertheless, *Ruminococcaceae* UCG-014 (2.882%), *Phascolarctobacterium* (1.496%) and *Oscillibacter* (1.426%) were more abundant in the SCCM11 group than the other three groups (P<0.05) (*Table 3*).

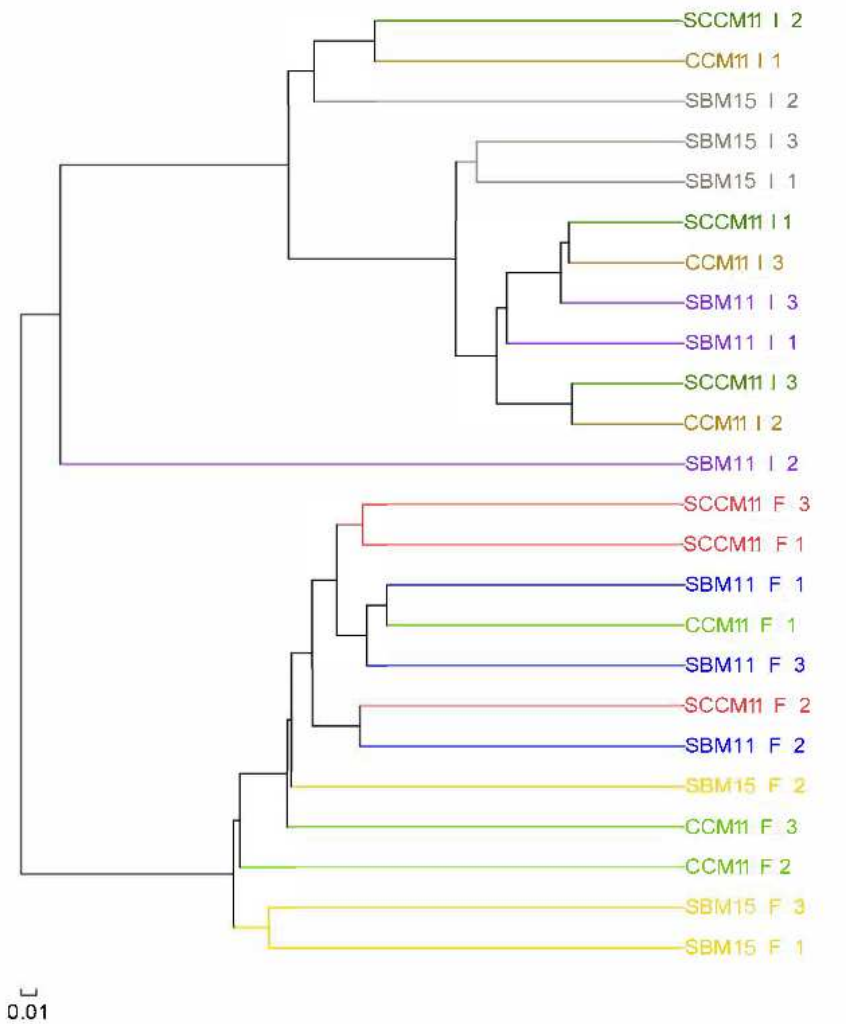

Figure 2. *Cluster analysis by the weighted unweighted pair group method with arithmetic means (UPGMA) for bacterial communities from different diets samples. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%. The "I" represent ileum; "F" represents feces. The number, in the end, is pig number (one to three pigs) and the same color means the same group. To make the figure clear, I did not put all pig number (one to six pigs)*

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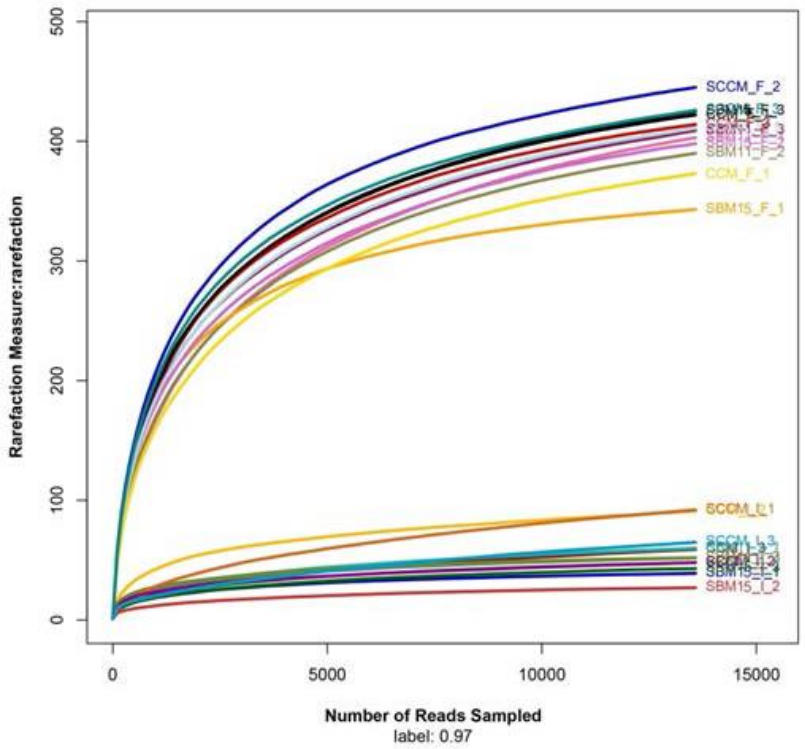

Figure 3. *Rarefaction curve of bacterial 16S rRNA sequences for different diets samples. OTUs are identified using 97 % cutoffs. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%. The "I" represent ileum; "F" represents feces*

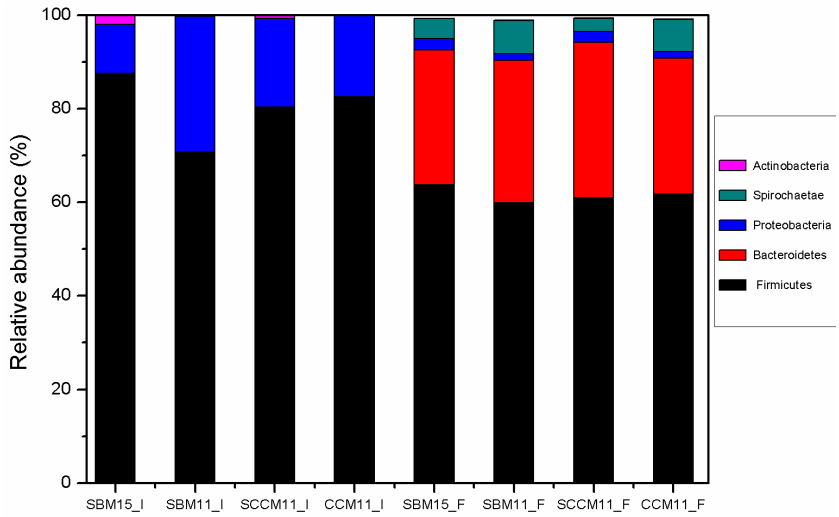

Figure 4. *The relative abundance of ileum and feces microbiota general (percentages) at the phylum level in the finishing pigs fed different diets. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%. The "I" represent ileum; "F" represents feces*

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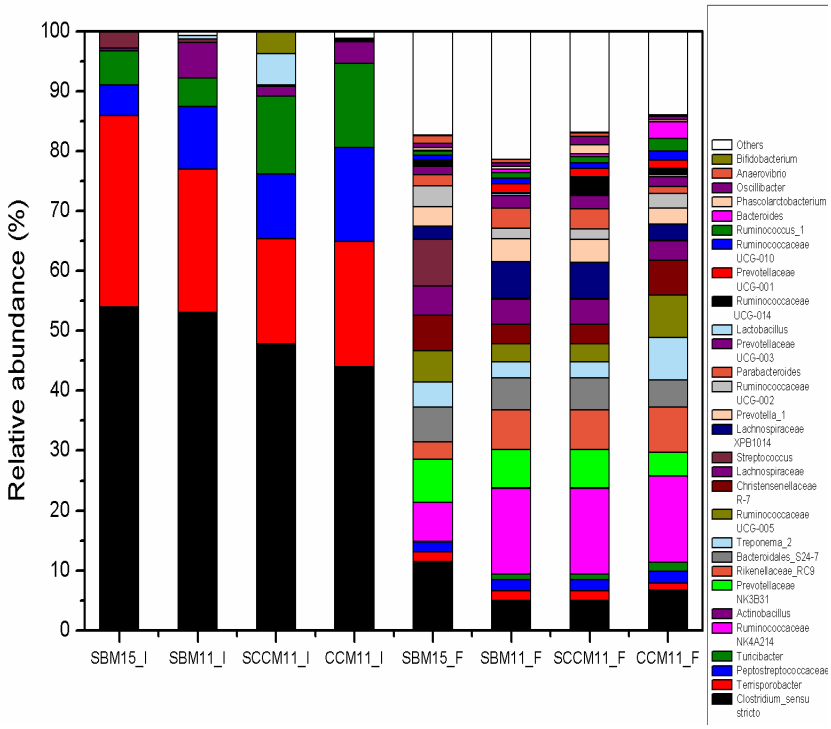

Figure 5. *The relative abundance of ileum and feces microbiota general (percentages) at the genus level in the finishing pigs fed different diets. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%. The "I" represent ileum; "F" represents feces*

Table 3. *The relative abundance of microbiota genera (%) that were significantly affected by the dietary treatment in the ileum and feces of finishing pigs (n=6)*

Items	Genus	SBM15	SBM11	SCCM11	CCM11	*P* value
Ileum	Actinobacillus	0.501±0.051^{d}	5.930±0.671^{a}	1.596±0.212^{c}	3.562±0.789^{b}	0.001
	Streptococcus	2.895±0.728^{a}	0.567±0.367^{b}	0.237±0.230^{b}	0.195±0.257^{b}	0.003
	Lactobacillus	0.017±0.017^{c}	0.589±0.580^{b}	5.214±2.768^{a}	0.194±0.082^{b}	0.006
	Bifidobacterium	0.000^{c}	0.026±0.023^{b}	3.923±4.739^{a}	0.241±0.196^{b}	0.029
Feces	Streptococcus	7.785±5.620^{a}	0.172±0.139^{b}	0.077±0.065^{b}	0.057±0.055^{b}	0.023
	Ruminococcaceae UCG-014	0.498±0.432^{b}	0.183±0.034^{b}	2.882±0.890^{a}	1.110±0.942^{b}	0.009
	Prevotellaceae UCG-001	0.243±0.109^{b}	1.857±0.904^{a}	1.739±0.923^{a}	1.695±0.993^{a}	0.022
	Ruminococcaceae UCG-010	0.862±0.478^{ab}	0.691±0.133^{b}	0.941±0.144^{b}	1.502±0.245^{a}	0.039
	Ruminococcus 1	0.414±0.013^{b}	0.555±0.344^{b}	0.987±0.585^{b}	2.125±0.262^{a}	0.002
	Phascolarctobacterium	0.508±0.177^{b}	0.545±0.455^{b}	1.496±0.158^{a}	0.360±0.231^{b}	0.004
	Oscillibacter	0.676±0.196^{b}	0.504±0.237^{b}	1.426±0.454^{a}	0.504±0.298^{b}	0.019
	Anaerovibrio	1.394±0.344^{a}	0.433±0.390^{b}	0.625±0.335^{b}	0.175±0.165^{c}	0.008

Different letters in the same column identify significant differences at 0.05 level among parameters. SBM15 represents soybean meal (SBM) and the crude protein (CP) level is 15%. SBM11 represents SBM and the CP level is 11%. SCCM11 represents soybean-cottonseed-corn germ meal (SCCM) and the CP level is 11%. CCM11 represents cottonseed-corn germ meal (CCM) and CP level is 11%

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Discussion

With the development of next-generation sequencing techniques, we know the density of bacteria in the pig intestine represents as much as 10^{11}-10^{12} cells for every gram of gut contents and belongs to thousands of bacterial taxa (Rinninella, 2019). This is necessary to determine intestine microbiota diversity in pigs.

In this study, the LP diet, in which the protein level is 11%, can affect the diversity of ileum and feces bacterial. This consists of previous reports (Rist et al., 2014). For ileum, the SCCM11 and CCM11 groups significantly increased the diversity of bacterial compared with the groups of SBM15 and SBM11. The possible reason is that the SCCM11 group is combined with SBM, CSM, CGM as the protein source, and the CCM11 group is combined with CSM, CGM. Different protein sources and combinations, due to their different protein structures, the amino acids released by digestion have a certain effect on microbiota diversity. It is beneficial for the small intestine microbe to utilize amino acids for metabolism activities. However, the bacterial diversity of the CCM11 group was less than the SCCM11 group. Gidlund et al. (2015) indicated that SBM has higher protein digestion than CSM. The reason may be due to lack of SBM, which is one of the protein sources, compared with SCCM11 group, the protein cannot be degraded well in the small intestine for CCM11 diet and intestine microbiota also cannot better-used amino acid for metabolism activities (Fan et al., 2015). For feces, the bacterial diversity of the SCCM11 group was significantly increased compared with the SBM15 group. The possible reason is that the SCCM11 group consisted of different sources not only enrich amino acid composition but also other abundant nutrition in the diet. The diversity of bacterial increased to adapt to the competition of bacterial for nutrition.

For bacterial structure, the present study showed that bacterial community structure is similar in the same intestine among the four groups. Zhao et al. (2010) indicated that intestinal microbiota structure is the result of natural choice. The reason may be that, during the long-term treatment, intestinal structure adapts to the competition relation of microbiota and the change of nutrition (Wu et al., 2011). However, contrary to the stable fecal microbiota, ileum microbiota displays pronounced compositional fluctuations. This is in agreement with the opinion of Jiang et al. (2013). The reason may be due to the different functions of the small intestine and large intestine. As we have known, the small intestine is the main place of nutrition digestion and absorption. It may enrich the activities of microbiota to used nutrition.

Recently, because of the potential impacts of host health, the effect of dietary protein on the intestinal microbiome in pigs is necessary to be attentive. This study focused on potential pathogenic and beneficial microorganisms. Also, the dietary treatment in the condition of this study modulated several abundances of gut microbiota in the phyla and genus levels.

At the phylum level, the present study showed that *Firmicutes* is the predominant bacterial in ileum among the four groups. This is in agreement with the opinion of Yang et al. (2018). Moreover, the relative abundance of *Proteobacteria* phyla in the ileum of the three LP groups was significantly increased compared with the SBM15 group, as previously shown in the microbiota study of pigs (Huang et al., 2020). The possible reason is that three LP diets adding more crystalline amino acids which are more easily to be absorbed by small intestinal compared with the normal protein diet. Meanwhile, it also increased the interest of *Proteobacteria* to utilize amino acid as a fermented protein. But the three LP groups had no effect on bacterial composition in feces

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compared with the SBM15 group. More studies demonstrate that a reduction in dietary protein content may result in a lower intestinal ammonia concentration, but not necessarily changes in the intestinal bacterial community of pigs (Heo et al., 2013; Rutherfurd et al., 2014). Our results may be insisted on the opinion. These results indicated that LP diets supplement crystalline amino acid has the potential to be available for large intestinal microbiota.

At the genus level, the abundance of *Lactobacillus* and *Bifidobacterium* of the SCCM11 group was increased in ileum compared to the SBM15 group. It is generally accepted that *Lactobacillus* is important to maintain intestinal health and can inhibit the growth of potential pathogenic groups (Zhang et al., 2018). Similar to *Lactobacillus, Bifidobacterium* was also shown to beneficial affect intestinal health (Zhang et al., 2020). This is in agreement with previous studies (Bindelle et al., 2010; Jiang et al., 2015). The reason may be that the SCCM11 group combined with different protein sources, which have a high-quality source can enrich amino acid composition and contribute to the development of *Lactobacillus* and *Bifidobacterium*.

In the feces, the relative abundance of *Streptococcus* and *Anaerovibrio* genus was higher and the abundance of *Prevotellaceae* was lower in the SBM15 group compared with three LP groups. *Streptococcus* genus also belongs to the lactic acid bacteria and some species have similar probiotic characteristics to *Streptococcus*. Also, some species are potential pathogens (Gómez-Gascón et al., 2016). The previous study reported that a reduction of dietary CP content diet from 230 to 130 g/kg was associated with lower counts of pathogenic bacteria, such as Coliforms (Ye et al., 2011). In the present study, the abundance of *Ruminococcus* and *Prevotellaceae* of the SCCM11 and CCM11 group were increased. This is in general agreement with Zhou et al. (2015), where feeding an LP diet to pigs increased *Prevotellaceae*. Moreover, it was previously reported that *Ruminococcus* and *Prevotellaceae* were two of the predominant fiber-degrading bacterial species in the intestinal tracts of pigs (Zhao et al., 2017). The abundance of *Bacteroides* was also increased in the SCCM11 and CCM11 groups. The reason may be that *Bacteroides* has a better ability to decompose and use substrate. It was revealed that adding CSM and CGM increased the decomposition and utilization of fiber as a substrate in the large intestine for Bacteroides. In the SCCM11 group, the reads assigned to the genera *Oscillibacter* were present in slightly higher proportions than the other three groups. *Oscillibacter* has been identified as a valerate-producing anaerobe (Le Roy et al., 2020). Valerate as organic acids can reduce pathogenic bacteria of the intestine and endogenous losses (Park et al., 2014; Rodrigues et al., 2020).

Conclusion

The present study indicated that support crystalline amino acid to LP diet, in which the protein level is 11%, may modulate the intestinal microbiota. Maybe due to the preferred location of some bacteria groups in the intestine, the effect on intestinal microbiota diversity, abundance, and composition are not consistent throughout the sampling sites in the current study. The present results showed that the SCCM11 group increased the bacterial diversity in ileum and feces and also increased the abundance of *Lactobacillus* which is important to maintain intestinal health and can inhibit the growth of potential pathogenic groups. However, the potential regulatory mechanism of the microbiota on the finishing pig health is still unclear, and further research is needed.

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Further studies are warranted to clarify the potential roles of this microbiota modulate to the health of finishing pigs according to DGGE technology.

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GROWTH DYNAMICS OF GRASS-SHRUB COMMUNITIES DURING EARLY FORMATIVE PERIOD

HUANG, G.[1,2*] – ZHENG, M. X.[1*]

[1]*Department of Civil Architecture College, East China Jiaotong University Nanchang, China*

[2]*Department of Civil and Architecture Engineering, Huanggang Normal University Huanggang, China*

[*]*Corresponding authors*
e-mail: huanggang0317@163.com; zhengmingxin0317@yeah.net; phone: +86-134-0990-2162

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Abstract. The formation of stable grass-shrub communities is the goal of highway slope ecological protection. However, the growth dynamics of grass-shrub communities during the early formative period is poorly explored, leading to a lack of available guiding. In this study, we carried out an ecological slope protection model of grass and shrubs, the following species were selected: *Cynodon dactylon* and *Magnolia multiflora.* We examined the dynamics of plant coverage (PD), plant density (PD), and plant height (PH) of the two plants affected by topographic factors (slope gradient and slope aspect) and seed schemes (grass-shrub ratio and seed density). Moreover, we attempted to optimize the four influencing factors according to various PC, PD, and PH. The results indicated that the PD of grass-shrub experimental group was significantly better than that of pure-grass and pure-shrub experimental groups during the early formative period. Significant spatial variation of PD was also found during the first two months. The peak PD for 45° is 1.26-1.46 times higher than that for the other two slope gradients, PD of *C. dactylon* differed significantly with grass-shrub ratio ($P< 0.05$). The PH of *C. dactylon* and *M. multiflora* was significantly affected by the slope aspect. In addition, the optimum conditions for the formulation of grass-shrub communities are as follows: slope gradient of 30°-45°, slope aspect of south-facing, seed density of 20 $g \cdot m^{-2}$, grass-shrub ratio of 1: 9.
Keywords: *slope, ecological protection, grass-shrub vegetation, topographic factor, seed schemes, plant height*

Introduction

With the rapid development of China's economic construction, a large number of mountains have been excavated in road construction projects (Cao et al., 2009). The original natural ecosystem has been seriously damaged, forming several exposed slopes (Cohen-Fernández and Naeth, 2013; Shao et al., 2014). When there is no vegetation cover and other engineering measures on the highway slope, ecological imbalance and geological disasters will occur frequently. To prevent the collapse, debris flow, soil erosion, and other geological disasters caused by the degradation of the ecological environment, it is necessary to restore and reconstruct the ecosystem under the guidance of human beings (Fan et al., 2013). In recent years, with raising awareness of environmental protection, ecological restoration with vegetation has been increasingly emphasized by some people (Yang et al., 2016). More recently, grass-shrub seed technology is gradually the hot spot of ecological protection for highway slope (Wang, 2014).

Several researchers have reported on research of grass and shrub growth in grass-shrub community formation (Chen et al., 2019). Many meaningful and interpretable growth

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parameters are often used to evaluate grass and shrub growth, such as plant coverage (PC), plant density (PD), and plant height (PH) (Allen, 1998; Gadi et al., 2019). Increasing PC can significantly control soil erosion (Fu et al., 2012). PC indicates the interception area of radial energy, which controls the evapotranspiration induced suction by leaves (Leung et al., 2015). Evapotranspiration induced suction governs the shallow stability of the vegetated slope (Liu et al., 2018; Ni et al., 2018). The PD determines competition intensity to change in community formation (Li et al., 2020). PH of grass and shrubs affects the biomass of vegetation, which affects the process of ecological restoration (Lei et al., 2016; Zhao et al., 2020). PH is presumed to be a constant growth factor in previous studies (Zhu and Zhang, 2015; Gadi et al., 2018). There are growth dynamics during grass-shrub community formation, and monitoring and assessing the status during vegetation restoration is essential when evaluating the restoration success for highway slope (Hobbs and Harris, 2001).

The growth dynamics of grass-shrub community formation are influenced by topographic factors and seed schemes. Researchers have reported that the growth of grass and shrub was affected by the slope gradient and slope aspect (Fang et al., 2018). Research revealed that the shrub had both positive and negative effects on the growth parameters of grass (Cavieres and Badano, 2009). Early survival of grass-shrub seeds was influenced due to competition between grass and shrubs (Manzaneda et al., 2005). The competition of seed density and grass-shrub ratio influences the PH (Olsen et al., 2006). The different grass-shrub ratio affected the number and diversity index of grass-shrub communities (Song, 2015). The spatial patterns of individuals were related to the PH of grass and shrubs (Zhao, 2020). Grass and shrubs have different survival rates at different grass-shrub ratios and seed density (Schob et al., 2013). Topographic factors (slope gradient and slope aspect) and seed schemes (grass-shrub ratio and seed density) are often accelerated or inhibited the formation of grass-shrub communities. Therefore, it is important to know how the four influencing factors affect grass-shrub community formation, because it will determine the successful use of grass-shrub communities in restoration projects. However, the current main research is about topographical factors affecting soil microbiological indicators (Teixeira et al., 2019) and radial growth of vegetation (Kim et al., 2017), and seed scheme focusing on the yield of agricultural crops (Freudenreich and Mußhoff, 2018). Little systematic attention has been paid to the effect of topographic factors and seed schemes on the dynamics of PC, PD, and PH.

In this paper, we carried out an ecological slope protection model of selected grass and shrubs, namely *Cynodon dactylon* and *Magnolia multiflora*. The objectives of the experimental study are the followings: (1) exploring the dynamics of PC, PD, and PH affected by two topographic factors (slope gradient and slope aspect) and two seed schemes (grass-shrub ratio and seed density); (2) optimizing the formulation of topographic factors and seed schemes for grass-shrub community formation or highway slope ecological restoration.

Materials and methods

Materials

The experiment was carried out at Hubei University of Technology, Wuhan, China. In this test model, 48 cement prefabricated plates ($1.5\times0.5\times0.05$ m^3) and 0.5 m^3 red brick were used to make the slope models. We designed four slopes (15^o, 30^o, 45^o, and 60^o) according to the slopes of the most practical engineering. Two slope aspects (north-facing and south-facing)

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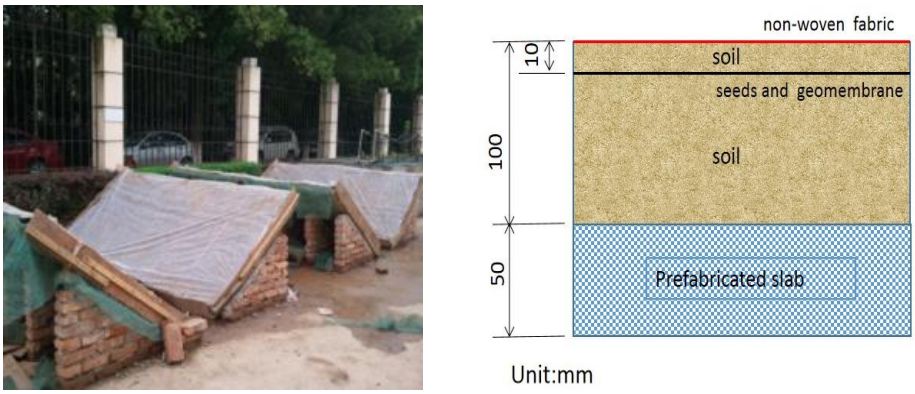
were arranged (*Fig. 1a*). The model of each slope gradient contains 12 experimental groups, of which three slope gradients (15°, 30°, and 60°) are 6 experimental groups in the two slope aspects, and the 45° slope is north-facing (N) of 12 experimental groups. The area of each experimental group was 1.5×0.5 m^2. The total thickness is 150 mm, which can be divided into three layers: the bottom layer is the prefabricated plate with a thickness of 50 mm; the middle layer is the soil layer with a thickness of 90 mm; the topmost layer is the soil overburden layer with a thickness of 10 mm. After investigation of plenty of engineering slopes, the grass was chosen (*C. dactylon)*, and the shrub was chosen (*M. multiflora)*. The vitality of these two plants is particularly strong, suitable for slope restoration. The soil used for the tests was excavated at a depth of 10 cm~30 cm below the surface, from the bank of the Xunsi River in Wuhan city, and the soil area was 40 m^2. To provide initial soil fertility, the same fertilizer was added to all experimental groups.



(a) (b)

Figure 1. *(a) Experimental group, (b) Profile of experimental group*

Full-factorial experiments

In this test, the ecological slope protection model was carried out under outdoor conditions. A full-factorial experiment method was adopted to explore the optimal ratio of the four components evaluated in this study using four test levels (*Table 1*). These full-factorial experiments produced forty-eight different mix compositions. The test design is shown in *Table 2*.

Table 1. *Factors and level values*

Test level	Factors			
	Slope gradient(°)	Slope aspect	seed density ($g \cdot m^{-2}$)	grass-shrub ratio
1	15	N	15	1:0
2	30	S	20	1:3
3	45	-	25	1:9
4	60	-	-	0:1

The soil needed to go through three procedures (dried, crushed, and screened for 10 mm) before it was placed on the prefabricated plates. The soil thickness was identical for each experimental group. Plant seed was first weighed and soaked in distilled water for 12 h, then evenly sowed on the soil. Finally, 10 mm thick soil and a layer of non-woven fabric covered the vegetation seeds. The profile of the experimental group is shown in *Fig. 1b*.

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Table 2. *Testing program for the experimental group*

Mix	Slope gradient (°)	Slope aspect	Seed density ($g \cdot m^{-2}$)	Grass-shrub ratio	Mix	Slope gradient (°)	Slope aspect	Seed density ($g \cdot m^{-2}$)	Grass-shrub ratio
1#	15	S	15	1:0	25#	45	N	15	1:0
2#	15	S	15	1:3	26#	45	N	15	1:3
3#	15	S	15	1:9	27#	45	N	15	1:9
4#	15	S	15	0:1	28#	45	N	15	0:1
5#	15	S	20	1:0	29#	45	N	20	1:0
6#	15	S	20	1:3	30#	45	N	20	1:3
7#	15	N	20	1:9	31#	45	N	20	1:9
8#	15	N	20	0:1	32#	45	N	20	0:1
9#	15	N	25	1:0	33#	45	N	25	1:0
10#	15	N	25	1:3	34#	45	N	25	1:3
11#	15	N	25	1:9	35#	45	N	25	1:9
12#	15	N	25	0:1	36#	45	N	25	0:1
13#	30	S	15	1:0	37#	60	S	15	1:0
14#	30	S	15	1:3	38#	60	S	15	1:3
15#	30	S	15	1:9	39#	60	S	15	1:9
16#	30	S	15	0:1	40#	60	S	15	0:1
17#	30	S	20	1:0	41#	60	S	20	1:0
18#	30	S	20	1:3	42#	60	S	20	1:3
19#	30	N	20	1:9	43#	60	N	20	1:9
20#	30	N	20	0:1	44#	60	N	20	0:1
21#	30	N	25	1:0	45#	60	N	25	1:0
22#	30	N	25	1:3	46#	60	N	25	1:3
23#	30	N	25	1:9	47#	60	N	25	1:9
24#	30	N	25	0:1	48#	60	N	25	0:1

Experiment monitoring design

The purpose of the test monitoring program was to quantify the dynamics of PC, PD, and PH. The monitoring period of the test was 32 months, from May 2013 to December 2015. Depending on the climate monitoring during the experiment, monthly rainfalls are shown in *Fig. 2*. In three years, the precipitation from January to May and August to December was less than 100 mm, in the dry period. The rainfall in June and July was more than 250 mm, which was a wet period.

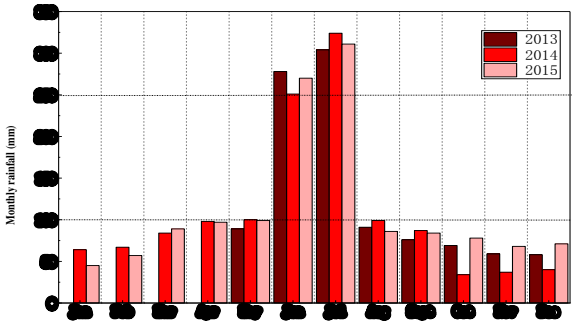

Figure 2. *Monthly rainfall*

The method of measuring the PC was to take photos of the test block with a SLR camera. To quantitatively analyze the spatial dynamics of PC, the green color was re-

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colored into black and the rest of the colors were re-colored into white using the software IPP 6.0 (Yang et al., 2019), as shown in *Fig. 3*. The PC in this study is the total PC generated by the grass and shrub canopy (the sheltered area of shrub to grass is not calculated repeatedly). The calculated black area by the software was the projected area of vegetation, which was used to calculate PC according to the formula:

$$C = \frac{\sum_{i=1}^{n} A_i}{A_S} \quad \text{(Eq.1)}$$

where, C denotes the area PC, A_s denotes the total area, A_i denotes the projected area of a vegetation.

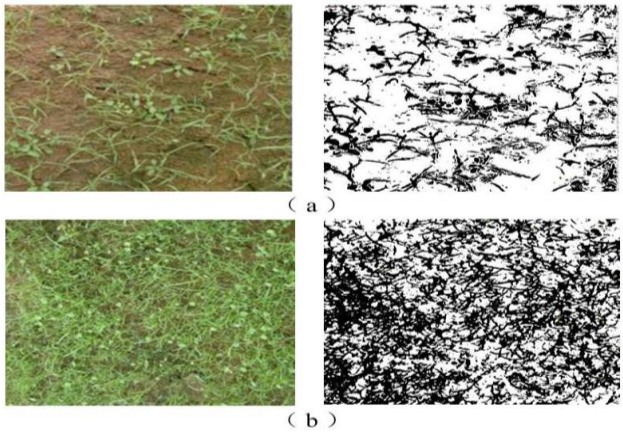

Figure 3. *Vegetation and their projected areas (test 2#). (a) Growth status in May30, 2013, (b) Growth status in June 30, 2013*

The method of obtaining PD was to measure the number of grass and shrubs in the range of 1 dm^2. PH of grass and shrubs needed to be measured with a tape. PC and PH were measured every three days for the first 20 days, every seven days for 20 days to three months, and every month after three months. PC and PH are the average of three measurements in each time monitoring.

Data analysis

Differences in PC, PD, and PH were determined using ANOVA with LSD test. Paired t-tests were used to test differences in PC, PD, and PH for December 2015 between the two topographic factors and two seed schemes. All statistical tests were performed using IBM SPSS statistics 23.

Results and Discussion

Dynamics of vegetation cover during the monitoring period

Fig. 4a-f shows the dynamics of vegetation cover in different stages. In May, the surface area covered by vegetation of the entire experimental group was relatively low (*Fig. 4a*). In June, the cover of *C. dactylon* increased rapidly, but the surface area was observed to be relatively low in pure-shrub experimental group (*Fig. 4b*). Grass-shrub experimental groups withered at a few locations, and *C. dactylon* withered more seriously than *M. multiflora*

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during July. Furthermore, we observed traces of rain erosion and exposure of the geonet in pure-shrub and pure-grass experimental groups. Unlike pure-shrub or pure-grass experimental groups, we noted relatively high resistance to erosion in grass-shrub experimental groups during heavy rain. This is probably because a combination of *C. dactylon* and *M. multiflora*. *C. dactylon* can slow runoff erosion. The stems and leaves of *M. multiflora* can play the role of rainfall interception and gravitational potential energy. In August, we observed the regrowth of *C. dactylon*. Changes in grass-shrub vegetation cover were relatively small from September to October. In addition, we observed that the cover area was relatively high in grass-shrub and experimental groups. This may be attributed to the relatively high water retention in grass-shrub experimental group during the monitoring period. The surface area of grass-shrub cover can avoid direct exposure to sunlight. The drought resistance of grass-shrub experimental group is stronger than that of the pure-shrub experimental group. The variation in grass-shrub experimental group cover was less during August and December, indicating that the grass-shrub communities tend to be stable. However, we observed wilting of *C. dactylon* from November to December (see *Fig. 4c-d*).

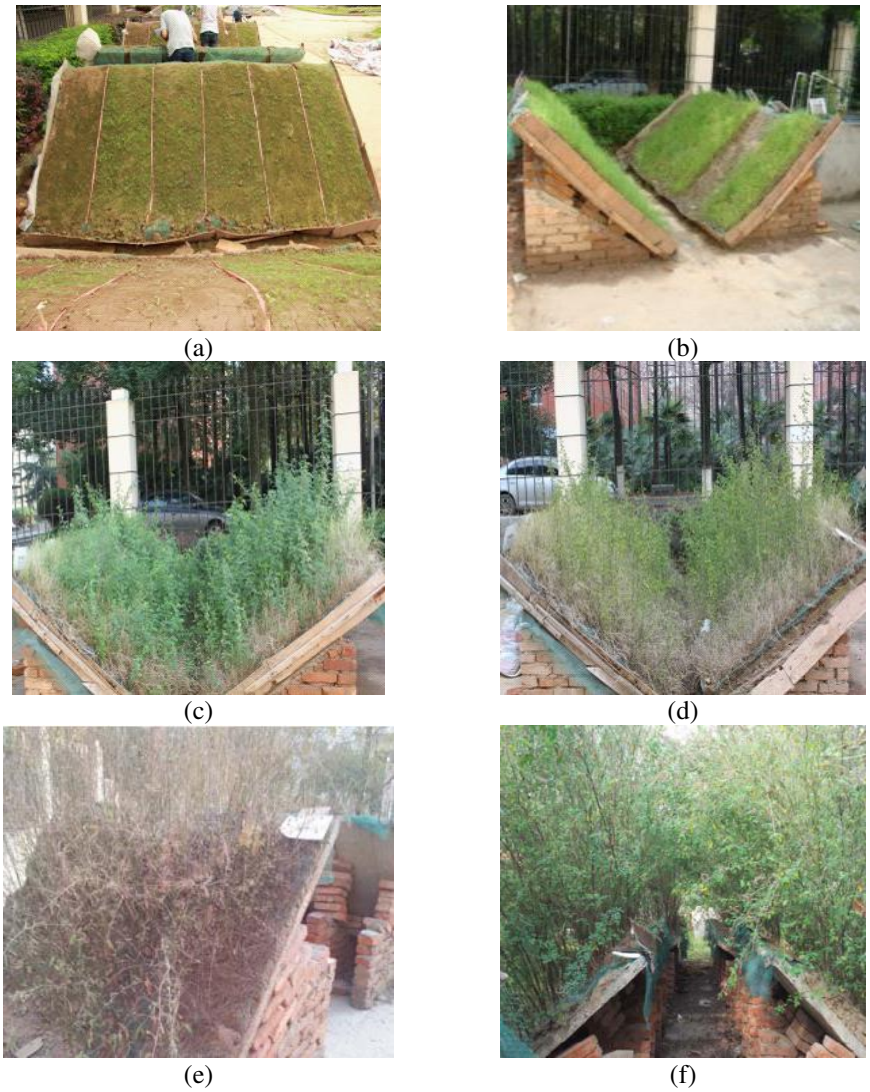

Figure 4. *Changes in vegetation cover at different times (a) May 30, 2013, (b) June 30, 2013, (c) October 30, 2013, (d) November 30, 2013, (e) December 30, 2014, (f) November 30, 2015*

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From March to May 2014, most of the withered *C. dactylon* regrow, and a certain number of *C. dactylon* sprouted. From November 2014 to January 2015, *C. dactylon* and *M. multiflora* withered, resulting in reduced stem and leaves (see *Fig. 4e*). This may be due to the lack of rainfall in the two months of the year and insufficient soil moisture, which caused grass and shrub plants to wither. Most of the *C. dactylon* and *M. multiflora* began to resurrect in March 2015, and a few *C. dactylon* sprouted. In December 2015, a small amount of *C. dactylon* withered, but *M. multiflora* did not wither (see *Fig. 4f*).

PC during the monitoring period

PC is an important indicator to measure the condition of surface vegetation. Spatial and temporal variations of the PC are plotted in *Fig. 5*. From *Fig. 5*, we note that the PC curves exhibit two stages: (1) The rapid rise stage: in the first two months, the PC of all experimental groups increases to 48.28-100% range. Rapid change of PC indicates that the germination and initial growth of vegetation are mainly concentrated in May and June 2013. (2) Small fluctuation stage: the PC curve changes with season. In winter (October to February of the next year), the PC decreased by less than 10%, while in the winter of 2014 (October to February of the next year), the PC dropped by 15-20%. This may be because the *C. dactylon* and *M. multiflora* partially wither in winter, especially when plenty of leaves of *M. multiflora* fall, the PC reduces. The winter rainfall in 2014 was 30% less than that in previous years, which led to the withering of the two plantations to a certain extent and reduced the PC. In the spring and summer of 2014 and 2015 (March to August), the germination and new leaves of *C. dactylon* and *M. multiflora* increased the PC.

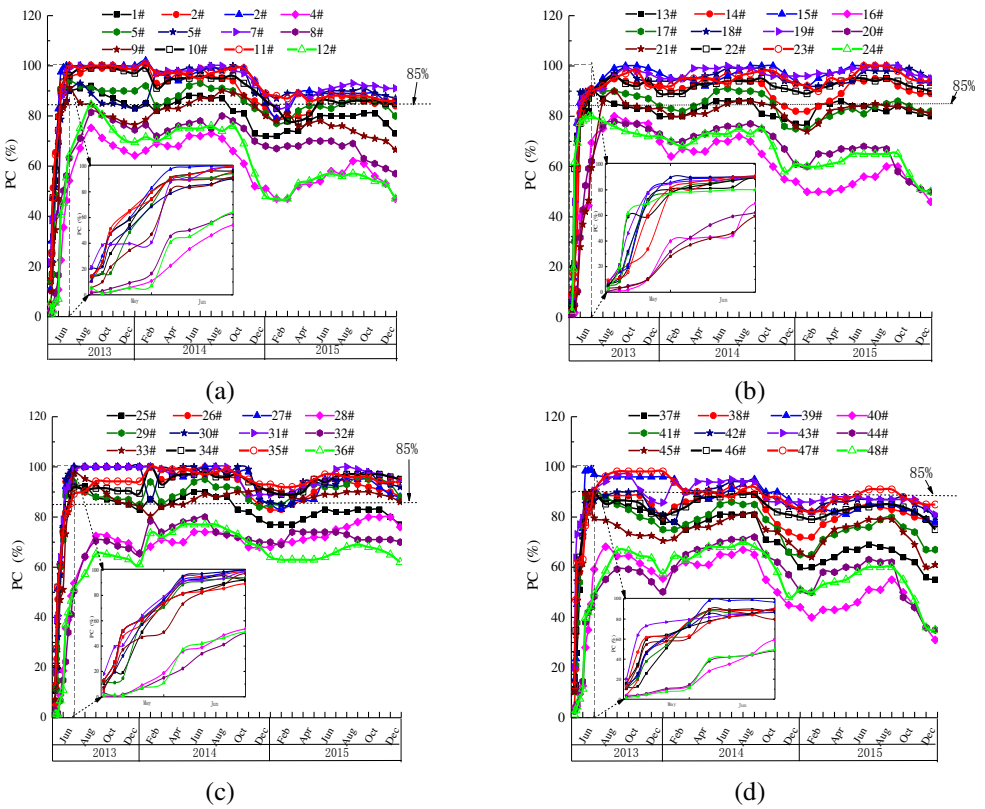

Figure 5. *PC of experimental groups during the monitoring period (a) 15°, (b) 30°, (c) 45°, (d) 60°*

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As can be observed in *Fig. 5*, the PC of grass-shrub experimental group is greater than that of pure-grass and pure-shrub experimental group, the PC of pure-grass experimental group is higher than that of pure-shrub experimental group. In December 2015, the slope gradients with the highest average PC were 30º (93.44%), followed by 45º (90.46%), 15º (87.42%), and 60º (78.41%) in grass-shrub experimental groups. According to the above analysis, the average PC first increases and then decreases with the increase of slope gradient. We use 85% PC to indicate the turfgrass establishment of the vegetated communities (Vietor et al., 2010). From July 2013 to December 2014, all grass-shrub experimental groups and some pure-grass experimental groups reached turfgrass establishment. After December 2014, only the grass-shrub experimental group reached turfgrass establishment, the PC of all pure-shrub experimental group and pure-grass experimental groups ranged from 32.18% to 83.95%, which has not reached the turfgrass establishment. Thus, we can conclude that the proper grass-shrub combination and proper slope gradient (30-45º) are beneficial to increase PC.

Next ANOVA method was applied to analyze differences in PC, PC differed insignificantly with slope gradient (P=0.233), slope aspect (P=0.09), and seed density (P=0.203). PC differed significantly with grass-shrub ratio (P< 0.05).

Dynamics of PD affected by topographic factors (slope gradient and slope aspect)

7#, 19#, 31#, and 43#experimental groups were selected (1:9 of grass-shrub ratio, N of slope aspect, and 20g·m^{-2} of seed density) to study the dynamics of PD affected by slope gradient. The curves of PD in experimental groups under different slope gradients are plotted in *Fig. 6*. It can be observed from *Fig. 6* that the germination rate of *M. multiflora* is faster than that of *C. dactylon*. The PD curves of the two plants reach the maximum in one month and then generally decrease slowly.

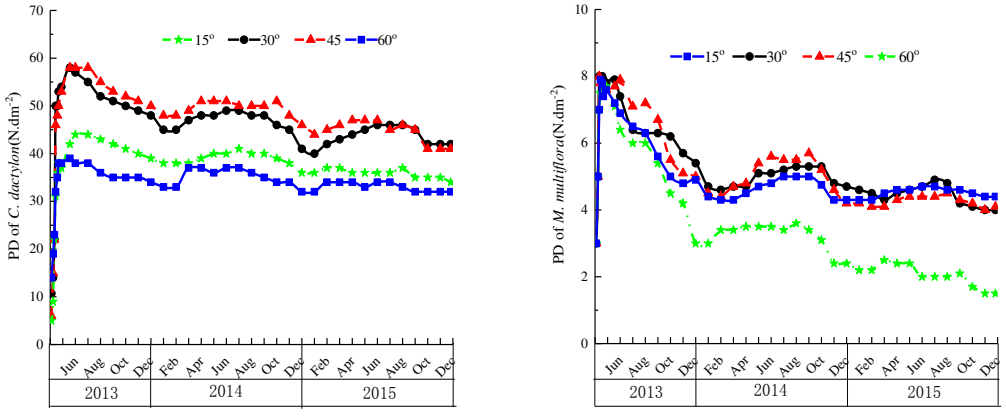

Figure 6. *Change of PD in experimental groups under different slope gradients (a) C. dactylon, (b) M. multiflora*

As can be seen from *Fig. 6a*, the PD of *C. dactylon* decreased in winter (from November to February) and increased in spring (from April to July), but the variation of PD is only within 15%. We note that the PD of *C. dactylon* first increases and then decreases with the increase of the slope gradient, and the slope gradient of *C. dactylon* highest PD was 45º. Furthermore, The PD of 45º (57 N·dm^{-2}) is 1.46 and 1.26 times

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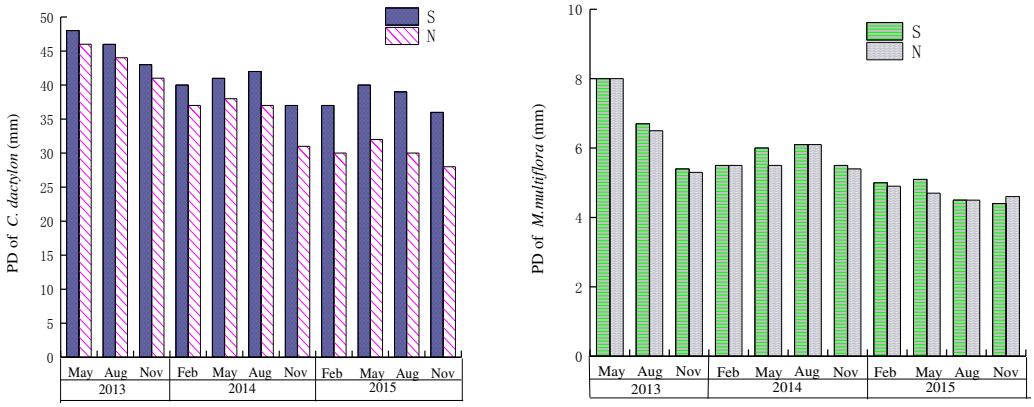
higher than that of 60° (39 N·dm^{-2}) and 15° (45 N·dm^{-2}) on June 30, 2013. This phenomenon is mainly due to increased sunlight. At medium and low slope gradients, the energy exposed to sunlight increases with the increasing of the slope gradient. When the slope gradient is greater than 45°, the germination and growth are hindered by insufficient water retention on the slope, thereby leading to reduced PD.

From *Fig. 6b*, the average PD of *M. multiflora* fell from 7.9 to 4.5 from August to December 2013. The low early survival rate of *M. multiflora* is mainly due to the dry period from August to December and the lack of water for the survival of *M. multiflora*. A small number of re-germinated *M. multiflora* appeared in the spring of 2014, a few *M. multiflora* died in the winter of 2014, the PD of *M. multiflora* became stable after 2015. The PD of the 60° slope is significantly lower than that of the other three slopes, indicating that the high slope is not conducive to the growth of *M. multiflora*. There were insignificant differences in PD of *C. dactylon* (P = 0.06) and *M. multiflora* at different slope gradients (P = 0.12).

Next, to explore PD variation affected by the two slope aspects, we considered the 18# and 30# experimental groups (1:9 of grass-shrub ratio, 30° and 45° of slope gradient, and 20 g·m^{-2} of seed density). As can be seen from *Fig. 7a*, the PD of *C. dactylon* with 18# (S) is greater than that with 18# (N), and the difference between the two experimental groups increases with time. There are significant differences in PD of *C. dactylon* at different slope aspects ($P < 0.05$). From *Fig. 7b*, the slope aspect has little effect on the PD of *M. multiflora*.



(a) (b)

Figure 7. *Variation of PD affected by two slope aspects (a) C. dactylon, (b) M. multiflora*

PD affected by seed schemes (seed density and grass-shrub ratio)

Fig. 8 depicts the histogram of PD for the 45° experimental slopes (25#-36#) in December 2015. We observed from *Fig. 8a* that PD of *C. dactylon* is observed to rise with increasing seed density. Seed density for the highest average PD of *C. dactylon* is 25 g·m^{-2} (41.67 N·dm^{-2}), which is 1.59 and 1.09 times higher than that of 15 g·m^{-2} (26.17 N·dm^{-2}) and 20 g·m^{-2} (38.07 N·dm^{-2}). From *Fig. 8*, comparing the PD of *C. dactylon* and *M. multiflora* with 25 g·m^{-2} and 20 g·m^{-2}, we note that the difference in average PD is very small. This result suggests that 25 g·m^{-2} cannot bring more *C. dactylon* PD. Thus, we can conclude that from an economic point of view, 20 g·m^{-2} is the best choice for the PD of *C. dactylon*.

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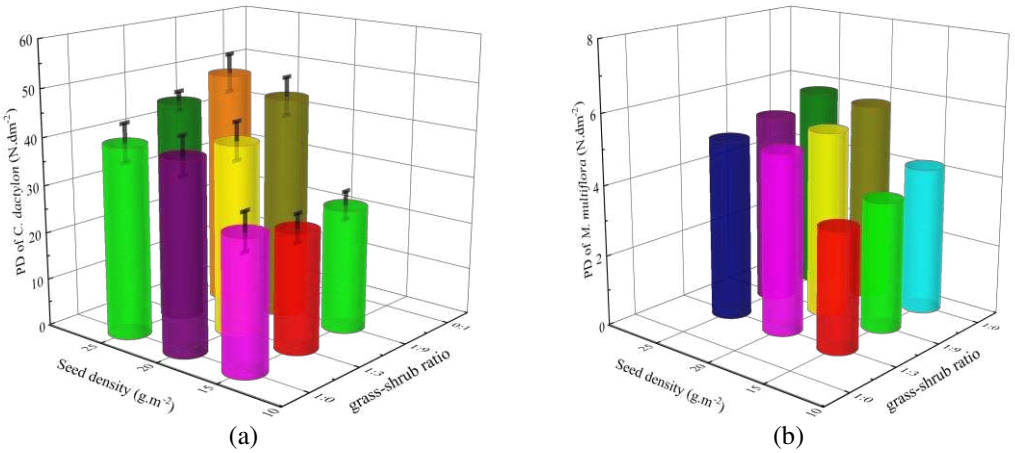

(a) (b)

Figure 8. *Variation of average PD affected by different seed density and grass-shrub ratio (a) C. dactylon (b) M. multiflora*

Meanwhile, PD of *C. dactylon* does not decrease significantly with the increasing of grass-shrub ratio, PD of *M. multiflora* increases with the increase of grass-shrub ratio. It can infer that more shrub seeds account for more total PD. PD differed significantly with grass-shrub ratio ($P< 0.05$).

Dynamics of PH affected by topographic factors (slope gradient and slope aspect)

We plot the PH curves of *C. dactylon* and *M. multiflora* obtained from 7#, 19#, 31#, and 43# experimental groups (N, 1: , and 20 $g \cdot m^{-2}$) in *Fig. 9*. From *Fig. 9*, *C. dactylon* grew faster before mid-May 2013, and the correlation between PH and time is linear. We found that PH of *C. dactylon* is relatively slow during mid-May and September 2013, which is only about 20 mm in three months. This result indicates that *C. dactylon* has stopped growing, which is consistent with the growth mechanism. We note that the PH of *C. dactylon* were reduced in July 2013. This is probably because the heavy rainfall seriously affected the seedling stage of the *C. dactylon*. Notably, the decrease of PH can be observed from October 2013 to February 2014, from October 2014 to February 2015, and from October 2015 to December 2015. Consequently, we can conclude that heavy rainfall and low temperature and dry climate have a great impact on the vegetation growth.

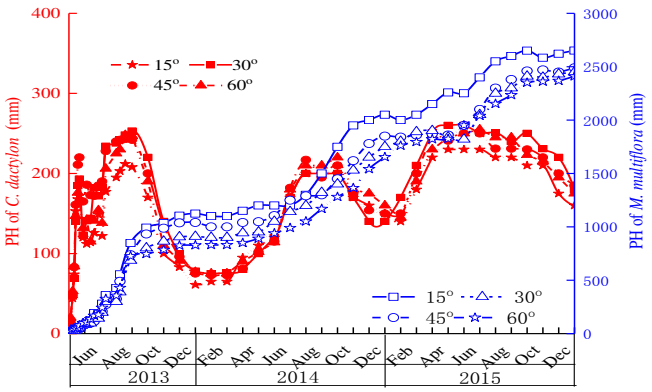

Figure 9. *Variation of PH affected by different slope gradients*

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From *Fig. 9b*, we observed that the PH curves of *M. multiflora* exhibit a four -stage nonlinear relationship: (a) the first stage is before July 2013, *M. multiflora* grows rather slowly, with a total growth of 580 mm. (b) The second stage is from August to September 2013 *M. multiflora* growing faster. Four experimental groups have grown 540 mm every month. (c) From October 2013 to February 2014 is the third stage, when *M. multiflora* growth slows down. *M. multiflora* grows 100 mm in five months. (d) The fourth stage is after May 2014, *M. multiflora* grows faster, but the growth rate is lower than that in the second stage and slows down in winter. This result indicates that the fastest growing time of *M. multiflora* is from the fifth month to the next spring/summer. In addition, the slope gradient with the highest average PH of *M. multiflora* is 15° (1064 mm) during September and December 2013, which is 1.06, 1.22, and 1.34 times higher than that of 30° (1000 mm), 45° (869 mm) and 60° (796 mm). PH of *C. dactylon* and *M. multiflora* differed insignificantly with the slope gradient ($P > 0.05$).

Next, to analyze the variation of PH affected by the two slope aspects, we considered the 18# and 30# experimental groups (1:9 of grass-shrub ratio, 30° and 45° of slope gradient and 20 g m^{-2} of seed density). From *Fig. 10*, we note that the slope aspect affects the PH of plants, and the difference of PH between the north-facing and south-facing increases with time. In December 2015, the PH of *C. dactylon* were 151 mm (south-facing), which is 1.13 times higher than that of 107 mm (north-facing). The PH (south-facing) of *M. multiflora* is 1.25 times higher than that (north-facing). The PH of *C. dactylon* and *M. multiflora* differed significantly with the slope aspect ($P< 0.05$), which can be attributed to the positive effects of the vailing winds from the southeast in summer (Zhou et al., 2016).

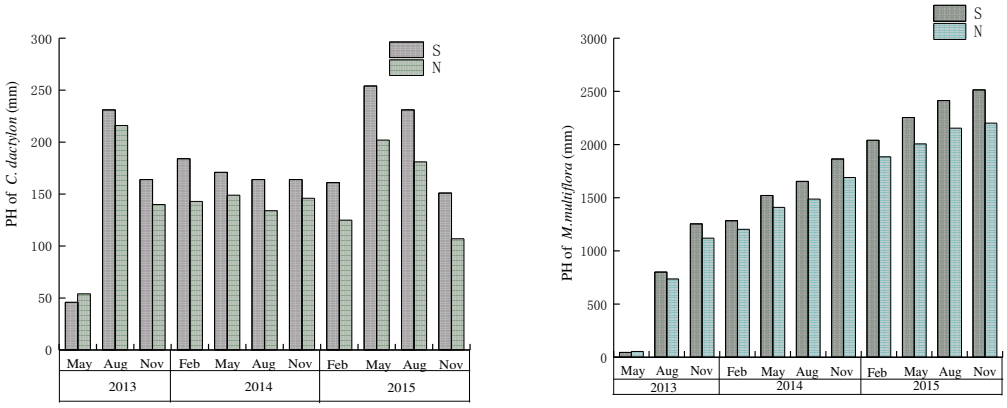

Figure 10. *Variation of PH affected by two slope aspects*

Dynamics of PH affected by seed schemes (grass-shrub ratio and seed density)

To understand the significance of the change in competition between grass and shrub, we considered four experimental groups (29#, 30#, 31#, and 32#) with the same factors (45°, N, 20g·m^{-2}). Dynamics of PH affected by the four grass-shrub ratios are plotted in *Fig. 11*. We found that the pH difference between *C. dactylon* and *M. multiflora* was very small in the four experimental groups before August 2013, but the difference between the PH of pure-grass experimental group and grass-shrub experimental group increased with time after September 2013. As the time and the grass-shrub ratio increase, the pH value of *M. multiflora* also increases. We observe that the PH of *C. dactylon* for 1:3 (1:9) was

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greater than 50 mm (60 mm) of 1:0 in December 2015. The PH of *M. multiflora* for 0:1 is 1.10 times that for 0:1 and 1.35 times that for 0:1. Consequently, we can conclude that the higher grass-shrub ratio is beneficial to the growth of *C. dactylon* and *M. multiflora*. PH differed insignificantly with grass-shrub ratio ($P > 0.05$).

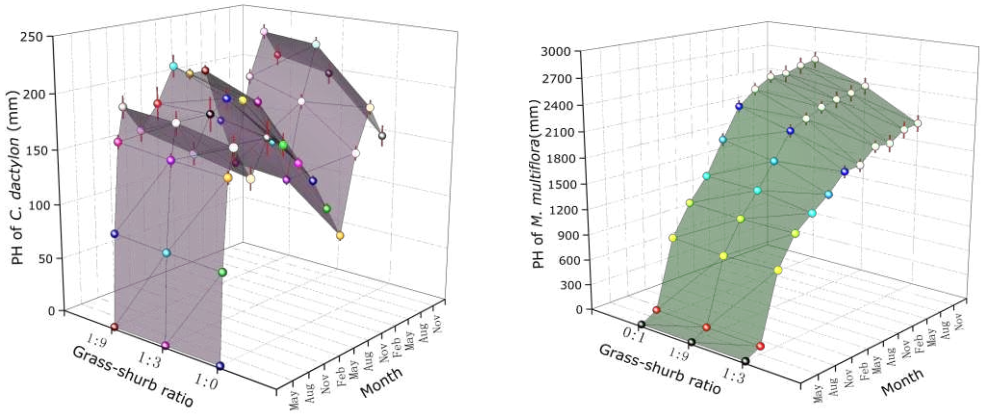

Figure 11. *Dynamics of PH affected by different grass-shrub ratio*

We plot the PH curves of *C. dactylon* and *M. multiflora* obtained from 27#, 31#, and 35# experimental groups (45°, N, 1:9) in *Fig. 12*. From *Fig. 12a*, we observed that the relationship between PH of *C. dactylon* with seed density was not as obvious during the first two months. The PH difference of *C. dactylon* with different seed densities was not significant, and the PH difference of different seed densities of *M. multiflora* increased with time (see *Fig. 12b*). PH of *M. multiflora* decreased with the increasing of seed density. Seed density with the highest average growth height of *M. multiflora* is $20g \cdot m^{-2}$ (2464 mm), followed by $25g \cdot m^{-2}$ (2286 mm) and $15g \cdot m^{-2}$ (1830 mm) in December 2015. The PH of *M. multiflora* to $20g \cdot m^{-2}$ is 1.07 times higher than that to $25g \cdot m^{-2}$. The results indicated that more than $20g \cdot m^{-2}$ of seed density had a negative effect on the PH of *M. multiflora*. Excessive seed leads to increased competition among vegetation. The nutrients and water obtained from the soil of vegetation may be reduced. Consequently, we can conclude that excessive seed density will have a negative impact on PH. PH differed insignificantly with seed density ($P > 0.05$).

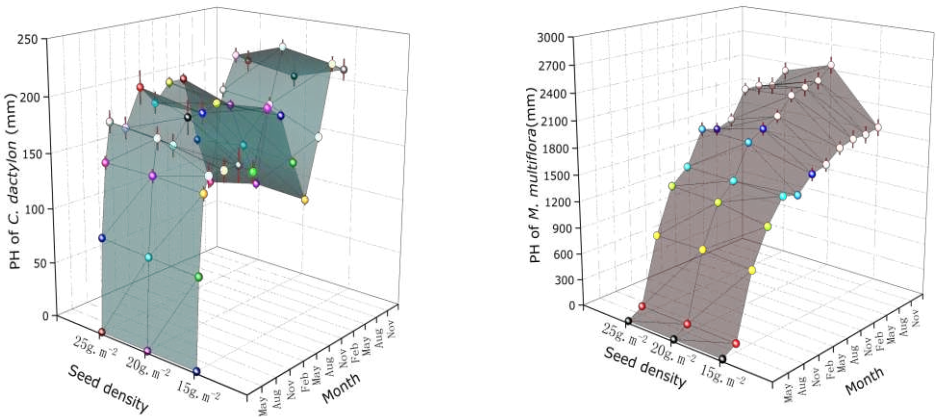

Figure 12. *Dynamics of PH affected by seed density*

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Discussion

It is a challenging task to stabilize and restore these damaged highway slopes by using suitable ecological restoration measures (Cao et al., 2010). The growth dynamics of different vegetation species has varying effects on community formation and may also reflect species differential responses to ecological restoration (Kadmon, 2016). PC, PD, and PH of pure-grass or pure-shrub revealed a sharp reduction, and consequently grass-shrub is beneficial to community formation. Our results showed that the PC grew very fast in a month is consistent with the conclusion of the studies on deciduous species (Gadi et al., 2019). The results indicate that the growth dynamics of *C. dactylon* and *M. multiflora* depend on the seed scheme, grass-shrub ratio has a critical influence on PC, which is consistent with the conclusion that grass and shrub significantly increased PC (Schäfer et al., 2018). It is clear from our data to reveal that shrub positively affected grass survival and the shrub had positive effects on PC, PD, and PH (He et al., 2014). First, shrub canopy has a facilitative effect on grass due to more shade, lower temperature, lower radiation, and higher soil moisture (Jankju, 2013). Second, pure-grass or pure-shrub has limited slope surface cover, unable to retain moisture. Seedlings of pure-grass or pure-shrub would be inferior to adaptation to harsh habitat conditions. Grass has advantages over deep-rooted shrubs in competition (Wang, 2014). However, grass had a negative feedback effect on the growth of the shrub and PC (Bai et al., 2018). The relationship between the shrub and the associated herbs was antagonistic. The final development is mainly herb-based or shrub-based communities, depending on the outcome of the confrontation between herbs and shrubs.

Previous studies showed that seed density played an important role in the stability and succession of ecological restoration (Bodziarczyk, 2001). The successful vegetation community establishment depends on the quality of seed density, germination conditions of seed, and growth conditions. Our results of more than $20 g \cdot m^{-2}$ of seed density have a significant negative effect on the PH of *C. dactylon* and *M. multiflora*, which is consistent with the conclusion of high seed density producing low PD (Xu et al., 2016). Seed density has positive and negative effects on grass-shrub PH. Only a little increasing amplitude of the average total PD of *C. dactylon* is emerged as the high seed density of *Vetiveria zizanioides* (Kozovits, 2014). This dynamic was linked to competition and soil nutrition of grass-shrub. High nitrogen fertilizer provided significant increases in PD at high seed density compared to low seed density (Elfadl et al., 2009). Therefore, PD can be improved by increasing fertility and high planting density.

It was proven in this study that topographic variables may have an impact on plant growth dynamics. Results indicated that the effect of topographic factors (slope gradient and slope aspect) on PD and PH is significant, which is consistent with the conclusion of slope aspect having significant effects on the PD of all plants and slope gradient only affecting the PD of shrubs significantly (Ou et al., 2011). In addition to topographic factors affecting growth dynamics, age cannot be ignored. The topographic factors did not influence newly sprouted culms during the stable period (Niu et al., 2020). The plants we studied are all in the growth period, especially the *M.multiflora* has substantial growth in the second and third years, which is greatly affected by the slope aspect and slope gradient. Climate influence on PC, PD, and PH determines whether ecological restoration can be successful (Fu et al., 2012). To improve the seed germination, the sowing time was chosen at 20-25^{o}C in May. Altitude is also a topographic factor, which has an important impact on the ecological dynamics of vegetation (Wagner and Mitschunas,

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2008). Altitude is not involved in this paper, and further research in this area should be strengthened in the future.

The results highlight that it is essential to optimize the formulation of the four influencing factors of topographic factors and seed schemes in the scheme design before construction, and ensure that community formation of slope ecological restoration is quicker and economically feasible.

Conclusion

In this study, we investigated the effects of topographic factors (slope gradient and slope aspect) and seed schemes (grass-shrub ratio and seed density) on the dynamics of PC, PD, and PH of grass-shrub communities. Based on our results, we drew the following conclusions:

(1) Vegetation cover increased rapidly during the first four months. The change in PC after the fifth month was small. The average PC first increases and then decreases with the increase of slope gradient. The community formation period differed significantly for grass-shrub ratio ($P< 0.05$).

(2) The PD reached its maximum during the first two months and then decreased slowly. The slope gradient and seed density with peak value of C. dactylon PD are 45° (57 $N \cdot dm^{-2}$) and 25$g \cdot m^{-2}$ (41.67 $N \cdot dm^{-2}$), respectively. PD differed significantly for grass-shrub ratio.

(3) The slope gradient has an insignificant effect on the PH of *C. dactylon*, but the slope aspect has a significant effect on the PH of *M. multiflora* ($P< 0.05$). The seed density and grass-shrub ratio with the highest average PH are 20$g \cdot m^{-2}$ and 1: 9, respectively.

(4) The optimal conditions for the formulation of grass-shrub communities are as follows: slope gradient of 30°-45°, slope aspect of south-facing, seed density of 20$g \cdot m^{-2}$, grass-shrub ratio of 1:9. The formulation can promote the formation of ecological restoration community of rock slope more quickly, which can be extended to practical projects.

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GROUND-DWELLING SPIDERS IN DIVERSE MOSAIC OF GARDEN HABITATS

KRUMPÁLOVÁ, Z.[1*] – ONDREJKOVÁ, N.[1] – IVANIČ-PORHAJAŠOVÁ, J.[2] – PETROVIČOVÁ, K.[2] – LANGRAF, V.[3]

[1]*Department of Ecology and Environmental Sciences, Faculty of Natural Sciences, Constantine the Philosopher University, Nitra, Slovakia*

[2]*Department of Environment and Biology, Faculty of Agrobiology and food resources, Slovak University of Agriculture, Nitra, Slovakia*

[3]*Department of Zoology and Anthropology, Faculty of Natural Sciences, Constantine the Philosopher University, Nitra, Slovakia*

[*]*Corresponding author*
e-mail: zkrumpalova@ukf.sk; phone: +421-917-201-036

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Abstract. The species composition of a biota in an urban environment can greatly depend on the characteristics of local and surrounding habitats. Research on the composition of spider communities was carried out in a private garden, in various places - stored wood, under thujas, on a mown lawn, in an orchard and in a garden with crops, to identify habitat variables associated with fluctuations in spider assemblages affected by human disturbance. Although gardens do not seem to be as diverse as the natural places, we found a surprisingly large variety of spiders, 74 species. Differences in the composition of spider communities between land use types were - a small number of species tolerant to the urban environment, a high number of specific species that showed different responses to habitat properties such as vegetation cover, light and humidity conditions and human activity. We found interesting records of new species that were spread by humans - species successfully adapted to synanthropic environments. In gardens, our records were the second one to report adult individuals of *Hoplopholcus forskali* and *Mermessus trilobatus*, so far found as juveniles in botanical gardens or *Tallusia vindobonensis*, previously only found in salt marshes. *Tegenaria hasperi* is a new spider to Slovakia.
Keywords: *urban environment, invertebrates, biodiversity, urban green space, human influence, bioindication, private garden*

Introduction

Urban gardens are a major component of urban green spaces in many countries (Loram et al., 2008; Edmondson et al., 2014). They are heterogeneous in structure, but despite their relatively small size they provide critical habitat resources and increase the connectivity of urban landscapes (Soanes, 2019). Urban gardens are popular green spaces that have the potential to provide essential ecosystem services, support human well-being, and at the same time foster biodiversity in cities (Tresch et al., 2019).

The garden is an important refuge for biota in an urban environment. It forms a diverse mosaic of interesting habitats, where man creates an important, structured and relatively diverse ecosystem. It is a space for a specific fauna of invertebrates. Gardens form separate micro-ecosystems with typical conditions, they are not only completely man-made, but also disturbed and altered. They are a special type of habitat, absolutely different from the surrounding nature, with the characteristic planting of non-native transported trees, plants and soil. On the one hand, some gardens are built with usefulness in mind, planting of agricultural crops and fruit trees (they are regularly

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disturbed - hoeing, selective watering, fertilization, loosening, weeding, they have a very short growing season, etc.); others are recreational-type gardens - with exotic trees and sown lawn, often without flowering plants (they are regularly disturbed by grass mowing, watering, fertilization and loosening; in the case of trees and trusses - pruning and crown formation, etc.) (Ondrejková and Purgat, 2019; Krumpálová et al., 2020b).

Spiders as suitable model animals provide us with important information about the environment in which we find them. Spiders, especially ground dwelling, are considered an important bioindication group. In connection with human activities, they react very sensitively to changes in the country (Růžička, 1987). In general, the decrease in spiders per unit area depends on the difference between the original state and the current one. This reflects the degree of anthropogenic influence on the habitat (Růžička, 1987). The spider may, for example, assess a microhabitat as a potential web site, oviposition site, overwintering site or as a safe haven from predators during the inactive phase of a diel cycle. Spider may need to seek temporary refuge in a favourable micro-habitat in order to maintain its physiological integrity. From this it follows that individuals of any spider population are harboured by specific micro-habitats which might vary over time, according to the current needs of the individuals (Samu et al., 1999). Selection of micro-habitat by individual spiders is likely to be in relation to a specific biological need or collection of needs or may reflect avoidance of some factor, such as interspecific encounters (Post and Riechert, 1977). Soil organisms are sensitive to environmental contamination, in which they occur and also called stress as bio-indicators. Their reaction may result to the environmental load in different ways – in a change in behaviour; a change in habitat; a quantitative change and composition of species spectrum; and into physiological or morphological deformation of the individual or of the whole community (Baranová et al., 2015). The soil edaphon is an important component of biocoenosis, reflects the burden on biotopes and is an important bio-indicator of environmental quality (Porhajašová-Ivanič et al., 2016).

In Slovakia, the research of invertebrates in gardens has not yet received much attention. Invertebrates' investigation in this environment is pioneering. Spiders were part of the study of the urban environment in the region of Nitra (Ondrejková and Purgat, 2019; Krumpálová et al., 2020a,b; Purgat et al., 2020), the authors concluded that some of them have adapted on urban conditions and occupy habitats with high human influence: *Lycosa singoriensis* on mowed lawns, *Allagelena gracilens* on modified shrubs, *Brigittea civica* and *Larinioides ixobolus* on walls of buildings. On the contrary, the progressive ruderalisation of the peripheral parts of the towns and the low anthropic influence create suitable habitats for *Cheiracanthium punctorium*. Attention was also paid to soil mites (Oribatida) in planted garden (Krumpálová et al., 2020b). In Hungary, Magura et al. (2010) studied the effects of urbanization on ground-dwelling spiders (Araneae) along an urban-suburban-rural forest gradient in Debrecen, and found that overall spider species richness was significantly higher in the urban sites compared to the suburban and rural ones. Tajthi et al. (2017) assumed a higher number of species in suburban habitats than in rural or urban ones, where they also confirmed the hypothesis of species susceptibility to disturbance. In Denmark, fragments of the suburban, rural and urban forest were observed by Horváth et al. (2014), so the highest number of common species was in suburban and urban habitats. Research shows that urbanization did not reduce biodiversity, but there were few common species with native forest habitats. Lowe et al. (2017) compared araneocenoses in private gardens, city parks and residual vegetation. The gardens excelled in relatively high diversity.

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Coenoses differed significantly between gardens and other urban areas. In Finland, Alaruikka et al. (2002) confirmed the significant influence of the locality, while spiders were affected on a small scale by the structure of the habitat itself; large urban areas had a greater influence.

We hypothesised that i. - intensive soil management will reduce the species diversity in garden; ii. – a higher diversity of plants will have a positive effect on higher diversity of spiders than in recreational garden; iii. – the gardens create conditions and offer space for specific species little known from the natural environment.

The aim of this study was to analyze the coenoses of spiders in different five micro-habitats of the garden, we expected that frequently disturbed places would have the lowest species diversity within (alpha diversity) and among (beta diversity) garden sites. Additionally, we tried to find differences between garden land-use types in analyzing of species diversity, and evaluate the data obtained as a whole with a view to emphasizing the importance of gardens in the urban environment.

Material and methods

This study took place in private garden of family houses of the village Machulince (Nitra region); 48°24´N, 18°25´E; in western part of Slovakia in the Danubian lowland.

We chose the garden, in the area of 880 m^2, and on the basis of two independent criteria: i. the recreational part of the garden and ii. garden with cultivated crops and old orchard. In the recreational garden, three sampling plots (2 traps in each plot) with different land management were selected. The first place was in the part of the garden with stored wood, shaded during the year and least affected by human influences; the second place was an artificially planted shrub vegetation of non-native trees (*Thuja occidentalis*) with minimal interference; and the third place was on an open artificially planted lawn, where the interventions in the form of irrigation, regular mowing and raking took place during the year. In the managed part of the garden with cultivated crops, two sampling plots (2 traps in each place) were selected - a study place in the orchard, where the original, sporadically mowed lawn was partially shaded by tree vegetation and the fifth study place was located in a cultivated garden with crops, in which the greatest human interventions and disturbances were present (plowing, planting, selective irrigation and fertilization) and vegetation cover occurred only during the active period of cultivated crops, in the remaining time only the soil was without vegetation. The distance between the sampling plots was about 30 metres.

Spiders were collected in each of the 5 places using traps. There were two traps at each studied microhabitat, approximately 3 m apart. Pitfall traps (glass cups; 6.5 cm in diameter) were filled with a fixative, a formaldehyde solution. Traps were exposed continuously throughout the year, from September 2017 to October 2018. Trapped spiders were picked up every month.

Collected individuals were identified by using the works of Heimer and Nentwig (1991), Miller (1971), or www.arachno.piwigo.com and Nentwig et al. (2020) - www.araneae.nmbe.ch. Juvenile and sub adult stages were determined at the genus level. Nomenclature followed the World Spider Catalog (2020). Habitat affinity, ecological demands of spiders (humidity or light requirements) of the collected species was designated from the literature (Buchar and Růžička, 2002).

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Statistical analyses

The analyses in the PAST-software, version 2.14 (Hammer et al., 2001) and Statistica, version 7 (2004) was focused on quantitative-qualitative methods. By Shapiro-Wilks W-test we tested the normality of data distribution of number of individuals and species of the spiders. Kruskal-Wallis test (ANOVA) we used to test the differences in number of individuals and species between microhabitats. Cluster analysis (Bray-Curtis index) in the algorithm (UPGMA) we found a similarity of spider assemblages. Spatial modeling was performed by multivariate analysis in the program Canoco 5 (Ter Braak and Šmilauer, 2012), with which we look for dependencies between objects species of spiders and biotopes (wood, thuja, lawn, orchard, garden with crops). We used Principal Component Analysis (PCA) to evaluate the dependence and similarity between studied coenoses.

Results

Habitats with a high diversity of spiders

The total spider catch consisted of 536 individuals representing 74 species (99 taxa, resp.) (*Table 1*). Many or most of species were found singletons and doubletons. *Erigone dentipalpis*, *Pardosa hortensis* and juveniles from genus *Pardosa* were dominant. Diversity indices of all collected garden spiders achieved a very high values - Shannon H´= 3.99, Margalef = 15.74 and Pielou = 0.86 (*Table 2*).

In the stored wood there were 105 individuals belonging to 43 species, under thujas we found higher number of individuals (124) and species (44), whereas in mowed lawn we collected 84 individuals of 30 species, in orchard were 116 individuals belonging to 31 species, and in garden with crops 107 individuals representing 26 species were captured (*Table 1*).

The normality data distribution (number of individuals) was violation (p-value = 0.00), based on we are used a nonparametric Kruskal-Wallis test (ANOVA) to confirm the statistically non-significant difference (p-value = 0.3112) between spider assemblages in microhabitats (*Fig. 1*). Number of individuals in the garden as a whole was relatively balanced; but the number of species in the coenoses varied and had significant differences (*Fig. 2*).

The normality data distribution (number of species) was violation (p-value = 0.01), based on we are used a nonparametric Kruskal-Wallis test (ANOVA) to confirm the statistically significant difference (p-value = 0.0131) between biotopes (*Fig. 2*). Most of the species were found under thujas and stored wood, lower number was in mowed lawn and orchard, and the lowest number of species was in the garden with crops.

Garden micro-habitats offer different living condition

In the recreational part of the garden with planted thujas, forming a barrier from the surroundings and stored wood, study site was characterized by small disturbing influence of humans, there were relatively low soil humidity and high shading, and there was no vegetation cover. Despite the fact, it was found that spider communities differ not only from the other three habitats, but these two habitats were dissimilar to each other in species composition and assemblages structure (*Figures 2-4*).

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Table 1. *Abundance of spiders in different habitats of the gardens*

Taxon – species/ study site	stored wood	thujas	mowed lawn	orchard	garden with crops	∑	Total dominance (%)
Scytotidae							
Scytodes thoracica (Latreille, 1802)	1		1			2	0.4
Pholcidae							
Hoplopholcus forskali (Thorell, 1871)	11					11	2.1
Pholcus opilionides (Schrank, 1781)	2	17	4			23	4.3
Pholcus sp.		6				6	1.1
Dysderidae							
Dysdera sp.	1					1	0.2
Harpactea rubicunda (C. L. Koch, 1838)	5	3				8	1.5
Harpactea sp.	2	1				3	0.6
Mimetidae							
Ero furcata (Villers, 1789)	1					1	0.2
Ero tuberculata (De Geer, 177)		1				1	0.2
Theridiidae							
Asagena phalerata (Panzer, 1801)			6		2	8	1.5
Euryopis sp.				1		1	0.2
Lasaeola tristis (Hahn, 1833)				1		1	0.2
Robertus arundineti (O. P. Cambridge, 1871)		1				1	0.2
Robertus lividus (Blackwall, 1836)	4	1				5	0.9
Steatoda sp.	3	0		1		4	0.7
Linyphiidae							
Agyneta cauta (P. O. Cambridge, 1902)			7	2	2	11	2.1
Araeoncus humilis (Blackwall, 1841)			1			1	0.2
Centromerus sp.			2			2	0.4
Centromerus sylvaticus (Blackwall, 1841)	1	3		2		6	1.1
Diplostyla concolor (Wider, 1834)	3	9	1			13	2.4
Erigone dentipalpis (Wider, 1834)			8	26	10	44	8.2
Leptyphantes angulatus (P. O. Cambr., 1881)	1					1	0.2
Leptyphantes leprosus (Ohlert, 1865)	1					1	0.2
Leptyphantes sp.	7	2			3	12	2.2
Leptyphantes tenuis (Blackwall, 1852)	1	1				2	0.4
Mermessus trilobatus (Emerton, 1882)	3	6	2		2	13	2.4
Micrargus subaequalis (Westring, 1851)		3				3	0.6
Oedothorax apicatus (Blackwall, 1850)				7	4	11	2.1
Oedothorax sp.				1		1	0.2
Palliduphantes pallidus (P. O. Cambr., 1871)					1	1	0.2
Porrhoma sp.				1		1	0.2
Porrhoma campbelli (F. O. P.-Cambr., 1894)	2	1				3	0.6
Porrhoma pygmaeum (Blackwall, 1834)		1				1	0.2
Sintula sp.		1				1	0.2
Stemonyphantes lineatus (Linné, 1758)		4			1	5	0.9
Tallusia vindobonensis (Kluczyński, 1898)		1	1			2	0.4
Tapinocyba biscissa (P. O.-Cambridge, 1872)	1					1	0.2
Trematocephalus cristatus (Wider, 1834)		3				3	0.6
Trichopterna cito (P. O.-Cambridge, 1872)				4		4	0.7
Walckenaria capito (Westring, 1861)	1	3				4	0.7
Tetragnathidae							
Pachygnatha degeeri (Sundevall, 1830)				4		4	0.7
Araneidae							
Araniela sp.				1		1	0.2

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Taxon – species/ study site	stored wood	thujas	mowed lawn	orchard	garden with crops	Σ	Total dominance (%)
Lycosiadae							
Alopecosa pulverulenta (Clerck, 1757)	1					1	0.2
Alopecosa sp.	3	4	3	1		11	2.1
Arctosa sp.			2			2	0.4
Aulonia albimana (Walckenaer, 1805)	2					2	0.4
Pardosa agrestis (Westring, 1861)	1	1	2	1		5	0.9
Pardosa agricola (Thorell, 1856)				2		2	0.4
Pardosa bifasciata (C. L. Koch, 1834)		1				1	0.2
Pardosa hortensis (Thorell, 1872)	4			7	19	30	5.6
Pardosa lugubris (Walckenaer, 1802)	1					1	0.2
Pardosa monticola (Clerck, 1757)			1	1	2	4	0.7
Pardosa palustris (Linné, 1758)			4		1	5	0.9
Pardosa prativaga (L. Koch 1870)		1		1		2	0.4
Pardosa pullata (Clerck, 1757)					1	1	0.2
Pardosa sp.	15	8	8	14	5	50	9.3
Trochosa robusta (Simon, 1876)	1		2		2	5	0.9
Trochosa ruricola (Simon, 1876)		1		3		4	0.7
Trochosa sp.			1	1		2	0.4
Xerolycosa miniata (C. L. Koch, 1834)				4	12	16	3.0
Xerolycosa nemoralis (Westring. 1861)					10	10	1.9
Xerolycosa sp.			1			1	0.2
Pisauridae							
Pisaura mirabilis (Clerck, 1757)		1				1	0.2
Agelenidae							
Agelena gracilens (C. L. Koch, 1841)	1	5				6	1.1
Agelena sp.		3	4			7	1.3
Coelotes sp.		1				1	0.2
Eratigena agrestis (Walckenaer, 1802)			2			2	0.4
Tegenaria domestica (Clerck, 1757)	1					1	0.2
Tegenaria hasperi (Chyzer, 1897)	1			1		2	0.4
Tegenaria sp.	1					1	0.2
Urocoras longispina (Kulczyński, 1897)	5					5	0.9
Hahniidae							
Hahnia helveola (Simon,1875)			4			4	0.7
Hahnia nava (Blackwall, 1841)		2			2	4	0.7
Hahnia pusilla (C. L. Koch, 1841)	1	1	1	3		6	1.1
Cicurina cicur (Fabricius, 1793)	1					1	0.2
Amaurobiidae							
Amarobius ferox (Walckenaer, 1830)		1				1	0.2
Liocranidae							
Liocranoeca sp.			1			1	0.2
Sagana rutilans (Thorell, 1875)	4					4	0.7
Clubionidae							
Clubiona sp.	1	1				2	0.4
Zodariidae							
Zodarion rubidum (Simon, 1914)		2		5	9	16	3.0
Gnaphosidae							
Civizelotes gracilis (Canestrini, 1868)		1	1	7	7	16	3.0
Drassodes lapidosus (Walckenaer, 1802)		2	5			7	1.3
Drassodes sp.			2		2	4	0.7
Drassyllus pumilus (C. L. Koch, 1833)		1			4	5	0.9
Drassyllus pusillus (C. L. Koch 1833)				6	2	8	1.5
Drassyllus sp.	2	10				12	2.2

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Taxon – species/ study site	stored wood	thujas	mowed lawn	orchard	garden with crops	Σ	Total dominance (%)
Drassyllus villicus (Thorell, 1875)	1	1				2	0.4
Drassylus pareficus (L. Koch, 1866)		1				1	0.2
Haplodrassus signifer (C. L. Koch, 1839)	2					2	0.4
Trachyzelotes pedestris (C. L. Koch, 1837)		2			1	3	0.6
Zelotes apricorum (C. L. Koch, 1876)	2					2	0.4
Zelotes electus (C. L. Koch, 1839)	1					1	0.2
Zelotes sp.		4	5	5	1	15	2.8
Zoridae							
Zora silvestris (Kulczyński, 1897)	1					1	0.2
Thomisidae							
Diaea dorsata (Fabricius, 1777)					1	1	0.2
Xysticus erraticus (Blackwall, 1834)			1			1	0.2
Xysticus kochi (Thorell, 1872)			1	1		2	0.4
Xysticus sp.	1			1		2	0.4
Salticidae							
Phlegra fasciata (Hahn, 1826)		1		1	1	3	0.6
Total number of individuals	105	124	84	116	107	536	

Table 2. *Diversity indices of spider assemblages in garden*

	wood	thuja	lawn	orchard	crop garden	Whole garden
Taxa_S	43	44	30	31	26	99
Individuals	105	124	84	116	107	536
Shannon (H´)	3. 36	3.37	3.13	2.89	2.82	3.99
Margalef (R)	9.03	8.92	6.55	6.31	5.35	15.74
Equitability (e)	0.89	0.89	0.92	0.84	0.86	0.86

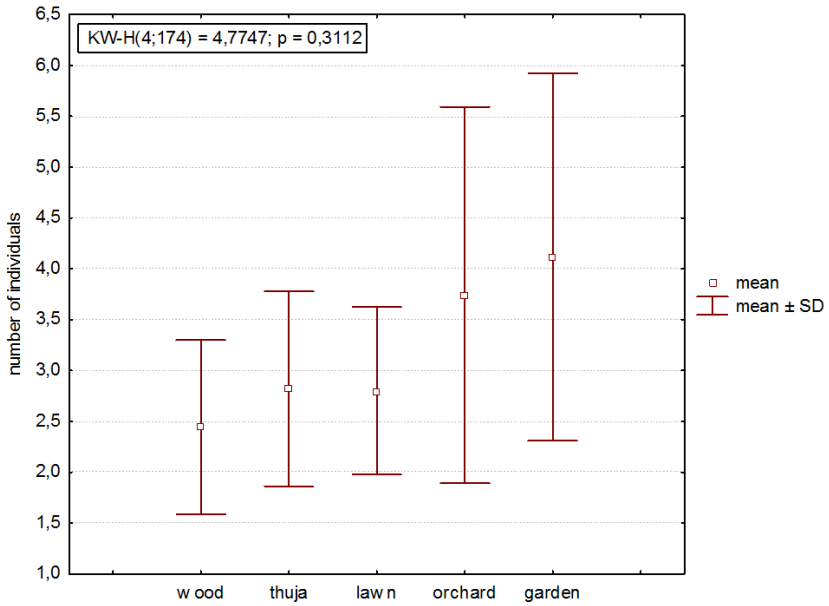

Figure 1. *Kruskal-Wallis test (ANOVA) of abundance among the spider communities in the microhabitats of garden*

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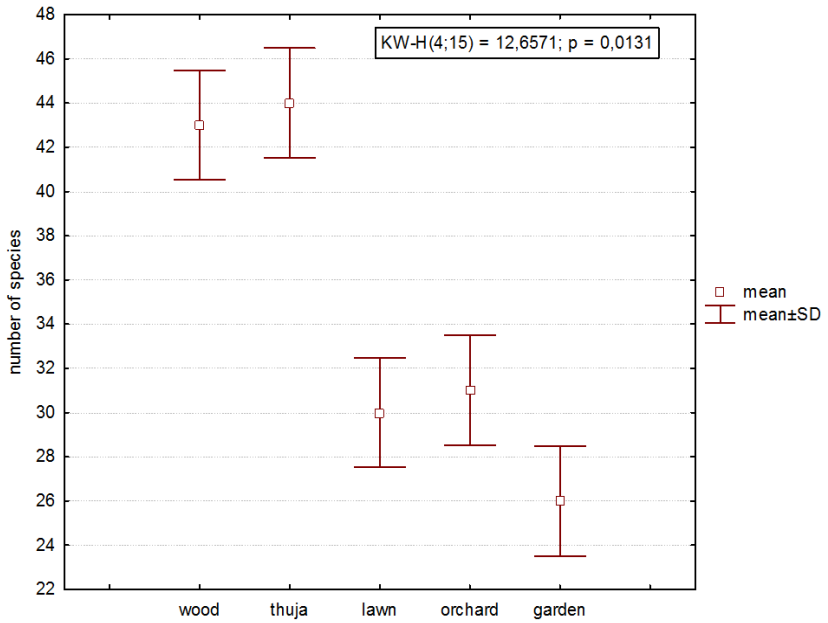

Figure 2. *Kruskal-Wallis test (ANOVA) of spider species spectrum in the garden microhabitats*

In the study site – stored wood we collected 105 individuals belonging to 43 species. Diversity indices were high (H´= 3.36), as well as species richness (R = 9.03) and equitability (e = 0.89) (*Tables 1 and 2*). *H. forskali*, *H. rubicunda*, *U. longispina* and juveniles from the genus *Lepthyphantes* (sensu lato) and *Pardosa* were dominant.

In the thujas site – was the spider assemblages the richest as in the number of individuals (124) as in the number of species (44). *P. opilionides*, *E. dentipalpis*, *D. concolor*, *M. trilobatus* and juveniles from the genus *Pardosa* and *Drassyllus* dominated there. Diversity, species richness and equitability were high at this study site (*Tables 1 and 2*).

Next study plots were grasslands, in which the human disturbance was higher. Mowed lawn in the recreational part of garden was regularly watered and maintained. In the recreational lawn we found 84 individuals only, belonging to 30 species. We noticed a co-dominance of *A. phalerata*, *A. cauta*, *L. angulatus*, *P. palustris*, *H. helveola*, *D. lapidosus* and juveniles from the gen. *Zelotes*, *Agelena*, *Pholcus* and *Pardosa* here; so the spiders' community in the lawn reached the highest equitability. This spider coenose in garden was absolutely dissimilar to others (*Figures 3 and 4*).

Second studied lawn in orchard was in the cultivated part of garden, but it was rarely taken care of, there were lower human intervention. In the grassland of orchard, we trapped 116 individuals belonging to 31 species (*Tables 1 and 2*), in which *E. dentipalpis* highly predominated; *O. apicatus*, *P. hortensis*, *C. gracilis*, *D. pusillus* and non-adults from the genus *Pardosa* were dominant spiders there.

Study site - the cultivated garden with planted crops was subject with the highest human disturbance all year round, in the form of loosening the soil, selective watering and early end of the growing season (after harvesting of the crops we did not collected any spiders there). All these facts resulted in lower number of species (26); there were the lowest values of diversity indices from all monitored communities of gardens (*Tables 1 and 2*). *P. hortensis* predominate at this study site, next species *E. dentipalpis*, *X. miniata*, *X. nemoralis*, *Z. rubidum* and *C. gracilis* were dominants.

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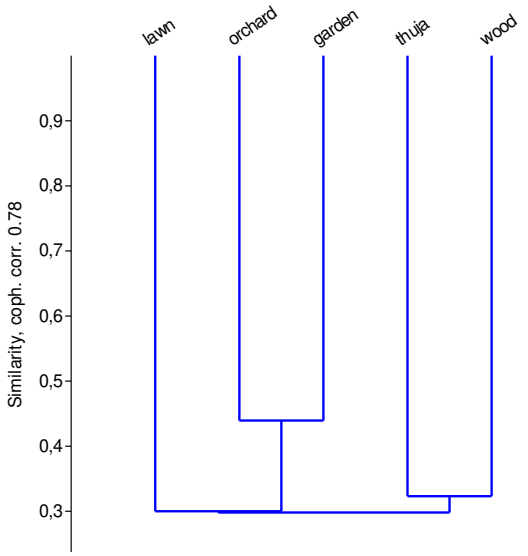

Figure 3. *Similarity of spider communities at five different microhabitats in garden (single linkage cluster analysis, Bray-Curtis)*

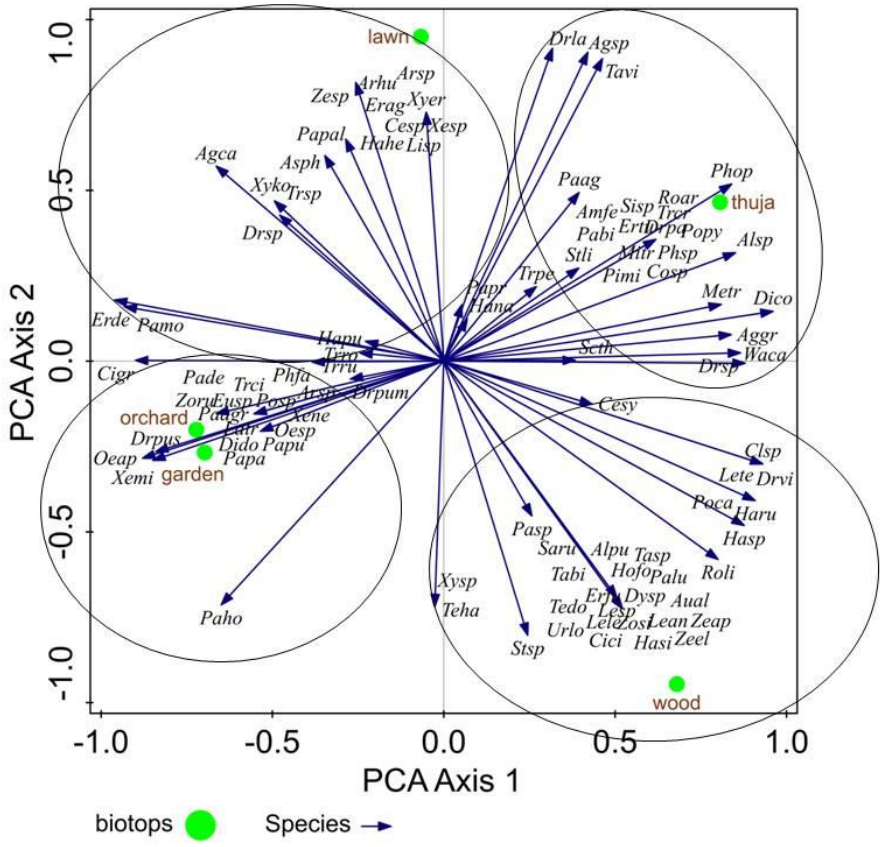

Figure 4. *PCA analysis of species distribution in the five different study sites of garden*

Cluster analysis revealed that the spider assemblages of garden habitats (single linkage cluster analysis, Bray-Curtis) were dissimilar (*Figure 3*; similarity level 0.3). A separate branch was created by two coenoses of dry and shaded habitats of the garden,

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under thujas and wood storage. Separate line represents the coenose of mowed lawn in recreational part of garden. Spiders in orchard and garden with crops, which preferred higher growth of plants, create more similar group.

If we evaluate the spiders of the garden as a whole, we can state that species with high demands on light conditions (direct light) accounted for 42%, with the largest number being in the garden with crops. The number of spiders with shading requirement the habitat was almost the same (41%), most of sciophilous individuals gathered in the recreational part of the garden, on the mown lawn, under wood and thujas. The demands of the spiders in terms of humidity conditions were as follows - xerophilous (42%), hemixerophilous (14%) and hemihygrophilous - 20%; while most xerophilous individuals were in the garden with crops, spiders with higher requirements on moisture were in the grassland of orchard.

Impact of human activities vs. specific micro-habitats in garden

Multivariate analysis of spiders at five garden micro-habitats was determined by Principal Component Analysis (PCA, SD = 3 was on the first ordination axis). The values of the explained cumulative variability of species data were 43.79% on the 1st ordination axis and 67.51% on the 2nd ordination axis. The ordination graph (biplot) contains species ordered into four clusters (*Fig. 4*).

Spider assemblages were characterized by certain number of specific spiders, which were found in the certain habitats, only. Under the wood we found 19 specific species, occurred only in this place; under thujas we recorded 12 specific spider species. Spider assemblages in moved lawn and orchard had 8 specific species each (*Table 1*). The lowest specific species that would characterize the habitat a microclimatic condition, we found in the garden with crops, four only. The garden as the urban environment is a space altered by anthropic activities with varying degrees of disturbance. Due to the highest human interventions in garden with crops, orchard or on the moved lawn we found there a lower species spectrum and lower number of specific spiders. While in undisturbed places (under thuja and stored wood) we found many specific species and the diversity was higher (*Figs. 2 and 4*). We can conclude that on a relatively small gardens´ area there was a diverse mosaic of micro-habitats have created suitable conditions for different types of arachnocoenoses, but these assemblages have been strongly influenced by human activities. The gardens seem to be quite an interesting space for the existence of many spiders with different requirements for a successful life.

Conditions and places for adaptation of a new spider species

Gardens seem to be an appropriate habitat (small place with many microclimatic conditions) for colonisation by aliens or newly established species. Here we found the adult spider individuals of both sexes, so far found as juveniles only in botanical garden. Significant research results include the findings of the following species - *Tallusia vindobonensis* (Kulczyński, 1898), we found two males under the thujas and in the lawn, this is a second record for Slovakia. Gajdoš et al. (2019) found this species in Pannonic salt marshes. Next interesting species was *Hoplopholcus forskali* (Thorell, 1871) - juveniles we collected in the interior of the house, males and females under thujas; this is the second record for Slovakia (so far juveniles were only found in the botanical garden). Males of *Mermessus trilobatus* (Emerton, 1882) we found under thujas; it is a second record for Slovakia (it was found only in the botanical garden by Šestáková et al. 2017), this North American species spreads to Central Europe via

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Germany. The first record for Slovak fauna was *Tegenaria hasperi* (Chyzer, 1897) from the family Gnaphosidae, males of which we collected under thujas (males were after mating).

Gardens can create very suitable conditions for the adaptation of non-original, resp. alien spider species as well. In studied garden we found a very high diversity, interesting records of species or spiders successfully adapted to urban conditions.

Discussion

Urban gardens are popular green spaces that have the potential to provide essential ecosystem services, support human well-being, and at the same time foster biodiversity in cities (Tresch et al., 2019). As species have different responses to anthropogenic habitat modification, the species composition of urban areas can depend greatly on the habitat characteristics of the local and surrounding areas (Lowe et al., 2017). We suppose the results of our study of the private gardens with five various micro-habitats are original and indicate creation the appropriate conditions for a high diversity of ground-dwelling spiders; we found there nearly one hundred of taxa. Species composition differed significantly between garden micro-habitats. The higher spider species diversity of gardens was also associated with specific soil cover (thujas, stored wood) as well as with lower disturbance on these study sites (orchard, thujas and wood), which suggests that local management had an impact on biodiversity. In contrast, Lowe et al. (2017) concluded that gardens are not as diverse as the surrounding vegetation areas with increased vegetation cover.

This study shows that using urban land in the form of private gardens support unique spider communities and the maintenance of this form of management in the urban matrix, it is necessary to support it in the cities. Differences in the composition of communities between types of land use have been caused by a small number of tolerant spider species, and guilds showed different responses to habitat characteristics such as vegetation cover and human activities (plowing the soil, planting, watering and selective intervention at harvest crops). Alaruikka et al. (2002) in Finland confirmed the significant influence of the locality. In Slovakia, we noticed a very high species diversity of spiders in the researched garden, although the abundance was not so high. Selected microhabitats offered suitable and appropriate specific living conditions. One quarter of collected individuals were in the immature stage of development (pullus or subadults) identified at the genus level. Nevertheless, all together 74 species we confirmed there.

Urban areas encompass a wide range of ecosystems, include regions of high native biodiversity, and are inhabited by rare and threatened species. Public and private gardens often provide novel resources that might not otherwise exist in the urban landscape (Davies et al., 2009). Based on our research we confirmed the hypothesis - the gardens create conditions and offer space for specific species little known from the natural environment. Surprisingly, we found the presence of four non-native spider species, or two soil mite species - *Corynoppia kosarovi* and *Mesoplophora pulchra* (Krumpálová et al., 2020b) in the investigated gardens. We agree with the works of Loram et al. (2008) and Edmondson et al. (2014) that urban gardens are a major component of green spaces in many countries; they are heterogeneous in structure, but despite their relatively small size they provide critical habitat resources and increase the connectivity of urban landscapes (Soanes et al., 2019).

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In our study, most of spider species we found in relatively small numbers. We obtained similar results in the research of soil mites in the garden - most oribatids occurred in very small amounts (Krumpálová et al., 2020b), probably due to habitat requirements of species and their trophic supply, as well as human activities in the garden. Horváth et al. (2014) concluded a high share of rare species (singletons) as a common phenomenon in spider assemblages. On the basis of the studies, no generalization is possible about rarity patterns. The highest number of singleton species was in the suburban zone in Denmark (Horváth et al., 2014); in Hungary Magura et al. (2010) confirmed in the urban habitat eight species and Alaruikka et al. (2002) in the rural habitat in Finland 17 species. The number of singleton species was high in urban forest fragments in all countries. This could be an effect of the matrix - the trees become a fragment in the urban setting, with extensive surrounding matrix areas, which influences the faunal composition of the fragments as well (Lövei et al., 2006).

The hypothesis that a higher diversity of plants will have a positive effect on higher diversity of spiders (in contrast of recreational garden) was not confirmed. In the two microhabitats in planted garden we found lower number of species as in recreational parts. This fact may be a result of high disturbance and seasonal human activities.

The increasing disturbance hypothesis suggests that species richness monotonously decreases as the disturbance increasing (Gray, 1989). Magura et al. (2010) summarised that in disturbed, thinned urban park with increased ground and air temperature contained several favourable microhabitats for open-habitat species. Habitat management that does not modify considerably the habitat structure but rather mimics natural processes could serve both the demands of humans and the maintenance of the diversity of habitat-specific species. We can confirm and document these findings. After the complete harvesting of crops and the removal of vegetation cover in the managed garden, we did not catch the spiders. Disruptions of area in this range cause the disappearance epigeic individuals for some time, even in conditions of private garden. Thomas and Jepson (1997) said the same conclusions many years ago - farming operations result in major habitat-scale disturbance for spiders. Harvesting, plowing, pesticide spraying and forest clearcutting are likely to affect most micro-habitats within a given habitat; and they are known to cause severe reductions in spider populations.

Braschler et al. (2020) assumed besides larger public green spaces such as parks, urban forests and greenways, domestic urban gardens in aggregate constitute a considerable share of the overall urban area. Habitat provided by public and private urban green space has an increased importance in supporting populations of animal and plant species. For example, urban green space could play an important role in mitigating insect declines. However, the few published studies surveying the ground-dwelling invertebrate biodiversity of urban domestic and community gardens, reported considerable numbers of individuals and species in various invertebrate groups if data of multiple gardens were combined (Braschler et al., 2020).

Taken together, a sample of private domestic urban gardens represents a wide range of habitat types, with various degrees of management intensity and a huge range of naturalness (abundance of native plant species, presence of wildlife friendly features such as dead wood or stone piles, extensive management of grassland, bushes and hedges). Thus, a sample of private domestic urban gardens offers niches for numerous species with very different requirements (Braschler et al., 2020). Recognizing the value of small spaces and unconventional habitats for native species, and the potential for creative conservation opportunities, opens up new avenues for managers in urban

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environments and will lead to better conservation outcomes. Our study suggests that private gardens (as part of the green infrastructure of an urban environment) increase diversity in terms of species richness and are important for regional diversity. Urban planners should consider the value of the biological mosaic of highly variable home gardens and upgrade their biodiversity.

Conclusions

The garden is a very important refuge of the urban environment, it is a significantly altered habitat, disturbed by man, yet it forms an interesting and diverse ecosystem. The presence of 99 taxa has been identified in the course of the research of private gardens' microhabitats in Slovakia. Taxonomic spectrum of families is dominated by Linyphiidae (25), followed by Lycosidae (20), Gnaphosidae (13), Agelenidae (8), Theridiidae (6), Hahnidae and Thomisidae (4 species on each), Pholcidae and Dysderidae (3 species on each) the remaining 11 families were represented by only one (two) species.

Different degree of disturbance and different microhabitat conditions (man-made) significantly affected the species diversity of coenoses and through PCA analysis we confirmed relatively large differences in the structure of araneocoenoses in studied microhabitats. The highest degree of disturbance was in the garden with crops (lowest values of diversity indices), so we confirmed the hypothesis that soil management will reduce the species diversity in garden.

The garden is also a refuge for many euryvalent and synanthropic species (e.g. *Scytodes thoracica*) and creates a potential space for the spread and acclimatization of non-native species (e.g. *H. forskali*, *M. trilobatus*, *T. vindobonensis*, or *T. hasperi*).

The results of this research, specifically the research of spiders in the private garden of a family house among various microhabitats, are unique and the first of its kind not only in Slovakia but also in Central Europe. We consider it important to continue research and monitoring of interesting populations of spiders in gardens.

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POTENTIAL HAZARDS OF AN INORGANIC FERTILIZER (WEATFERT) FOR THE BROWN GARDEN SNAIL (*EOBANIA VERMICULATA* MÜLLER, 1774): GROWTH, HISTOLOGICAL AND BIOCHEMICAL CHANGES AND BIOMARKERS

ATTIA, L.[1] – TINE, S.[1,2] – TINE-DJEBBAR, F.[1,2*] – SOLTANI, N.[2]

[1]*Laboratory of Water and Environment, University of Larbi Tébessi, Tébessa, Algeria*

[2]*Laboratory of Applied Animal Biology, University of Badji Mokhtar, Annaba, Algeria*

**Corresponding author*
e-mail: fouzia.djebbar@univ-tebessa.dz; phone: +213-66-355-7273

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Abstract. Land snails are often used to monitor soil pollution. The present study aimed to investigate the effects of Weatfert R complex mineral nitrogen, phosphorus, and potassium (N: P: K) fertilizer with high phosphorous content (NPK: 8/36/15 + SO_3:13) on *Eobania* (syn. Helix) *vermiculata* used as a bioindicator species. Moreover, the histological and biochemical changes in the digestive gland were examined and biomarkers of environmental stress (glutathione peroxidase and lactate dehydrogenase activity) measured. Results showed that the fertilizer tested has a negative impact on the growth rate of juvenile snails. They also revealed alterations in the structure of the digestive gland and changes in biomarker response values in the field, accompanied by significant correlations with bioconcentration. Meanwhile, the biochemical parameters showed varying results representing an increase in the protein rate and a decrease in lipid and carbohydrate rates after treatment with weatfertin. Overall, our study showed that the fertilizer tested could have potential hazards on E. vermiculata and this snail was a suitable sentinel organism and the selected biomarkers are efficient for soil assessment.
Keywords: *chemical fertilizers, bioindicator organism, growth rate, digestive gland, biochemical changes, biomarkers of oxidative stress*

Introduction

Increasing food production is the main concern of all countries, as world population is expected to grow to nearly 10 billion by 2050 (Gill and Garg, 1998). It has led to the application of a wide variety of chemicals, pesticides and inorganic fertilizers to agricultural land (Yahiaabadi et al., 2018). The usage of inorganic fertilizers has grown in various parts of the world (Lalthanzra and Ramanujam, 2010) and the impact of intensive use is seen not only in terms of the soil quality but also on the survival of soil organisms dwelling there in (Rai et al., 2014). In literature, some researchers have shown that chemical fertilizers are harmful for soil organisms, but on the contrary, others have found that they are beneficial for their food supply (Rai et al., 2014).

Soil is home to different life forms and a reservoir of all the agrochemicals (Yahiaabbadi et al., 2018). To test the effects of chemicals on soil organisms, several biological methods have been applied to nematodes, earthworms, collembolans, and snails (Druart et al., 2011). Mollusca are major part of the world fauna, and the second-largest phylum of the animal kingdom. The terrestrial Gastropoda is one of the most diverse groups of animals, both in shape and habit (Sallam and El-Wakeil, 2012). These animals are of great importance not only due

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to their food value but also for their use in road construction, lime production, preparation of feed for poultry, and finally they indirectly contribute to organic matter decomposition (Bhowmik, 2005).

Several studies have been carried out to determine their activity, especially fertilizers that reach water streams during agricultural activities and may kill snails (Ragab and Showrky, 2006; Ismail, 2009) or make their environmental conditions unsuitable for their life (El-Deeb, 2007). The toxicity endpoints of most of the bioassays are survival, growth, and reproduction (Roh et al., 2010).

Land snails have also been widely used as a sentinel species for the assessment of pollution in terrestrial ecosystems (Douafer et al., 2020). They accumulate various contaminants in their soft tissues, especially the digestive gland, as well as they are helpful species in monitoring the exposure to trace metals, agrochemicals, urban pollution and electromagnetic agents (Regoli et al., 2006). The impact on cellular and biochemical alterations of digestive glands as biomarkers of toxicants have been investigated (Suyman et al., 2006).

Eobania vermiculata (Müller, 1774), the brown garden snail, belongs to the family of Helicidae and is distributed worldwide especially in the Mediterranean area (Radwan et al., 2008). Commonly used as test organism in ecotoxicology studies, it presents several advantages, since it is relatively easy to adapt and maintain in the laboratory conditions, and its physiology is well known (Yousef, 2011).

Terrestrial snails are well known for their capacities to accumulate different classes of chemicals in their tissues, particularly, the hepatopancreas (digestive gland) (Regoli et al., 2006). The possible use of cellular alterations on the gastropods' hepato-pancreas as biomarkers for the exposure to xenobiotics have been investigated (Radwan et al., 2008). The digestive gland is the key organ of chemicals detoxification.

The biochemical responses in organisms exposed to toxic contaminants have been used as biomarkers (Abd-El Azeem and Sheir, 2018). These biomarkers measure the interaction between a biological system and an environmental agent, which might be chemical, physical, or biological (WHO, 1993). In vivo, their inhibition or their induction is a good environmental tool to assess the exposure and the effects of xenobiotics on organisms (Porte et al., 2005).

The use of oxidative stress biomarkers is of potential interest for the assessment of the pollutants impact (Versclar et al., 2008). Moreover, the interaction between xenobiotics and the components of the antioxidant defense systems play an important role in the ecotoxicological response of an organism to its environment (Regoli et al., 2006).

This study was designed to measure the effects during a chronic exposure (3 months) of an inorganic fertilizer with high phosphorus content (Weatfert^{R}) on survival and growth rates of *E. vermiculata.* The main biochemical components (proteins, carbohydrates and lipids) and the biomarker responses biomarkers were also determined in the digestive gland of the snails. In addition, the histological alterations of the digestive gland due to fertilizer accumulation were examined.

Materials and methods

Snail's collection and maintenance

E. vermiculata was collected from untreated area in Bekkaria (35° 22′ 20″ N; 8° 14′ 32″ E) (Tebessa, Northeast Algeria) in autumn and winter of 2017-2018. The collection

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was always made manually. The collected snails (1.90 ± 0.029 mm in the shell diameter and 3.43 ± 0.04 g in body weight) were transferred in glass boxes (20 ×20 ×20 cm) with 5 cm layer of moistened soil at the bottom. They were identified according to the key of (Godan, 1983). The animals were fed with lettuce leaves and the food was renewed on alternate days. The snails were acclimatized for 2 weeks under laboratory conditions (16 ± 1 °C; 31%) relative humidity and a 18:6 h light-dark cycle before the beginning of experiments (Gomot, 1994).

Chemicals and treatment

Weatfert (Profert, Bejaia, Algeria) is a trade name of an inorganic fertilizer. It contains a mixture of the three principal nutrients (8% N + 36% P_2O_5 + 15% K_2O) + 13%SO_3. Thereafter, 16 snails were added to each box and the sets were subdivided as follows after 14 days of adaptation; three boxes were applied for a control test. Six boxes were treated with two doses of Weatfert, (i) the first treatment with a recommended agricultural dose (D_1 = 500 mg/400 cm^2) normally used by farmers (1.5 quintals per hectare) and (ii) the second treatment with a recommended agricultural dose x2 (D_2 = 1000 mg/400 cm^2). The amount of fertilizer required was determined by the total area of the experimental box (400 cm^2). These were added to the soil surface and then mixed thoroughly with enough water to ensure a homogeneous mixture. For the control treatment, distilled water was used. Treatment was done for 3 months. The tested fertilizer was replaced weekly using the same concentration.

Determination of growth inhibition

Weight growth is assessed weekly by weighing the snails with an analytical balance (Ohaus® Analytical, Switzerland). The inhibition percentage of average weight (W_{ip}) (ISO 15952) (*Eq. 1*) is calculated to compare the average weight of the groups treated with that of the control group.

$$Wip\ of group\ Gx = \frac{(WTn - WT0) - (WGn - WG0)}{(WTn - WT0)} \times 100 \quad \text{(Eq.1)}$$

where:
W_{ip} inhibition percentage of average weight
Group G_X represents the groups of treated snails G_1, G_2
W_{Tn} is the mass of the snails in the control group at time t = n weeks
W_{T0} is the mass of control group snails at the start of the experiment
W_{Gn} is the mass of snails in the G_X group at time t = n weeks
W_{G0} is the mass of G_X group snails at the start of experience

Biochemical composition of digestive glands

Digestive glands from control and treated snails were dissected out at different times (0, 1, 2 and 3 months). The main biochemical components (proteins, carbohydrates and lipids) were extracted following the procedure of Shibko et al. (1966). Fragments (100 mg) of the digestive gland were extracted in 1 mL of TCA (20%). In brief, quantification of proteins was carried following the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (1976) with bovine serum albumin as a standard. The absorbance was measured at 595 nm. Carbohydrates were determined as described by Duchateau and Florkin (1959) using anthrone as reagent

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and glucose as standard. Lipids were measured by the vanillin method of Goldsworthy et al. (1972). Data were expressed in µg per mg of fresh tissue and assays conducted with three replicates per treatment each containing 100 mg of digestive gland fragment.

Biomarker assays

Two doses (500 and 1000 mg/400 cm^2) of Weatfert were orally applied on *E vermiculata* and its effects examined on LDH and GPx activities measured at various times (0, 1, 2 and 3 months during treatment. GPx activity was determined according to the method of Flohe and Gunzler (1984). Fragments of digestive gland (100-200 mg) were homogenized in 1 ml of phosphate buffer (pH 7.8). The homogenate was centrifuged (3000 rpm for 10 min) and then the supernatant recovered for use as enzyme source. The assay was performed with 200 µl of supernatant added to 400 µl of GSH solution (0.2 mM, pH 10). Absorbance reading was done after 5 min at 412 nm. The LDH assay was based on the conversion of lactate to pyruvate or pyruvate to lactate. The lactate dehydrogenase activity (LDH) was spectrophotometrically measured according to the method of Hill and Lévi (1954) as previously described (Sifi and Soltani, 2019). It uses NAD (nicotinamide adenine dinucleotide) as substrate. Fragments of digestive gland (100-200 mg) were homogenized in 1 ml of Tris/HCl (0.1 M, pH 7.2). The homogenate was centrifuged (3000 rpm for 5 min) and then the supernatant recovered for use as enzyme source. The assay was performed with 50 µl of supernatant added to 675 µl of substrate buffer (0.2 M, pH 10) and 50 µl of NAD solution. The absorbance reading was done every minute for 5 min at 340 nm. The protein content was evaluated according to Bradford (1976) using bovine serum albumin as standard (BSA, Sigma). The activity was expressed as µM/min/mg protein.

Histological procedure

The histological procedure was performed according to the method of Gabe (1968). In brief, 16 snails from each control and treated series (D_1 and D_2) were randomly collected (at two periods: 0 day and 3 months) and the digestive glands were dissected. Each whole digestive gland was fixed in formalin solutions (30%) for 24 h, and dehydrated in baths of alcohol with increasing concentrations (70, 80, 90 and 95%). Then, specimens were cleared in xylene and embedded in paraffin wax. Serial sections (4 µm) were prepared by a Leitz microtome and stained using the hematoxylin and eosin. Sections were then mounted and covered with glass cover. Histological sections were examined under the light microscope (Leica DM LB2).

Statistics

Data are presented as a mean value ± SEM (standard error mean) in each treatment group. The normality of data was verified using the Kolmogorov-Smirnov test, and the homogeneity of variances was checked by Levene's test. Comparison of the experimental groups was tested by analysis of variance (ANOVA), and means were tested for statistical significance by a post hoc Tukey's honestly significant difference test. The statistical tests were performed using GraphPad Prism, version 7.00 (GraphPad Software, San Diego, CA, USA), where $p < 0.05$ indicates a statistically significant difference.

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Results and discussion

Effects on growth of snails

The growth of juvenile snails is noted monthly during the treatment period (3 months). The tested fertilizer resulted in an increase of growth inhibition (%) of snails without dose-response relationship and a decrease during the tested time. The growth of these snails is inhibited after treatment with two doses respectively, and displays percentages ranging from 69 and 78% for the first month, from 55 to 61% for the second month and from 49 to 54% for the third month (*Fig. 1*).

The use of change in body mass as a biomarker is ecologically relevant, as high body mass losses are believed to have negative effects on survival and reproduction (Dittbrenner, 2010). Treatments with three types of NPK fertilizers (high nitrogen, high phosphorus and balanced) (El-Deeb, 2017) or with urea product (Ragab and Shoukry, 2006) were found to influence snail growth. Meanwhile, Abdel Hamid et al. (1998) reported that urea and ammonium nitrate reduced the growth of juvenile *B. alexandrina* snails. Schuytema et al. (1994) reported a significant reduction in weight and shell diameter of *Helix aspersa* snails treated with Aminocarb, Methyl parathion and Paraquat; the responses observed varied according to the type of pesticides and dose administered. Furthermore, the overall results seem to indicate that the reduction in growth is linked to the mode (Sallama et al., 2005; Radwan et al., 2008) and to the duration of treatment exposure (Coeurdassier et al., 1957). Similarly, growth of snails (*Helix aspersa*) exposed to pesticides (thiamethoxam and tefluthrin) was affected (Ait-Hamlet, 2012). Afomezie et al. (2011) found that NPK soil treatment affects snail's performance by reducing their final weight and weight gain. Bhattacharya and Sahu (2016) showed clearly that the recommended dose of NPK did not cause earthworm mortality.

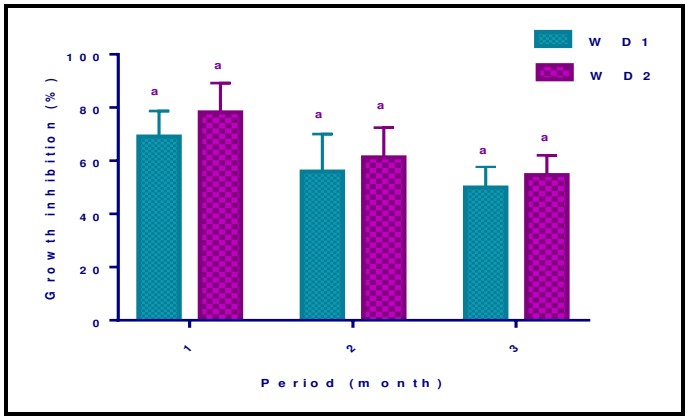

Figure 1. *Effects of fertilizer administrated at two doses on growth inhibition (%) in juvenile snails of E. vermiculata (mean ± SEM, n = 5 repeats, each containing 10 individuals): Same lowercase letters indicate no-significant differences at the same time based on T student test (p > 0.05)*

Effect on biochemical composition of digestive glands

The fertilizer Weatfert was applied to juveniles of *E. vermiculata* during 3 months. Its effects were evaluated on the main biochemical components (carbohydrates, lipids,

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proteins) of digestive glands at different times (0, 1, 2 and 3 months) after treatment (*Table 1*).

Results of the protein amounts showed a significant increase in treated series with the highest dose (dose 2) at the first ($p = 0.007$) and the third month ($p = 0.008$) as compared to control series. At the second month, the two applied doses induced a significant increase (control vs dose 1: $p = 0.032$; control vs dose 2: $p = 0.013$) of this component, without dose-response relationship.

Table 1. *Effects of Weatfert on protein, lipid and carbohydrates rates in digestive gland (µg/mg of fresh tissue; n = 3 pools each containing 100 mg of fresh tissue) at different periods during treatment in the juveniles of E. vermiculata (mean ± SEM)*

Components	Times (month)	Control	Dose 1	Dose 2
Proteins	0	57.73 ± 3.27a, A	56.07 ± 3.27a, A	65.78 ± 4.19a, A
	1	59.25 ± 3.55a, A	72.91 ± 5.00a, B	88.64 ± 7.12b, B
	2	69.25 ± 2.48a, A	87.46 ± 5.61b, C	91.57 ± 5.84b, B
	3	89.96 ± 11.31a, B	97.25 ± 8.20a, C	130.66 ± 1.85b, C
Lipids	0	36.69 ± 3.31a, A	36.33 ± 1.17a, A	36.11 ± 3.16a, A
	1	38.28 ± 1.77a, A	27.88 ± 1.24b, B	19.84 ± 2.46c, B
	2	39.61 ± 1.49a, A	27.00 ± 2.57b, B	19.39 ± 1.32c, B
	3	57.73 ± 1.78a, B	18.57 ± 2.28b, C	14.65 ± 0.70b, B
Carbohydrates	0	61.28 ± 1.49a, A	63.40 ± 1.12a, A	63.81 ± 1.83a, A
	1	61.74 ± 1.88 a, A	59.31 ± 2.29a, A	52.61 ± 3.57a, B
	2	64.02 ± 1.79a, A	57.92 ± 0.89a, A	40.81 ± 3.41b, C
	3	74.28 ± 0.60a, B	48.94 ± 4.49b, B	22.92 ± 1.18c, D

Mean values followed by different lowercase letter indicate significant differences between control and treated series

Mean values followed by different capital letter indicate significant differences between periods based on Tukey's HSD test at $p < 0.05$

Concerning the lipid rate, a significant reduction was observed in the first (control vs dose 1: $p = 0.005$; control vs dose 2: $p < 0.001$; dose 1 vs dose 2: $p = 0.0184$), the second (control vs dose 1: $p = 0.0019$; control vs dose 2: < 0.001; dose 1 vs dose 2: $p = 0.0218$) and the third month (control vs dose 1: $p < 0.001$; control vs dose 2: $p < 0.001$) in the treated series with the two tested doses compared to controls.

Finally, our results also revealed a significant decrease in carbohydrates amounts with the highest dose at the first month (control vs dose 2: $p = 0.0496$), the second (control vs dose 2: $p = 0.0002$; dose 1 vs dose 2: $p = 0.001$), and the third month (control vs dose 1: $p = 0.0003$; control vs dose 2: $p < 0.0001$; dose 1 vs dose 2: $p = 0.0003$) as compared to control series (*Table 1*).

Biochemical and enzymatic parameters in organisms exposed to toxic contaminants have been used as biomarkers and may be considered an important diagnostic tool to assess the exposure and effects of xenobiotics (Mclouglin et al., 2008). The increasing demands of organisms to energy during stress to detoxify, bio-transform and excrete the toxicants is achieved by the use of carbohydrate as the principal and immediate energy source (Umminger, 1977).

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Glycogen breakdown and increased glucose are among the well-known mechanisms to provide energy in mollusks in stressful environments (Tunholi et al., 2017). The energy is used to cope with the cytotoxicity induced by pesticides. In our research, the tested fertilizer, weatfert caused a significant reduction in the energy reserves as evidenced by a reduction in carbohydrate rates. In this regard reported a decrease in tissue glycogen levels and an increase in hemolymph glucose concentration in the freshwater snails (*Bulinus truncates*) exposed to glyphosate herbicide (Bakry et al., 2012). Similarly, the reduction of tissue glycogen has been reported in the mud snails (*Amphibola crenata*) exposed to waterborne cadmium (De Silva et al., 2018). The results of Mohammadein et al. (2013) and Radwan et al. (2008) disagree with our results, who found a significant decrease in the glycogen content of the digestive gland in land snails treated with heavy metal and chemicals (methomyl and methiocarb), respectively. This may result from an increased rate of glycogen breakdown (glycogenolysis). Moreover the decrease in glycogen content may have an indirect effect on the protein and lipid reserves in *E. vermiculata*. The results of Banaee and Taheri (2019) indicated significantly lower levels of glycogen and total antioxidant in the cells in the freshwater snail, *Galba truncatula* exposed to sewage compared to controls.

Proteins are one of the major groups of biological materials comprising the chief nitrogenous elements of the body tissues. The concentration of free amino acids in mollusk varies with pollution levels (Bishop, 1983). Proteins are mainly involved in the architecture of the cell (Radwan et al., 2008). During chronic periods of stress, the snails needed more energy to detoxify the toxicants and to overcome the induced stress (El-Shenawy et al., 2012) especially when they have a limited amount of carbohydrates and lipids (Radwan et al., 2008). Our experiment showed a significant increase in protein ratesin *E. vermiculata* treated with Weatfert fertilizer. The results of the study of Khalil (2016) suggest that the exposure to sublethal doses of pesticide, methomyl-lannate may influence total protein metabolism. Previously, Bakry et al. (2011) reported that two pesticides, deltamethrin and malathion had qualitative and quantitative effect on the protein patterns of *Helisoma duryi* snails. Furthermore, the electrophoretic profil of total proteins from *Biomphalaria alexandrina* snails treated with diazinon and profenfos showed less number of protein bands, indicating that the pesticides were thought to induce damage in these snails (Bakry et al., 2013). Padmaja and Rao (1994) suggested that the decrease in snail proteins treated with pesticides could be due to several mechanisms namely (a) the formation of lipoproteins which are utilized for the repair of damaged cells and tissue organelles, and (b) the direct utilization by cells for energy requirements (Radwan et al., 2008).

Lipids play extremely important roles in the normal function of a cell. They not only serve as highly reduced storage form of energy, but they also play an intimate role in the structure of cell membranes and the organelles found in the cell (Kandil et al., 2009). Indeed, lipids are the preferred energy fuel offered to tissues when needed after carbohydrates. During periods of chronic stress, they also constitute another source of energy (Moussard, 1999). When snails have a limited amount of carbohydrates and fat, the alternative energy source to meet the increased demand for energy is protein (Moussard, 1999). A decrease in total lipids was noted in *E. vermiculata* snails treated with Weatfert fertilizer compared to control snails. In addition, these results are similar to those of Aït Hamlet et al. (2012) showed that the Thiamethoxam causes a disturbance in the lipids content in the digestive gland of treated snails compared with controls.

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Moreover, depletion of some long chain and short chain fatty acids may be explained on the basis that reduction in rates of glucose metabolism in the snails was balanced through the stimulation of triglyceride hydrolysis and fatty acid oxidation (Ait-Hamlet et al., 2012).

Effects on biomarkers responses

Results of the specific activities of antioxidant defense enzyme (GPx and LDH) in *E. vermiculata* exposed to two doses of fertilizer are shown in *Figures 2* and *3*. The results showed a significant increase in GPx activity at the first ($p < 0.001$), the second ($p < 0.001$) and the third month ($p < 0.001$) respectively as compared to control series, with dose-response relationship at the first (dose 1 vs dose 2: $p = 0.016$) and the third month (dose 1 vs dose 2: $p = 0.006$) (*Fig. 2*).

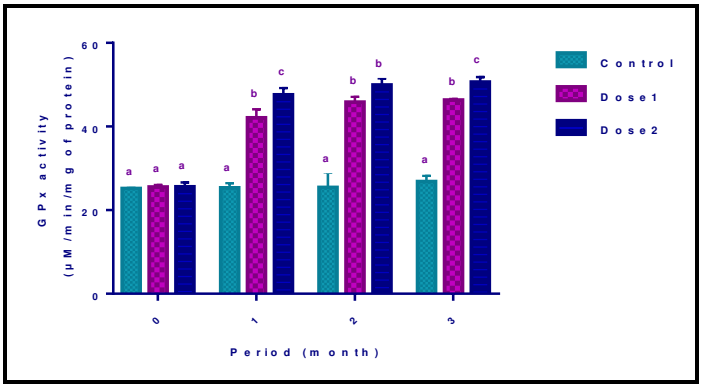

Figure 2. *Effect of Weatfert on LDH activities (µM/min/mg of proteins) in the juvenile snails of E. vermiculata at different times during treatment (mean ± SEM, n = 3). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test ($p < 0.05$)*

Finally, our results also showed a significant increase in the activity of LDH in the treated series (dose 1 and dose 2) at all tested periods: the first (control vs dose 1: $p = 0.0031$; control vs dose 2: $p < 0.001$), the second (control vs dose 1: $p = 0.0015$; control vs dose 2: $p < 0.001$) and the third month ($p < 0.001$) as compared to control series respectively (*Fig. 3*). In addition, we note a significant difference between the two tested doses only at the first month (dose 1 vs dose 2: $p = 0.036$).

Biomarkers are essential for ecosystem health assessment and management (Boyd, 2010). The study of the antioxidant defense system is increased because of its potential utility to provide biochemical biomarkers that can be used in environmental monitoring systems (Ballesteros, 2009).

Glutathione peroxidase (GPx) known as the most important peroxidase ensures the detoxification of peroxide and hydroperoxides to water and hydroxyl compounds, respectively (Pinto et al., 2003) so it play a protective role against oxidative stress (Van Der Oost et al., 1998; Van Der Oost et al., 2003).

Our results show a significant increase in GPx activity was found after both the first and second weeks in snails exposed to Weatfert fertilizer. These are in agreement with those of Farid et al. (2009) which reveal an increase of GPx in *Lymnaea natalensis* after treatment with niclosamide. Similar observations have been reported by El-Shennawy et al. (2012) who observed elevated GPx levels in snails exposed to cadmium, lead, copper

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and iron. This increase may be attributed to free radical production (Hermes et al., 2020) as shown by Orbea et al. (2000) and Radwan et al. (2010) on other snail species exposed to other pollutants. In contrast, a slight inhibition was observed in GPx activity with high phosphorus fertilizer treatment in *Biomphalaria alexandrina* snails (El-Deeb, 2017).

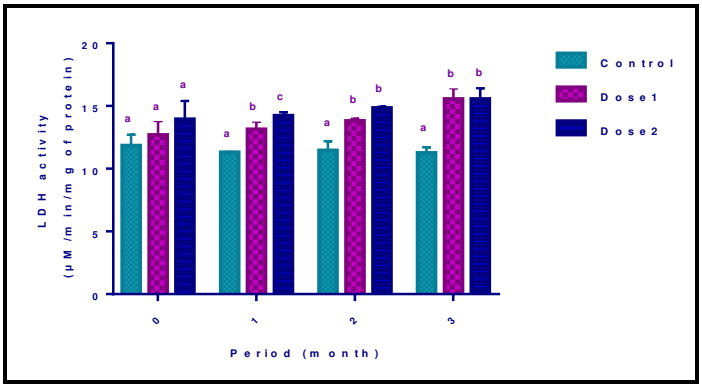

Figure 3. *Effect of Weatfert on GPx activities (µM/min/mg of proteins) in the juvenile snails of E. vermiculata at different times during treatment (mean ± SEM, n = 3). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test ($p < 0.05$)*

Lactate dehydrogenase (LDH) is an enzyme found in nearly all living cells (animals, plants and prokaryotes). Lactate dehydrogenase converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply and it performs the reverse reaction during the Cori cycle in the liver. Our results are in accordance with Banaee et al. (2019) found that there was a significant increase in the activity of LDH in *G. truncatula* exposed to cadmium and dimethoate. A reduction was shown in LDH acitvity in *B. alexandrina* treated with atrazine and Roundup (Bakey et al., 2012). In the study of Banaee and Taheri (2019) a significant increase was observed in the activity of LDH in the *G. truncatula* exposed to sewage, which could be a physiological response to the stressful environmental. Increased LDH activity in the soft tissues of snails could be attributed to hypoxia and increased anaerobic glycolysis. This enzyme is involved in the metabolism of carbohydrates in cells and plays a key role in maintaining the balance between the catabolism and anabolism of carbohydrates in mollusks (Chen et al., 2011). In a stressful environment, LDH converts pyruvate into lactate, which in turn leads to the enhanced concentration of carboxylic acid (lactic acid) in tissues and hemolymph. Therefore, the pH of tissues and hemolymph decreases and causes specific physiological changes (Tunholi et al., 2017). In a study by Abdel-Halim et al. (2013), the LDH activity was observed to increase, which indicate a physiological response to the hypoxia-induced stress following the exposure of *Helix aspersa* to heavy metals. In addition, a significant increase has been reported in the LDH activity in the land snails (*E. vermiculata*) exposed to methomyl (Khalil, 2016). A significant increase in the activity of LDH was observed in *Deroceras reticulatum* exposed to Caselio fertilizer (Abd-El Azeem and Sheir, 2018). LDH was chosen as a biomarker of the oxidative stress induced by the tested material (fertilizer, PMR). Increased oxidative stress biomarkers as lipid peroxidase in fertilizers polluted areas was documented by Yousef et al. (2017). Salama et al. (2005) recorded significant increase of the level of LDH in

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the land snail, *Helix aspersa* after exposure to several chemicals such as methomyl, carbofuran and chlorpyrifos. The increased release of LDH in mantle tissue is an indicative of cellular or membrane damage (Abd-El Azeem and Sheir, 2018).

Effect on digestive gland structure

Figure 4A, B and *C* represents the structure of digestive glands in control and treated snails after 3 months of exposition**.**

In control snails, the digestive gland epithelium presented a normal appearance at 0 day (*Fig. 4A*) and after 3 months (*Fig. 4B*) experimentation. The cells were readily distinguished, together with less abundant excretory and calcium cells.

In treated series, the examination of histological structure shows modifications. As shown in *Figure 4C* and *D,* the epithelium of snails treated presented a significant hyperplasia of the gland, with the presence of fat cells. In addition, no necrosis and cell congestion were observed.

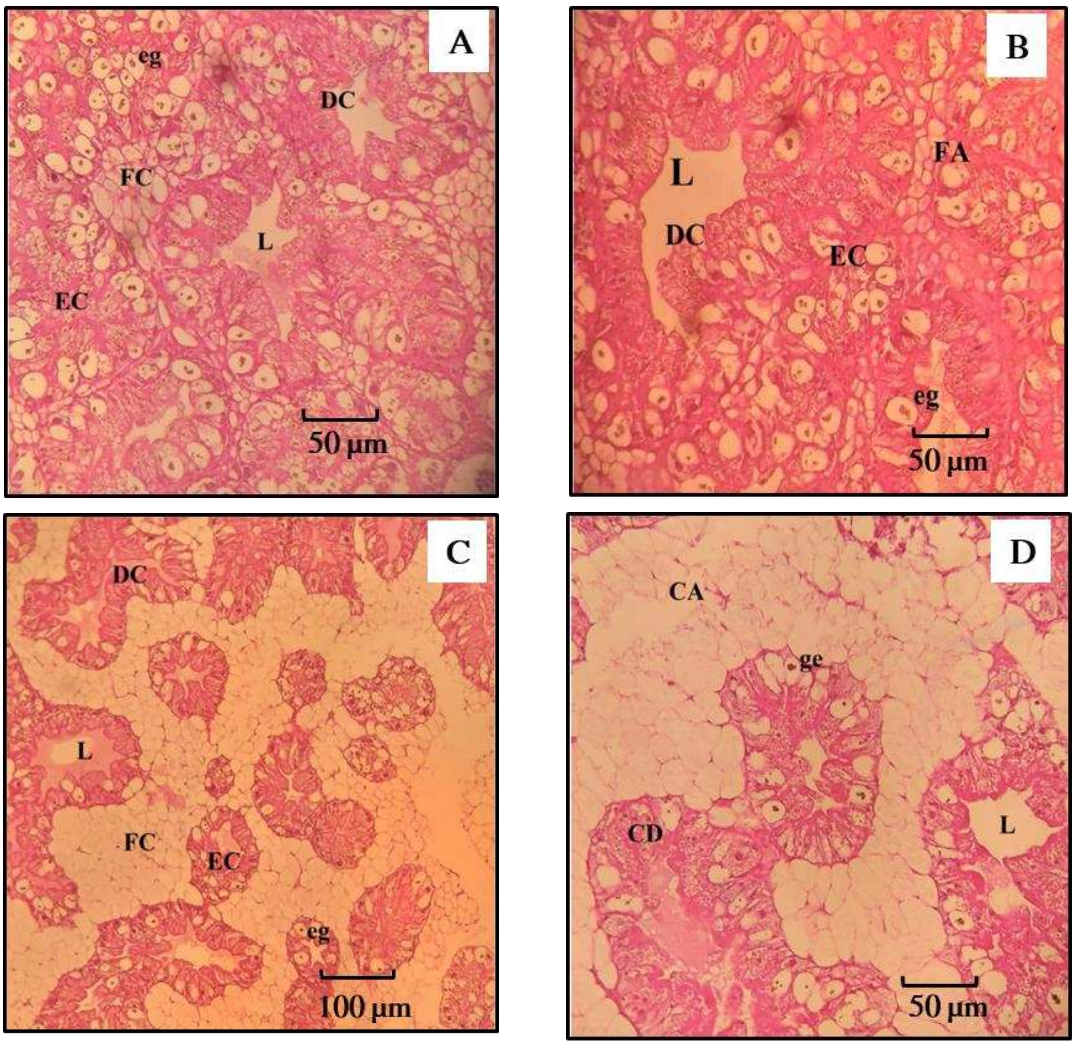

Figure 4. *Transverse sections of digestive glands: in control snails (0 day) (A), in control snails (3 months) (B), in treated snails (D1) (3 months) (C) and in treated snails (D2) (3 months) (D). Calcium Cell (CA); Digestive cell (DC); Excretory cell (EC); Excretory granules (eg); Fatty cell (FC); Tubular lumen (L)*

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Beside the role of fertilizers in the growth of the plant it has a molluscidal effect. Sheir (2015) found that Caselio (plant fertilizer) caused tissue damage in the digestive tubules as necrosis, fusion and increased lipofuscin pigment in the freshwater snail, *Lanistes carinatus*. El-Deeb et al. (2015) exposed *Biomphalaria alexandrina* snails to the inorganic fertilizers (high phosphorus and high nitrogen content) and recorded histological alterations in the digestive glands. Triebskorn and Ebert (1989) studied the effect of carbamate and metaldehyde molluscicides on the digestive tract of *Deroceras reticulatum*. He described that mucus deficiency in the digestive tract due to metaldehyde exposure. Dummee et al. (2012) exposed the golden apple snail, *Pomacea canaliculata* to contaminated sediment with the metals (Fe, Mn, Cu and Zn) for two months. The results showed metals accumulation in the digestive tract and digestive gland higher than in the foot muscles. Some of pathological signs were cellular degeneration, necrosis, inflammatory responses and leaky intestine (gaps) (Abd-El Azeem and Sheir, 2018). Hamed et al. (2007) found a severe vacuolization in digestive cells of *E. vermiculata* treated with molluscicidal carbamates; methiocarb and methomyl. However, the histological examination of the hepatopancreas of the treated snails showed alterations as a response to all the treatments, and revealed the degeneration of the digestive tubules and the breakdown of the basement membrane in a dose-dependent manner, leading to a severe deterioration of the tissues in the concentration of 200 mg/L thiametoxam (Ait-Hamlet et al., 2012). Hepatocyte necrosis, kidney failure, kidney cell necrosis, muscular dystrophy and anemia, heart disease could causes by an increase in LDH in snails treated with a combination of Pirimicarb and lead acetate (Chen et al., 2011).

Conclusion

The data obtained show significant changes in the growth, biochemical parameters and enzymatic activity of the soft tissues of *E. vermiculata* snail's exposed to a fertilizer (Weafert). The fertilizer tested led to the depletion of energy reserves (lipid and carbohydrate levels) and an increase of proteins in the digestive gland of snails. An activation of the detoxification system was evidenced by an increase in the activity of GPx and LDH in the digestive gland. Lastly, structural alterations were observed in digestive gland from treated snails.

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DYNAMICS OF SOIL NUTRIENTS AND STOICHIOMETRY IN MONGOLIAN SCOTS PINE (*PINUS SYLVESTRIS* VAR. *MONGOLICA)* PLANTATION, IN A SEMIARID AREA OF CHINA

KHAN, A.[1,2,3] – ZHANG, X.[2] – YANG, X.[2*] – ZHANG, K.[3*] – HAYAT, M.[3] – XU, S.[2] – MUHAMMAD, J.[4] – SAEED, S.[5] – SHAH, S.[5] – IQBAL, S.[3]

[1]*Department of Forestry, Shaheed Benazir Bhutto University, Sheringal, Dir (U), Pakistan*
(e-mail: alamgir.forester@gmail.com (Khan, A.))

[2]*Institute of Desertification Studies, Chinese Academy of Forestry, Beijing 100091, China*
(e-mail: zhangxiao978@caf.ac.cn (Zhang, X.), xsxkytjl@163.com (Xu, S.))

[3]*School of Soil and Water Conservation, Beijing Forestry University, Beijing 100083, China*
(e-mail: muhammadhayat66@gmail.com (Hayat, M.), sundasiqbal90@gmail.com (Iqbal, S.))

[4]*Department of Environmental Science, Shaheed Benazir Bhutto University, Sheringal, Dir (U), Pakistan*
(e-mail: juma@sbbu.edu.pk (Muhammad, J.))

[5]*Department of Forestry and Wildlife management, University of Haripur, Haripur 22620, Pakistan*
(e-mail: Sajjad_saeed222@yahoo.com (Saeed, S.), shah@uoh.edu.pk (Shah, S.))

[*]*Corresponding authors*
e-mail: yangxh@caf.ac.cn; phone: 86-10-62824087; fax: 86-10-62889007 (Yang, X.);
e-mail: ctccd@126.com; phone: 86-133-6665-5775 (Zhang, K.)

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Abstract. The aim of this study was to investigate about soil nutrients and stoichiometric ratio in Mongolian scots pine plantation forests. Our results showed that along 5 depth increments (0-20, 20-40, 40-60, 60-80, and 80-100 cm), the concentration and storage of total soil organic carbon (TSOC), total nitrogen (TN), and total phosphorous (TP) decrease. A significantly higher TSOC, TN, and TP concentration and storage was recorded at 0-20 cm, indicating that more C, N, and P were found in the upper soil profile. Similarly, the C:N and C: P stoichiometry and soil TSOC concentrations were significantly higher than soil N:P and TN and TP concentrations, indicating that coniferous plantation forests store more C compared to N and P. TSOC, TN, and TP ($p<0.01$) concentrations and storage were positively correlated at depth of 0-20 and 20-40 cm, showing a relatively constrained C:N:P ratio in this plantation forest. Furthermore, stand density, basal area, and total biomass carbon affected TSOC, TN, and TP concentration. Our findings provide key references to further research studies in Mongolian scots pine plantation regarding TSOC, TN, as well as TP and their variations and storage along other parameters (tree age, soil texture, ground flora, seasons etc.) at the Research Station of Liaoning Institute of Sand-fixation and Afforestation (42◦420 N, 122◦290 E, 220.67 m above sea level), Zhanggutai region, Liaoning Province, southeastern Horqin sandy region, China.
Keywords: *plantation forests, stand characteristics, depth increment, stoichiometric traits, Horqin sandy land*

Introduction

Soil nutrients play an important role in plant growth and development and are essential in vegetation succession, nutrient cycling and ecological management of terrestrial ecosystems (Li et al., 2017a), among which carbon (C) is the basic structural element of plant and contributes one-half of the plants biomass (Yang et al., 2007a,b; Kim et al., 2011;

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Sardans et al., 2011). Nitrogen (N) is an essential component of plants and the majority of which are completely dependent on soil N due to lacking of symbiotic bacteria that fix the N (Li et al., 2017a), while phosphorous (P) affects photosynthetic assimilation and dry mass in plants (Ågren et al., 2012) and represents the basic element of DNA, RNA and ATP. The lackage of these nutrient elements can limit the plants growth and development (Kim et al., 2011). The amount and concentration of these nutritional elements and their stoichiometry in plants reveal nutrient uptake, utilization ability and adaptation to the environment during different growth stages (Wright and Westoby, 2003; Yang and Luo, 2011).

In plantation forests, variation in nutrient concentrations with stand developmental stages have attracted attention due to their importance for the development of nutrient management practices (Sardans et al., 2011; Yang et al., 2014). So, the relationship between tree growing stages and soil nutrient concentrations based studies provide a basic information regarding management of plantation forests. Many studies have been conducted on variation and distribution of SOC, N, and P along the stand characteristics, and variation in these elements have been ascribed to the fact that the forest stand can potentially vary these nutrients through the amount and decomposition of litter, canopy composition, roots uptake and basal area (Yuan et al., 2013; Xia et al., 2015; Jiang et al., 2017). As an integrative approach that involves the study of different nutrientional elements in ecological relations and involves determining elemental engagements of living organisms (Li et al., 2017a), nutritional stoichiometry is affected by ecological conditions (elevation, preciptation, and temperature), plantation age and sampling time (Han et al., 2005; Sardans et al., 2011). It has been reported that SOC, N, and P are strongly correlated with the stand characteristics, soil texture and topography too (Jiang et al., 2017). Therefore, the determination of C, N, and P dynamics with stand characteristics and development and soil depth can facilitate the quantification of the pattern and distribution of C:N:P stoichoimetry in the plantation forest ecosystem. Furthermore, it is not clear whether C, N, and P concentrations and its stoichoimetric ratios in plants are controlled over time, stand characteristics and ecological factors in plantation forests.

In arid and semi-arid regions of China, currently much attention has been given on large scale afforestation and reforestation of degraded land, such as that related with "Grain for Green" and "Three Norths Shelter Forest Systems" Projects. Mongolian scots pine (*Pinus sylvestris* var. *mongolica*), only one recommended needle-leaved tree species, had been planted at large-scale in these programs for windbreak and sand stabilization in these regions (Zhang et al., 2019). Despite of the fact that Mongolian pine plantation in these regions has a great role in the combating desertification and controlling land degradation, this plantation forest ecosystem is also acting as carbon, nitrogen, and phosphorous sinks. There is a dire need to study the variation of nutrients in the study area, not only because of the fact that the average soil nutrients concentrations and storage are lower in semi-arid area of China than the global mean, resulting from low nutrients input due to water runoff and soil erosion (Cao and Chen, 2017; Zhang et al., 2017), but also because in semi-arid areas of China the growth of vegetation is restricted due to limited water resources (Chen et al., 2008; Deng et al., 2016). So far fewer information about the role of stand characteristics and depth increment on TSOC, TN, and TP budget in arid and semi-arid areas of China is available, especially in the study area. Thus, this study was conducted with the overall objectives of: 1) to estimate the changes in TSOC, TN, and TP concentration and storage along the depth increment; 2) to determine the TSOC, TN, and TP stoichiometry and their characteristics and nutrients limitations in soil; and, 3) to investigate the effect of stand characteristics on TSOC, TN, and TP concentration and their storage in the Scots Pine

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plantation forests. We hope that this study will be a baseline for further studies in monitoring and mapping of TSOC, TN, and TP in Mongolian Scots pine plantation forests.

Materials and methods

Description of the study area

The study was conducted in 2017 at the Research Station of Liaoning Institute of Sand-fixation and Afforestation (42°420 N, 122°290 E, 220.67 m above sea level), located in Zhanggutai region, Liaoning Province, southeastern Horqin sandy region, China (*Fig. 1*). The climate of the area is a typical temperate with continental monsoon, with the mean annual temperature of 7.7ºC (for 1954-2010) and mean annual precipitation of 474 mm that mostly (67%) occur during June-August (Song et al., 2016; Zhang et al., 2019). The area faces approximately three times more annual evaporation than the precipitation, with the annual frost-free period of 150-160 days. The geography is categorized by distribution of sand dunes with low-lying land affected by wind erosion. The key soil category is Aeolian sandy soil (89.4%). In 1954, an experimental based trial plantation of Mongolian Scots pine was introduced and planted in this region. Here a large number of Mongolian Scots pine plantation forests with different densities, diameter and age ars intersected by other woody plants like *Populus* L. *Pinus tabuliformis* Carr and *Ulmus pumila* L. Understory vegetation includes *Setaria viridis* (L.), *Cleistogenes squarrosa* (Trin.) Keng, *Eragrostis pilosa* (L.) *Setaria viridis*, *Elymus dahuricus* Turcz*, Geranium wilfordii* Maxim*, Lespedeza bicolor* and *Portulaca oleracea* L (Zhang et al., 2019).

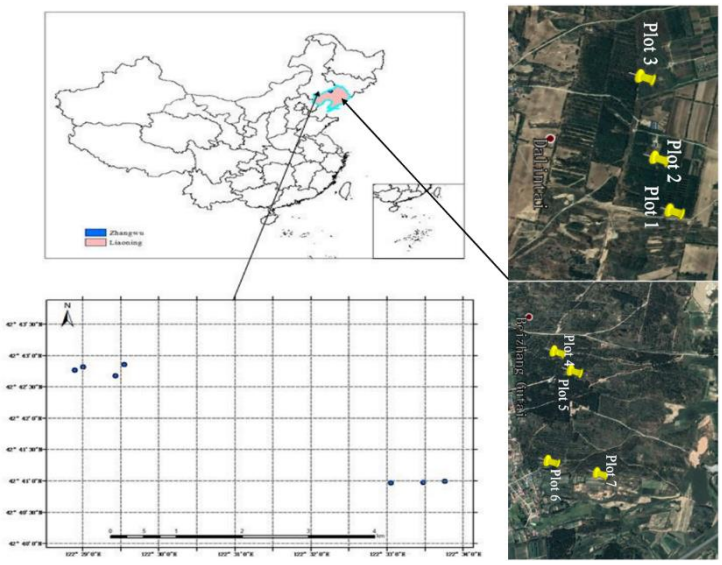

Figure 1. *Location of the study area in China*

Stand survey and soil sampling and analysis

7 sites were selected with different stand age and density (*Table 1*). In each site 50 m × 50 m standard plot was setup and then stand variables as diameter at breast height (DBH), height (H), stand density (SD), basal area (BA), and tree cross sectional area (CS) in sample plots were measured (*Fig. 2*).

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Table 1. *Stand characteristics and location of Mongolian Scots Pine (Pinus sylvestris var mongolica) plantation forests*

Plot No	Location	Altitude (m)	Total Density (ha^{-1})	Mean Diameter (cm)	Mean Height (m)	pH					Bulk Density (g/cm^3)				
						0-20 (cm)	20-40 (cm)	40-60 (cm)	60-80 (cm)	80-100 (cm)	0-20 (cm)	20-40 (cm)	40-60 (cm)	60-80 (cm)	80-100 (cm)
1	42.6832° N, 122.5627° E	204.3	700±35.16	19.5±9.38	9.23±3.04	5.86	6.42	6.44	6.26	6.76	1.54	1.56	1.58	1.57	1.55
2	42.6828° N, 122.5579° E	204.3	572±21.54	19.55±3.04	10.61±1.03	5.48	6.39	6.57	6.46	6.4	1.50	1.59	1.53	1.55	1.56
3	42.6827° N, 122.5508° E	197.3	248±12.52	20.15±2.88	10.19±0.89	6.34	6.45	6.74	6.57	6.32	1.59	1.58	1.59	1.60	1.60
4	42.7127° N, 122.4815° E	247.5	244±11.33	22.97±3.78	11.07±1.05	5.9	8.26	6.67	6.68	6.77	1.55	1.53	1.58	1.60	1.61
5	42.7137° N, 122.4833° E	225.5	312±12.30	23.02±4.31	13.45±11.30	5.73	6.59	6.69	6.85	6.59	1.59	1.58	1.57	1.57	1.56
6	42.7112° N, 122.4904° E	235.4	364±17.60	16.69±3.00	7.95±0.98	6.36	6.55	6.72	6.75	6.7	1.59	1.56	1.56	1.56	1.59
7	42.7143° N, 122.4924° E	230.4	608±62.13	9.81±1.60	4.18±0.56	6.16	6.52	6.53	6.87	6.94	1.63	1.63	1.61	1.58	1.56

Note: Values are mean ± standard deviation

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Figure 2. *Sampling/experimental design, site habitat and data collection*

In each sample plot, the soil samples at five depth levels (0-20 cm, 20-40 cm, 40-60 cm, 60-80 cm and 80-100 cm) (*Fig. 2*) were collected using a steel core (dimensions = 5 cm) and were packed and brought to a laboratory for further analysis (Nelson and Sommers, 1982; Bremner, 1996). All the soil samples were sieved through a 2 mm wire mesh after removing the litter plant roots and stones, and ovened for 24 hours at 105°C and the dry weight of each sample was measured. Soil pH value was measured using FE20 pH meter (Mettler Toledo, Shanghai, China) at soil-to-water (deionized) 2:2.5. $K_2Cr_2O_7/H_2SO_4$ method, a Semimicro-Kjeldhl method and sodium hydroxide (NaOH) fusion and Mo-Sb colorimetric method were applied to determine SOC, N, and P respectively (Ouyang et al., 2017).

Stand and soil parameter calculation

Soil bulk density

Soil bulk density (SD) (g cm^{-3}) of each sample at the respective depth was calculated as:

$$pb = M_s/V_t \quad \text{(Eq.1)}$$

where; ρb is the bulk density of the soil (g cm^{-3}), M_s (g) is an oven-dry mass and V_t is the core volume (cm^{-3}).

Stand density, basal area and biomass carbon

Stand basal area was calculated from the calculated stand density and cross sectional area of each tree at respective diameter following *Equation 2* (Ahmad et al., 2018)

$$BA = SD \times CS \quad \text{(Eq.2)}$$

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where, BA is the basal area (m^2 ha^{-1}), SD is the stand density (ha^{-1}), and CS is the cross sectional area of each tree.

For calculating the above and below ground biomass the experimental model was used for different organs (stem, branch, foliage, and root) of Mongolian Scots pine (Xing et al., 2017). To determine the carbon stock for tree layers, the total carbon concentration was used to the biomass estimates in the different stand diameter classes, then summed up and scaled on the basis of total area (ha^{-1}). For calculation of carbon concentration, following *Equation 3* was used (Hoover, 2008; Lorenz and Lal, 2010).

$$Cc = B \times \rho_c \quad \text{(Eq.3)}$$

where Cc is carbon concentration (Mg ha^{-1}) and B is biomass (Mg ha^{-1}), $\rho_c$is conversion factor as 0.5.

TSOC, TN, and TP

Following equations were used to calculate the mass storage per area (Mg ha^{-1}) of TSOC (C_S), TN (N_S), and TP (P_S) for each individual soil profile (Ouyang et al., 2017).

$$C_s = \sum_i^n [\, D_i \times TSOC_i \times BD_i \times (1 - G_i)/100 \,]/100 \quad \text{(Eq.4)}$$

$$N_s = \sum_i^n [\, D_i \times TN_i \times BD_i \times (1 - G_i)/100 \,]/100 \quad \text{(Eq.5)}$$

$$P_s = \sum_i^n [\, D_i \times TP_i \times BD_i \times (1 - G_i)/100 \,]/100 \quad \text{(Eq.6)}$$

where n is the number of soil layers; i is the ith soil layer; $TSOC_i$, TN_i, and TP_i are the TSOC, TN, and TP concentrations (g kg^{-1}) in the *i*th soil layer, respectively. Similarly, BD_i, G_i and D_i are the soil bulk density (g cm^{-3}), the proportion (%) of coarse (> 2 mm) fragments, and the thickness (cm) in the ith layer, respectively. In our study, TSOC, TN, and TP were calculated for depth of 100 cm, divided in to five soil layers (0-20, 20-40, 40-60, 60-80, and 80-100 cm).

Statistical analysis

Soil pH, soil bulk density (g cm^{-3}), TSOC (t ha^{-1}), TN (t ha^{-1}), TP (t ha^{-1}), C:N, C:P, and N:P were tested with soil profile layers by using ANOVA. Similarly, stand density (ha^{-1}), basal area (ha^{-1}), and total biomass carbon (t ha^{-1}) were fitted with TN (t ha^{-1}), TSOC (t ha^{-1}), TP (t ha^{-1}), C:N, C:P, and N:P as regression analysis (polynomial cubic). All the statistical analysis was performed using statistical software packages Statistix 8.1 and SigmaPlot 12.5.

Results

Variation in soil pH and bulk density along the depth increment

Depth increment and interaction affected the soil pH but there was no significant effect on bulk density value along depth (*Fig. 3A,B*). Soil pH was found statistically lower in 0-20 cm layer, while no significant difference among the other layers was recorded (*Fig. 3A*). The bulk density showed no significant change with increasing depth (*Fig. 3B*).

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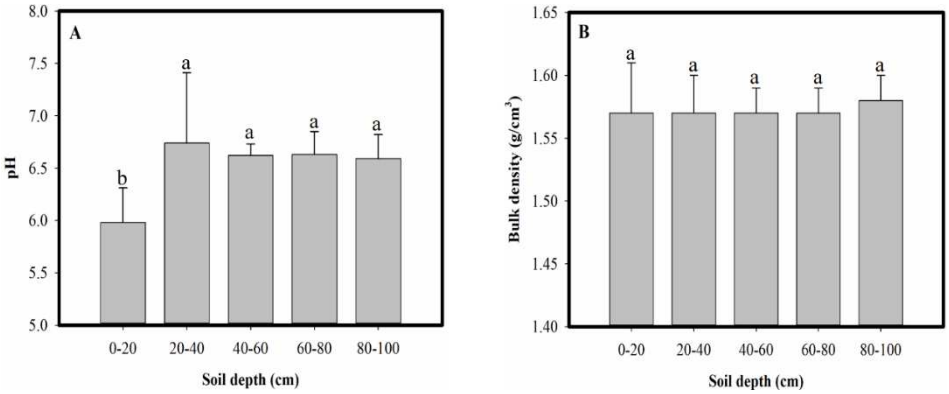

Figure 3. *Mean values of soil pH (a) and bulk density (b) along the depth increment. Values are mean ± standard deviation, different alphabets on each error bar denote the significant differences (p < 0.01, n=7) at a given soil depth*

Variation in soil TSOC, TN, and TP along the depth increment

The results showed that the soil TSOC, TN, and TP followed a decreasing trend with the depth increment (*Fig. 4*). Among the different layers the maximum values for TSOC, TN, and TP storage was found in 0-20 cm layer. The results presented in *Fig. 4* revealed that a significantly higher TSOC, TN, and TP storage occurred at the first layer increment (0-20 cm). Nevertheless, there was slight or no significant variation in their values among the other layers.

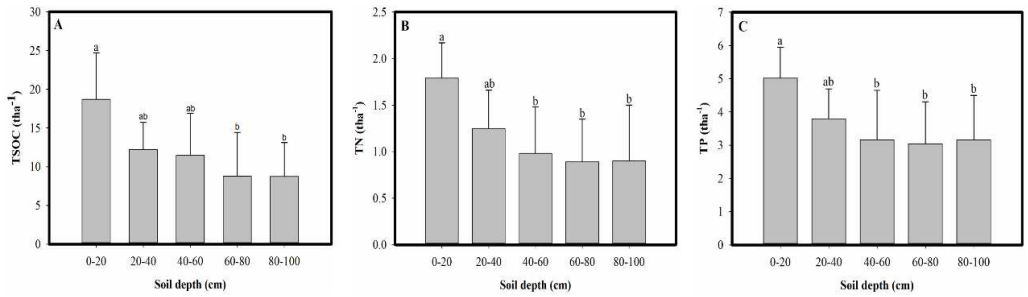

Figure 4. *Mean values of total soil organic carbon (t ha^{-1}) (a), total nitrogen (t ha^{-1}) (b) and total phosphorous (t ha^{-1}) (c) along the depth increment. TSOC, TN, and TP indicate the total soil organic carbon, total nitrogen, and total phosphorous. Values are mean ± standard deviation, different alphabets on each error bar denote the significant differences (p < 0.01, n=7) at a given soil depth*

Variation in soil C:N, C:P, and N:P ratios along the depth increment

Fig. 5 showed the values of C:N, C:P, and N:P along the depth. Results of the figure highlighted that among all the depths, C:N and C:P showing no significant variation, however, the highest C:N was found in 40-60 cm layer and lowest in 20-40 cm (*Fig. 5A*). Similarly, the maximum C:P was recorded in 0-20 cm layer while it was minimum in 80-100 cm layer (*Fig. 5B*). In contrast to C:N and C:P, a significant variation in the N:P was observed across the depth. A significant larger ration was recorded in the 0-20 cm and lower in 80-100 cm layer. However, no significant variation was recorded 20-40, 40-60 and 60-80 cm layer for N:P (*Fig. 5C*).

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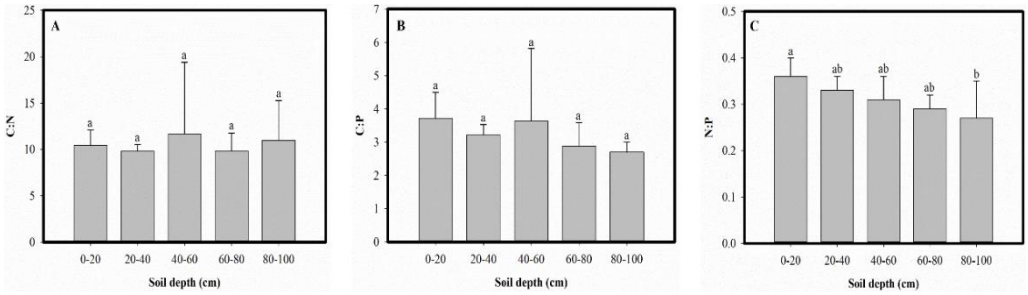

Figure 5. *Stoichiometric ratios of soil C, N, and P along the depth increment in Mongolian Scots pine plantation. Values are mean ± standard deviation, different alphabets on each error bar denote the significant differences ($p < 0.01$, $n=7$) at a given soil depth*

Regression analysis (polynomial cubic) between TSOC, TN, and TP with the stand characteristics

The values of TSOC, TN, and TP were positively correlated with stand different characteristics with R^2 value more then 0.5 (*Fig. 6a,b,c*). The relationship of stand density with TSOC, TN and TP was a polynomial cubic. The results of the regression analysis (*Fig. 6b*) showed that the amount of TN increased with increasing stand density, and reached to a maximum value of 9.48 (t ha^{-1}) at high stand density of 572 (ha^{-1}). However, a decreasing trend in TN was recorded with increasing density and reached to a minimum value of 4.02 (t ha^{-1}) at 700 (ha^{-1}) (*Fig. 6b*). Similarly, TP, TSOC followed nearly the same pattern and presented a high value i.e. 26.93 (t ha^{-1}) (*Fig. 6c*) and 93.64 (t ha^{-1}) (*Fig. 6a*) of TP and TSOC at high density 572 (ha^{-1}), respectively.

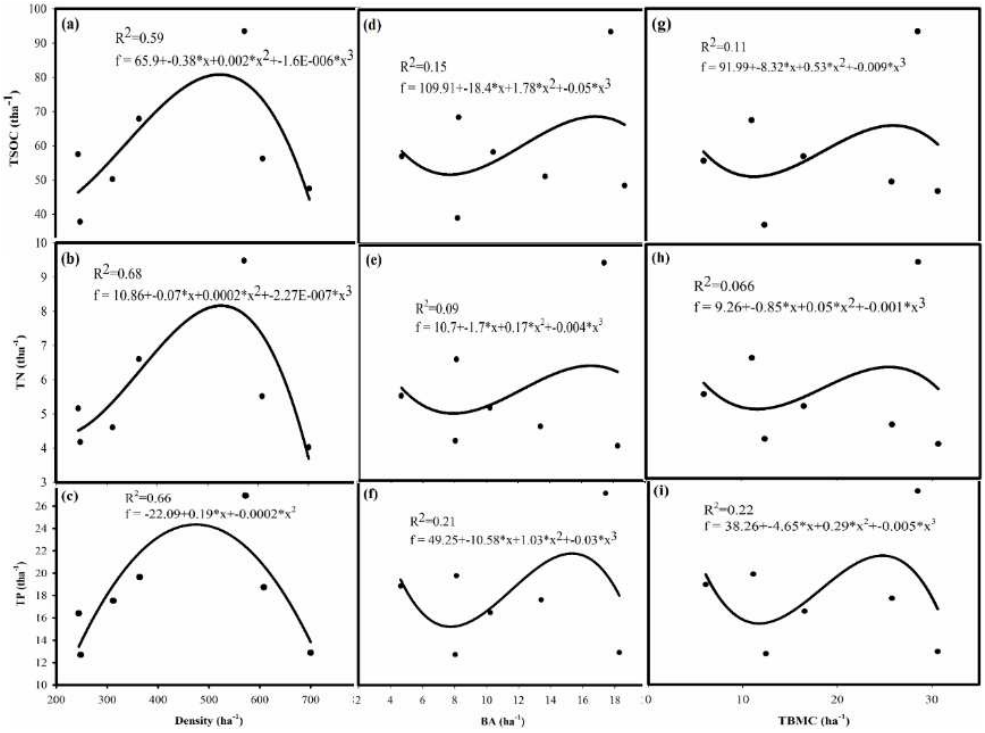

Figure 6. *Regression analysis (Polynomial Cubic) of total soil organic carbon (TSOC), total nitrogen (TN), and total phosphorous (TP) with stand density (a, b, c), stand basal area (d, e, f) and total biomass carbon (g, h, i), ($p < 0.05$, $n=7$)*

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Consistently, TSOC, TN and TP though positively correlated with basal area and biomass carbon, but a week (*Fig. 6d,e,f,g,h,i*) compared to stand density. The amount of TSOC, TN, and TP were found maximum at basal area of 17.43 (ha^{-1}) and minimum at basal area of 18.28 (ha^{-1}) (*Fig. 6d,e,f*), respectively. Similarly, the amount of TSOC, TN, and TP was recorded higher at biomass carbon of 28.52 (t ha^{-1}) and lower at biomass carbon of 30.63 (t ha^{-1}) (*Fig. 6g,h,i*), respectively.

Regression analysis (polynomial cubic) between C:N, C:P, and N:P ratios with the stand characteristics

The results showing the values of regression analysis between C:N, C:P, and N:P ratios with different stand characteristics are given in the *Fig. 7*. The results highlighted that C:N and C:P (*Fig. 7a,b*) showed a maximum value of 11.79 and 3.69 with maximum value of 700 (ha^{-1}) stand density and with minimum value of 9.04 and 2.86 at stand densities of 248 (ha^{-1}) and 312 (ha^{-1}), respectively, but N:P (*Fig. 7c*) showed a different pattern from C:N and C:P with the maximum and minimum value of 0.35 and 0.26 with respective stand density of 572 (ha^{-1}) and 312 (ha^{-1}). Similarly, maximum and minimum value of C:N and C:P and N:P (*Fig. 7d,e,f*) were found at their respective maximum and minimum stand basal areas of 18.28 (ha^{-1}) and 8.04 (ha^{-1}). *Fig. 7g,h,i* highlighted the regression analysis between C:N, C:P and N:P with total biomass carbon and figured out the maximum and minimum value of 11.79, 9.04 and 3.69, 2.86 and 0.35, 0.26 with their respective total biomass carbon of 30.63 (t ha^{-1}), 12.44 (ha^{-1}) and 30.63 (t ha^{-1}), 25.78 (t ha^{-1}) and 28.52 (t ha^{-1}), 25.78, respectively.

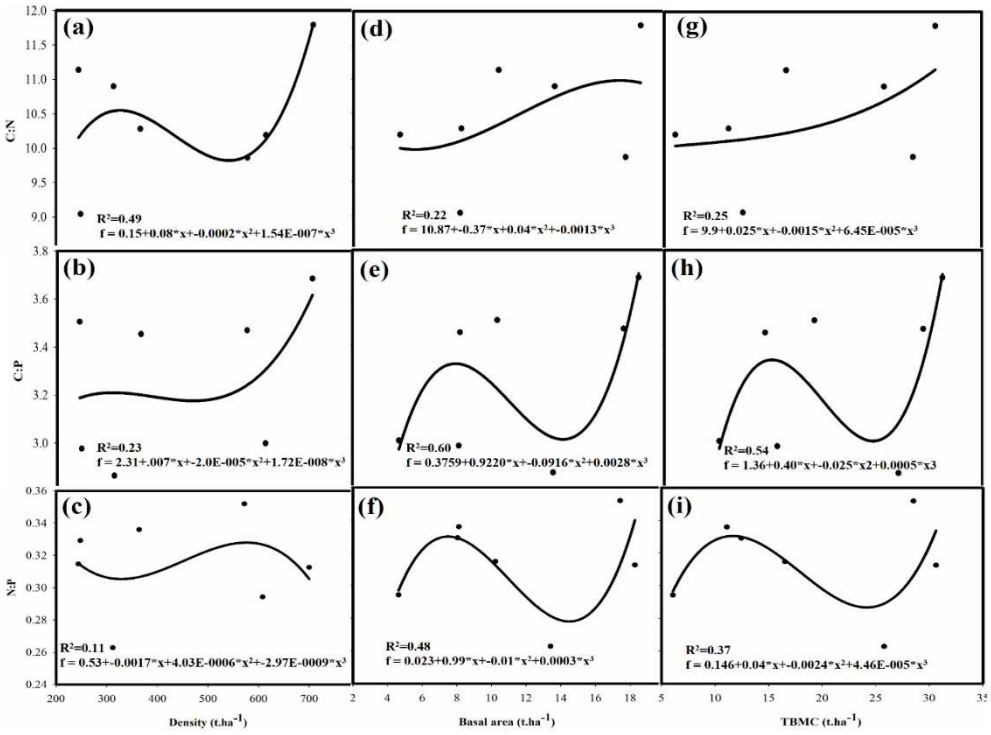

Figure 7. *Regression analysis (Polynomial Cubic) of C:N, C:P, and N:P with stand density (a, b, c), stand basal area (d, e, f) and total biomass carbon (g, h , i), ($p < 0.01$, $n=7$)*

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Discussion

Effect of soil depth on soil TSOC, TN, and TP storage and concentration

Variation in soil TSOC, TN, and TP storage and concentration are the key indicators of changes in soil fertility and long-term ecosystem sustainability and management and variation in soil organic carbon concentration have consequences for the influence of land cover change on atmospheric carbon dioxide concentration and global warming (Ross et al., 1999). Our Results show that depth and soil nutrients are significantly correlated and the nutrients concentration decreases significantly with depth increment under scots pine plantation (*Pinus sylvestris* var *mongolica*) (*Fig. 4*). These results match the statement of some previous observations in arid and semi-arid regions of China (Hu et al., 2008; Chen et al., 2010). There are many reasons affecting the variation of TSOC, TN, and TP concentration along the soil depth. For instance, the balance between inputs and outputs can affect the concentration of these nutrients status, and variation in the amount and quality of plant litter contribution with land cover change may be important factors to change the ecosystem practices and eventually ecosystem properties (McKinley et al., 2008). Soil disturbances during plantation establishment in the area may also result in the faster mineralization of soil nutrients and increase the potential for nutrients loss (Guo and Gifford, 2002). The plant community, species composition, topography and soil texture can also alter the soil nutrients status (Guo and Gifford, 2002; Paul et al., 2002; Archer et al., 2004) and the coarse soil in this region might have limited soil carbon accumulation after the establishment of Scots pine plantation (Richter et al., 1999). TSOC concentration decreased with depth because of the unavailability of easily decomposed organic matter in the deep soil (Liang et al., 2010) and the soil carbon mineralization is mostly controlled by easily available decomposable soil organic carbon. Similarly, the soil nutrient concentration and availability in the top soil are mostly observed after planation of arid and semi-arid sites (Billings, 2006; McKinley et al., 2008). Furthermore, Zeng et al. (2009) reported that after conversion of arid and semi-arid regions of China to Mongolian Scots pine plantation, the concentration and mineralization of soil nutrients increased in the upper soil because of increased annual root biomass and litter input and its decomposition in the upper soil as compared to the grassland and Savana.

The concentration and seasonal distribution of soil total carbon is governed by the size and quantity of soil microbial biomass (Wei et al., 2009). Soil TSOC and the depth increment significantly correlated (*Fig. 4a*), suggests that soil depth was an important factor influencing the soil TSOC in our study area. Difference in the amount of soil organic matter added to the soil under forest vegetation can affect the soil organic carbon (Chen et al., 2010). The significant higher TSOC concentration in the top soil of Mongolian pine plantation reflected the greater amount and availability of organic matter accumulated in the top soil and the same was reported for pine plantation (Zeng et al., 2009). Similarly, some other studies also concluded that with the development of plantation on arid and semi-arid regions, the more TSOC and nutrients are being released from accumulation and decomposition of microbial biomass and litter input into the top soil, which could result in higher TSOC and related nutrients concentration in the upper soil surface (Cleveland and Liptzin, 2007; Li et al., 2017b). Although, initially plantation consistently resulted in the loss of some SOC from top soil in this region by altering the soil properties, but later on with the development of plantation, the C concentration gradually increased (Chen et al., 2010).

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Our results for total soil nitrogen concentration was consistent with the previously reported for Keerqin sandy land (Chen et al., 2006; Holst et al., 2007; Zeng et al., 2009). Soil depth, litter fall, land cover change and sampling season has a valid interaction with the TN storage and can significantly alter the available nitrogen concentration (*Fig. 4b*), consistent with many other studies of soil nitrogen changes following land cover changes (Owen et al., 2003; Parfitt et al., 2003; Zeng et al., 2009).

Our results (*Fig. 4c*) demonstrated that afforestation in semiarid region of China significantly reduced TP stocks, which is consistent with the previously reported global scale study stating that the TP concentration below 20 cm significantly decreased (Deng et al., 2017). As it has also been explained in many studies that the microbial biomass, mineralization of organic P and phosphate activities and susceptibility of organic matter to microbial attack and enzymes hydrolysis take place in top surface and decreases gradually as the depth increases (Magid et al., 1996). The same phenomenon was also demonstrated for Mongolian pine plantation (Zhao et al., 2007). In our study, the pH level is low in the top soil (*Fig. 3a*), which may also improve organic P mineralization by increasing the susceptibility of organic P to enzyme hydrolysis in the top soil, and the same was also reported for Mongolian scots pine in Keerqin sandy land of China (Zhao et al., 2007). The decrease in the concentration of TSOC, TN, and TP with increase in the soil depth under Mongolian scots pine can be attributed to the development of juvenile plantation that require more nutrients in top soil than leaching down as the same was reports in other studies too (Laclau et al., 2003; Zhao et al., 2007).

Effect of depth increment on soil nutrients stoichiometry

The soil mean P and N concentrations and its stoichiometry in our study is lower than the global level (Zhang et al., 2005), may be because of weathering and soil erosion in the in Mongolian Scots pine (*Pinus sylvestris* var. *mongolica*) plantation forests in semiarid areas of China. It can also be attributed to the lower P concentration in China soil than global value (Han et al., 2005). Similarly, the C: N and C: P stoichiometry and soil C concentration was significantly higher than soil N and P concentration and stoichiometry, indicating that coniferous forests store more C, consistent with other studies for coniferous forests (Güsewell and Koerselman, 2002; Zeng et al., 2009). In addition, our study findings reveal higher C: N ratio than C: P and N: P, which might be the result of higher human influence in the plantation, and the same was reported (Zhang et al., 2017). The soil nutrient concentrations and stoichiometric ratios in our study were significantly correlated with depth increment, consistent with a previous study (Zeng et al., 2016), due to a large amount of the nutrients in litter and deadwood were released into the soil as litter is a main source of soil nutritional elements.

This study demonstrated the same trend of C:N>C:P>N:P, which is also reported in other studies (Tian et al., 2010; Ouyang et al., 2017). Different studies (Li, 2012; Ren et al., 2016) showed a different CNP rations along the soil profile increment because of difference in land use practices and management system, but followed the same trend as presented in this study. Li et al. (2012) studied that difference in C: N: P ratios might be the result of different vegetation cover and land use management practices. Zhao et al. (2015) demonstrated that forest types and plant communities can effect soil nutrients stoichiometry. Similarly, Fan et al. (2015) concluded that together, soil depth and successional stage significantly influence soil CNP stoichiometry. Globally, a well-balanced CNP stoichiometry for top 0-10 cm profile is 186:13:1 (Cleveland and Liptzin, 2007; Wang, 2014), while a general CNP stoichiometry for rich organic nutrients soil for

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0-10 cm depth is 134:9:1 and for the entire soil depth i.e., 0-250 cm is 60:5:1 in China (Tian et al., 2010). In our study, the average C:N:P ratio was 10.53:3.24:0.32 for 0-100 cm soil depth, while for 0-20 cm and 20-40 cm the C:N:P ratios were 10.44:3.2:0.36 and 9.80:3.22:0.33, respectively. Our estimates for C:N:P ratios are lower than the estimated average value of C:N:P as reported above. Our C:N:P value for 0-20 cm was much lower than reported for China soil (Tian et al., 2010). These differences might be due to the soil samples containing more humidified litter in Tian et al. (2010), resulting in relatively higher C:N:P ratios compared to our estimates. In this study the average value for C:N ratio was nearly >10, and a low (<25) C:N ratio suggests that the soil organic matter accumulation is slower than its decomposition (Yang et al., 2007b).

Relationship between soil nutrients and stand characteristics

Stand characteristics and soil property has an encouraging interaction and soil nutrients can vary along stand characteristic variations (Jiang et al., 2017; Xu et al., 2018). We investigated that the stand density has a significant effect on the biomass carbon production that can bring variation in TSOC, TN, and TP concentration and storage. Our results show that TSOC, TN, and TP followed an increased pattern with increase in stand characteristics (stand density, basal area and total biomass carbon) up to some extent and then dropped (*Fig. 6*). This difference in nutrients distribution and fluxes among plantations along different stand characteristics have implications for management of successive *Pinus sylvestris* var *mongolica* plantations forests in the study area in order to maximize nutrients concentration and cycling. It has been investigated that stand density has an impacts on soil nutrients and can alter the amount of soil TSOC, TN, and TP concentration (Ma et al., 2007) and the stand characteristics like, Density, basal area and total aboveground biomass have a key role in soil nutrients concentration in plantation forests because of the litter fall and fast decomposition rate (Little and Shainsky, 1995; Sheng and Yang, 1997). Our study findings are consistent with previous study (Ma et al., 2007), stating that the soil nutrients decreased with the competition among Chinese fir plantation increased. It could also be explained that with highest basal area, density and biomass, higher accumulation of litter with lower decomposition rate due unavailability of sun light and proper aeration (Jiang et al., 2017). However, the mean TSOC and TP concentration in our results were higher compared to Jiang et al. (2017). It could be attributed that there is more SOC and P input (litter and deadwood decomposition) from plants and rapid turnover of litter (Tong et al., 2012; Yang et al., 2014). In contrast, Yang et al. (2014) attributed higher soil TSOC and TN but lower TP concentration in broadleaved deciduous forest and our study shows high TSOC and TP as compared to TN. These differences might be due to the differences in forest nature and topography because Yang et al. (2014) studied a broadleaved plantation while we studied a Pine plantation forests. Polynomial regression analysis showed that the variations in soil TSOC, TN, and TP in the plantation investigated were positively and significantly correlated with the stand characteristics, including stand density, basal area and total biomass carbon. This shows a potential influence of stand characteristics on the variation of soil nutrients in the forests (Xia et al., 2015). Yuan et al. (2013) studied that the basal area and canopy structure can alter the soil nutrients concentration because it may affect the temperature and soil moisture contents of forest floor soil, which are important factors affecting the litter and deadwood decomposition process. Similarly, our results demonstrated a high value of C:N but slightly low C:P and N:P with high stand density, basal area and total biomass carbon (*Fig. 7*). It could be attributed that in soil, mostly TP contents is available

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in inorganic forms, that show little or no variation along the stand characteristics and may lead to the high ratios of C:N (Jiang et al., 2017). Soil nutrients stoichiometric ratios didn't show any good correlation along different stand characteristics (*Fig. 7*) might be because that stoichiometry is a combine result of multiple activities, like elemental uptake, excretion and storage, thus it cannot fully specify the ability of plants to achieve limiting resources. Furthermore, it has been investigated that stoichiometric and elemental compositions of vegetation vary significantly along stand characteristics (Yang et al., 2011; Liang et al., 2018). However, there might be other factors like, over grazing, land use change practices etc., that might also alter the soil nutrients and its stoichiometry.

Conclusion

Our findings support the hypothesis that depth increment and stand characteristics have a significant effect on TSOC, TN, and TP storage, stoichiometry, and concentration. Stand characteristics like stand density, basal area, and total biomass carbon could significantly affect the TSOC, TN, and TP concentration and storage. Furthermore, soil C:N and C:P ratios increase more as compared to N:P with depth, showing the organic N and P limitation for plant growth in the region. This study is the first attempt to indicate the TSOC, TN, TP and its stoichiometry at the vertical profile scale and along the stand characteristics. Similarly, this study will provide useful information for sustainable management of Mongolian Scots Pine plantation forest. For future, it is strongly recommended to conduct more research studies on climate change, soil nutrients dynamics and growth response relationship.

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A COMPARATIVE ANALYSIS OF DE NOVO TRANSCRIPTOME ASSEMBLY TO UNDERSTAND THE ABIOTIC STRESS ADAPTATION OF DESERT PLANTS IN SAUDI ARABIA

BAESHEN, M. N.[1*] – AHMED, F.[2*] – MOUSSA, T. A. A.[1,3] – ABULFARAJ, A. A.[4] – JALAL, R. S.[1] – NOOR, S. O.[5] – BAESHEN, N. A.[5] – HUELSENBECK, J. P.[1,6]

[1]*Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia*

[2]*Department of Biochemistry, College of Science, University of Jeddah, Jeddah, Saudi Arabia*

[3]*Botany and Microbiology Department, Faculty of Science, Cairo University, Giza 12613, Egypt*

[4]*Department of Biological Sciences, College of Sciences and Arts - Rabigh Campus, King Abdulaziz University, Jeddah 21589, Saudi Arabia*

[5]*Department of Biology, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*

[6]*Department of Integrative Biology, University of California, Berkeley, USA*

**Corresponding authors*
e-mail: mnbaeshen@uj.edu.sa, fahmed1@uj.edu.sa

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Abstract. *Rhazya stricta, Senna italica,* and *Zygophyllum simplex* are important desert plants of Saudi Arabia with great economic and medicinal value. However, their tolerance and survival mechanisms under combined abiotic stresses such as high temperature, high salinity, and drought are not well understood. In order to investigate the potential molecule mechanism of abiotic stress tolerance in these plants, we used de novo transcriptome assembly and their comparative analysis. This study used leaf tissues to construct three cDNA libraries of these plants and then generated RNA-seq data by the Illumina HiSeq2000 platform. Sequencing reads were de novo assembled to generate: (a) 71,116 unigenes in *R. stricta; (b)* 59,274 unigenes in *S. italica;* (c) 70,300 unigenes in *Z. simplex*. Furthermore, the unigenes from these plants were annotated and analyzed with different databases. A comparative analysis of KEGG pathways identified several common pathways induced in these plants, including "Plant-pathogen interaction", "Plant hormone signal transduction", "Spliceosome", "RNA transport", and "Protein processing in endoplasmic reticulum", which may play an important role in combined abiotic drought, heat, and salinity stress. Finally, a comparative analysis of transcriptional regulators identified C2H2, C3H, CCHC(Zn), MYB-HB-like, PHD, WD40-like, and bHLH as common Transcription Factors responsible for abiotic stress tolerance in these plants. Our study revealed key factors involved in abiotic stress tolerance, which could be applied to develop high-yield transgenic crops capable of growing under combined abiotic stresses in the field.
Keywords: plant transcriptome, GO, KEGG, simple sequence repeats, heat stress, drought stress, salinity stress, transcription factors

Introduction

Abiotic (non-biological) stresses, including drought, hot climate, salinity, and nutrient are the leading limiting factors in agricultural productivity in the desert (Fahad et al., 2017). These factors negatively impact plant growth and development, cause a significant decrease in biomass, crop yield, and quality resulting in extensive losses of agriculture production. The combined stress of drought and heat are the most important abiotic stress widely encountered by crops in the field of Saudi Arabia. The Kingdom of

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Saudi Arabia covers a land area of approximately 2,150,000 square kilometers (http://www.mofa.gov.sa/), with an estimated population of 32.55 million by 2017 (https://www.stats.gov.sa/). However, due to continuous drought, heat, and salinity stresses in the field, a big land of Saudi Arabia is deserted and not suitable for agriculture farming. Therefore, Saudi Arabia is unable to use its vast land for agriculture to meet domestic food demands and depends on importing them. For example: In 2013, 660,145 tons of wheat has been locally produced under 102,613 hectares cultivation, while 2,117,052 tons of wheat were imported (Fiaz et al., 2016). In the same year, 11,267 tons of barley has been locally produced in 1502 hectares of land, while 10,446,332 tons of barley were imported (Fiaz et al., 2016).

Researchers showed that a variety of plants have evolved their strategy to respond to different abiotic stresses, including drought and heat encounter in their extreme natural environment. These plants adapted effectively by activating the expression of various stress-responsive genes producing different metabolites, inducing signaling and biochemical pathways to mitigate stress-induced damages (An et al., 2013). These stress induced gene expression consequences on the: (a) morphological modifications in plants, including stomatal conductance, size of leaf and roots; (b) physiological and molecular modifications, such as the production of antioxidant compounds and cellular osmotic adjustment to develop abiotic stress tolerance. Studies found that each stress's molecular response, such as drought or heat, is unique (Rizhsky et al., 2002). Furthermore, combinations of various stresses show different molecular responses and cannot be extrapolated from their individual response (Rizhsky et al., 2002). This is the reason why transgenic plants developed for drought stress under experimental conditions failed tolerance when tested in the field under a naturally occurring environmental condition such as a combination of drought and heat stresses. During drought stress, stomata are close to avoiding water loss, while during heat stress, the stomata are open to reducing leaf temperature. In this way, drought and heat stresses have opposing physiological changes (Rizhsky et al., 2004). However, during combined drought and heat stresses, stomata are close (Rizhsky et al., 2002; Prasch and Sonnewald, 2013). In response to drought stress, proline synthesis and its degradation pathways are activated and repressed, respectively (Krasensky and Jonak, 2012). This results accumulation of proline in the leaf to protect plants against osmotic stress due to drought (An et al., 2013). Heat stress results in the accumulation of unfolded protein in the cytoplasm, which induces the expression of heat shock proteins (HSPs). HSPs bind and stabilize misfolded proteins and inhibit protein aggregation (Al-Whaibi, 2011). Thus, HSPs act as molecular chaperones. Interestingly, HSPs expression is controlled by various heat shock factors (HSFs). Overexpression of HSFA2 in transgenic *Arabidopsis thaliana* shows greater tolerance of combined heat and high light stress than control (Nishizawa et al., 2006). Expression of Ethylene Response Factor1 (ERF1) (AT3G23240) in *A. thaliana* is greatly induced by salt and drought stress, and its overexpression enhanced the drought, heat, and salt tolerance in *A. thaliana* (Cheng et al., 2013). In response to various abiotic stress, ERF1 up-regulates a different set of stress-related genes by binding to GCC box and DRE/CRT elements in their promoter region (Cheng et al., 2013). Transcription of the wheat ERF1 (TaERF1) gene was also induced by different biotic and abiotic stresses (Xu et al., 2007). The study found that the BT2 acts as a central stress regulator in *A. thaliana* and mediate different biotic and abiotic stress the responses. Furthermore, diverse microbial communities were

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identified in the soils, which help abiotic stress tolerance in plants through association with plants' roots (Baeshen et al., 2020).

Interestingly, several wild plants remarkably adapted to grow in the desert area under combined stress of heat, drought and high salt. However, the molecular mechanism, and pathways underlying stress response and tolerance has not been well understood in these plants. The advancement in next generation sequencing (NGS) technology gives ample opportunities to analyze the transcriptomic and genomics data to understand the abiotic stress tolerance in non-model plants without a reference genome (Grabherr et al., 2011; Li et al., 2017; Park et al., 2014; Sabir et al., 2016). For instance: de novo genome assembly of *Thellungiella parvula* that adapted the saline and resource poor environment (Dassanayake et al., 2011); Transcriptome analysis of *R. stricta* that grows in arid zone (Yates et al., 2014); transcriptomes analysis of desiccation-tolerant *Craterostigma plantagineum* (Rodriguez et al., 2010); and abiotic stress tolerance *Rhizophora mangle* and *Heritiera littoralis* (Dassanayake et al., 2009).

In order to investigate the molecule mechanism to adapt combined abiotic stress tolerance, this study used de novo transcriptome sequencing and assembly of three different plants *Rhazya stricta, Senna italica,* and *Zygophyllum simplex.* These plants grow in their natural environment, having combined abiotic stresses of high temperature, drought, and high salt conditions in Saudi Arabia. The assembled unigenes were annotated with NR, SwissProt, NT GO, KEGG, and GOC databases to elucidate the expressed genes and pathways involved in the abiotic stress tolerance. Finally, the comparative analysis of unigenes among three plants identified the common genes and TFs involved in abiotic stress tolerance. The finding of this study would be applied to develop high-yield transgenic crops with improved abiotic stress resistance. The present study would also provide transcriptome resources for further study of the molecular mechanism of abiotic stress tolerance in plants.

Materials and methods

Plant sample collection

The leaf shows significant physiological and biochemical changes than root in drought-tolerant wheat, which supports that the leaf is a more abiotic stress-sensitive part of the plant (Kang et al., 2019). Therefore, in our study, we consider leaves from three different plants. The plants' leaves were collected during the morning as the temperature was 38 °C from a public site at Hadda, Mecca-Jeddah Road, Saudi Arabia (N21° 45′ 04.03", E39° 53′ 88.92") (*Table 1*). The leaf samples of three plants *R. stricta*, *S. italic*a, and *Z. simplex* were taken in plastic bags and stored at -80 °C for RNA extraction.

Table 1. *Location of plant samples with their GPS coordinates*

Plants	Family	Photosynthesis	Sample codes	GPS coordinates	
				N	E
Rhazya stricta	Apocynaceae	C3	1A	21° 45′ 04.03"	39° 53′ 88.92"
Senna italica	Fabaceae	C4	4A	21° 45′ 04.03"	39° 53′ 88.92"
Zygophyllum simplex	Zygophyllaceae	C4	5A	21° 45′ 04.03"	39° 53′ 88.92"

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RNA extraction, cDNA library construction and Illumina sequencing

The total RNA was extracted from 100 mg of leaf tissue by using TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. Briefly, mRNAs were isolated using magnetic beads with Oligo (dT) then sliced into short fragments by mixing them in fragmentation buffer. The mRNA fragments were used as templates to synthesized cDNA using random hexamer primers. Short fragments were purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition, followed by connecting them with adapters. The suitable fragments size was selected for the PCR amplification as templates. Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used to quantify and qualify the sample library. Finally, three libraries were prepared from three plants *R. stricta*, *S. italic*a, and *Z. simplex*, each library coming from a single plant. Subsequently, these libraries were sequenced using Illumina HiSeq™ 2000 platform. The sequencing machine gives the raw sequence image data, which was transformed by base calling into sequence data and stored in the FASTQ format. The paired-end raw reads contain adapters and low-quality bases (Q-value < = 20) were removed to get a clean read (Mbandi et al., 2014).

De novo transcriptome assembly

The high-quality reads were used for de novo transcriptome assembly using Trinity sequence assembler, which consists of three independent software modules: Inchworm, Chrysalis, and Butterfly (Grabherr et al., 2011). Clean sequence reads were assembled into larger contigs by Inchworm; Contigs were clustered to create a de Bruijn graph for each locus (component) by Chrysalis; the Butterfly then processes each de Bruijn graph and reports unique transcripts (unigenes).

Gene annotation

In order to identify the potential function of unigenes, sequence alignment was performed against a series of protein databases: NR, SwissProt, KEGG (Kyoto Encyclopedia of Genes and Genomes), and COG (Clusters of Orthologous Groups of proteins) with BLASTx (E-value < 10^{-5}). In addition, sequence alignment of unigenes was performed against nucleotide databases NT with BLASTn (E-value < 10^{-5}). With NR annotation, the Blast2GO program (v 2.5.0) was used to get GO (Gene Ontology) annotation of unigenes (Conesa et al., 2005). Finally, GO functional annotation were classified and plotted using WEGO software (Ye et al., 2006). The Venn diagrams were plotted using (http://bioinformatics.psb.ugent.be/webtools/Venn/)

SSR and SNP analysis

SSR (simple sequence repeat) detection is carried out with software MicroSAtellite (MISA) using unigenes as reference (Beier et al., 2017), while SNP (single nucleotide polymorphism) analysis was carried out with SOAPsnp (Li et al., 2009).

Protein-coding region prediction (CDS)

Unigenes was firstly aligned by the BLASTx (E-value < 10^{-5}) to protein databases in the priority order of NR, Swiss-Prot, KEGG, and COG. Unigenes aligned to a higher priority database will not be aligned to a lower priority database. The alignments end

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when all alignments are finished. Proteins with the highest ranks in blast results are taken to decide the coding region sequences of Unigenes. Then the coding region sequences are translated into amino sequences with the standard codon table. So, both the nucleotide sequences (5'->3') and amino sequences of the Unigene coding region are acquired. Unigenes that could not align with any of the above-mentioned databases are scanned by ESTScan, producing nucleotide sequence (5'->3') direction and then translated into the amino acid sequence of the predicted coding region.

Unigene expression analysis

To identify genes associated with abiotic stresses, the clean reads were mapped to Unigenes using Bowtie2, and then the gene expression levels were measured with the number of Fragments Per Kilobase of a given transcript per million mapped reads (FPKM) according to the given formula (*Eq. 1*). FPKM normalized the reads counts based upon library sizes and the length of the transcripts.

$$FPKM = \frac{10^6\ C}{NL/10^3} \quad \text{(Eq.1)}$$

where C is the number of reads uniquely aligned to a unigene; N is the total number of reads that are uniquely aligned to all unigenes; L is the base number in the CDS of one unigene.

Unigene transcription factor and transcriptional regulator prediction

In order to predict the transcription factor and transcriptional regulator, Unigenes of *R. stricta* (1A), *S. italica* (4A), and *Z. simplex* (5A) were analyzed with online tool PlantTFcat (http://plantgrn.noble.org/PlantTFcat/) (Dai et al., 2013). Then the output results were analyzed and the most abundant TF were selected using an in-house program.

Results and discussions

RNA sequencing

In order to understand the comprehensive transcriptome of *R. stricta* (1A), *S. italica* (4A), and *Z. simplex* (5A), RNAs were extracted from leaf samples, and three cDNA libraries were constructed. Paired-end sequencing of these libraries was performed on an Illumina HiSeq 2000 platform, which generated a total of 56,965,920; 54,266,528; and 54,062,860 raw reads from *R. stricta* (1A); *S. italica* (4A); and *Z. simplex* (5A), respectively. After pre-processing of reads (trimming of the adaptor and filtering for the low-quality region), 54,201,896; 51,152,532; and 51,449,822 high-quality reads were obtained from *R. stricta* (1A); *S. italica* (4A); and *Z. simplex* (5A), respectively (*Table 2*).

De novo transcriptome assembly

In absence of whole genome sequencing data, de novo transcriptome assembly is employed to study the gene expression profiling, to identify novel transcripts and genetic markers, and to understand the signaling pathways in different physiological conditions (Deng et al., 2019). The clean reads from each library were used for de novo assembly

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with Trinity programs that generated: (a) 98,278 contigs and 71,116 unigenes for *R. stricta* (1A); 107,175 contigs and 59,274 unigenes for *S. italica* (4A), and 116,444 contigs and 70,300 unigenes for *Z. simplex* (5A). The assembly statistics, the length distribution of contigs and unigenes of each plant are provided in *Table 3* and *Figure 1*. The sequence file of contigs and unigenes are provided into the supplementary dataset [*Tables S1* and *S2* for *R. stricta* (1A); *Tables S3* and *S4* for *S. italica* (4A); and *Tables S5* and *S6* for *Z. simplex* (5A)]. A previous study used the leaves transcriptome of *R. stricta* and, using de novo assembly generated only 28018 unique transcript sequences (mean length = 1643 bp; N50 = 2199 bp) (Yates et al., 2014).

Table 2. *Statistical description of sequencing data from R. stricta (1A), S. italica (4A), and Z. simplex (5A)*

Samples	*R. stricta (1A)*	*S. italica (4A)*	*Z. simplex (5A)*
Total raw reads	56,965,920	54,266,528	54,062,860
Total clean reads	54,201,896	51,152,532	51,449,822
Total clean nucleotides (nt)	4,878,170,640	4,603,727,880	4,630,483,980
Q20 percentage	97.36%	97.42%	98.01%
N percentage	0.12%	0.09%	0.00%
GC percentage	43.94%	44.67%	43.01%

Q20 percentage is proportion of nucleotides with quality value larger than 20; N percentage is proportion of unknown nucleotides in clean reads; GC percentage is proportion of guanidine and cytosine nucleotides among total nucleotides

Table 3. *Statistical of assembly quality of high-quality reads from R. stricta (1A), S. italica (4A), and Z. simplex (5A)*

Samples	*R. stricta (1A)*		*S. italica (4A)*		*Z. simplex (5A)*	
	Contig	**Unigene**	**Contig**	**Unigene**	**Contig**	**Unigene**
Total number	98,278	71,116	107,175	59,274	116,444	70,300
Total length (nt)	47,232,016	101,448,447	45,217,296	60,317,437	49,262,775	81,584,604
Mean length (nt)	481	1427	422	1018	423	1161
N50	1175	2276	903	1671	945	1830
Total consensus sequences	-	71,116	-	59,274	-	70,300
Distinct clusters	-	34,830	-	23,405	-	33,142
Distinct singletons	-	36,286	-	35,869	-	37,158

Total consensus sequences represents all assembled unigenes; distinct clusters represents the cluster unigenes. The same cluster contains some high similar (more than 70%) unigenes, and these unigenes may come from same gene or homologous gene; distinct singletons represents this unigene come from a single gene

Function annotation and classification of unigenes

Functional annotation and classification of all expressed unigenes were carried out to understand their protein functions (Kerr et al., 2019). Initially, unigenes were searched against protein databases including NR, SwissProt, GO, KEGG, and COG by BLASTx, and then retrieved proteins with the highest sequence similarity (E-value < 10^{-5}) with unigenes. In addition, the expressed unigenes were searched against the NT nucleotide database by BLASTn and retrieved the highest sequence similarity (E-value < 10^{-5}). The number of unigenes annotated with each database is provided in *Table 4*.

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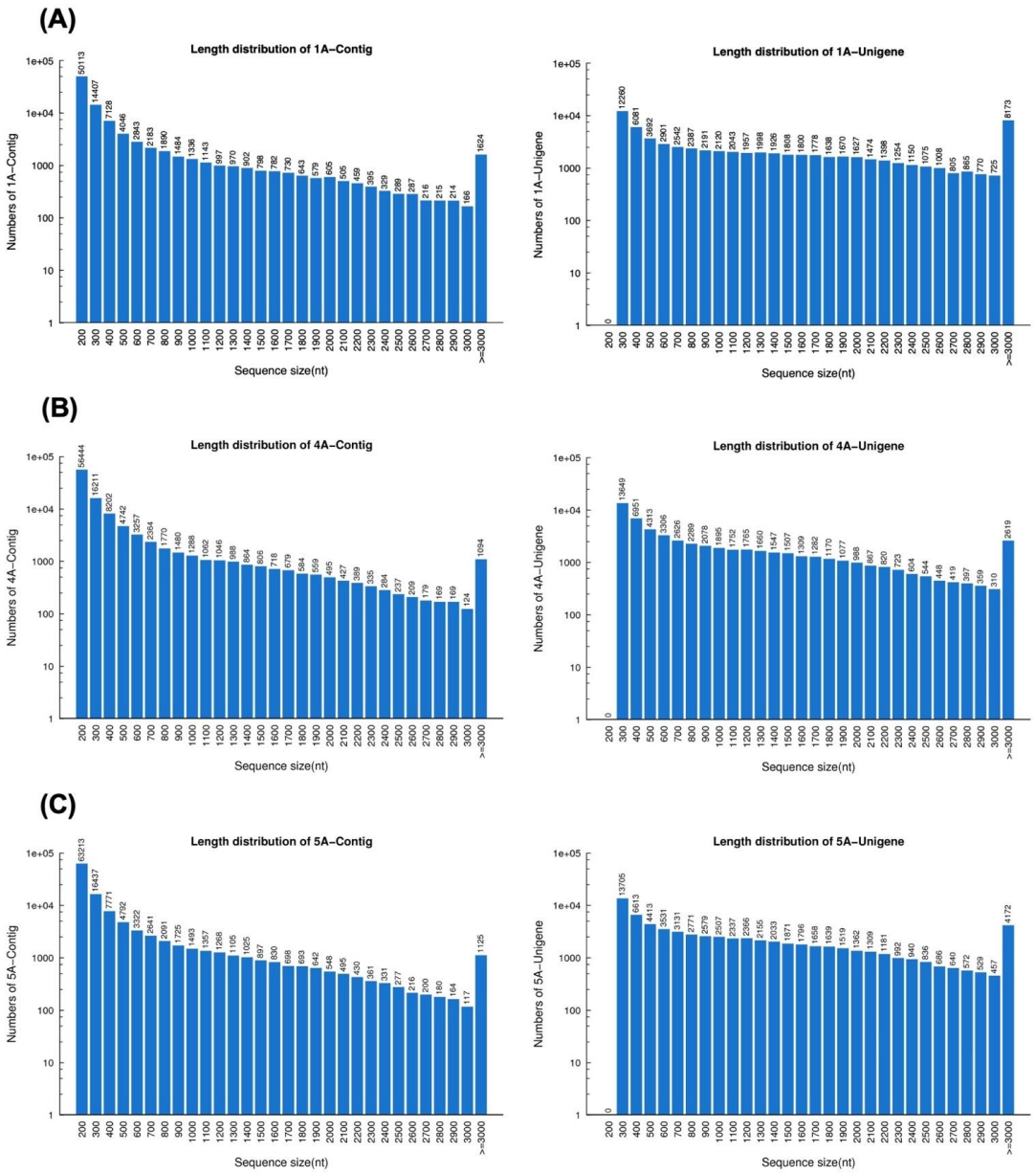

Figure 1. *Distribution of contig and unigene length of Trinity transcriptome assembly. (A) R. stricta (1A); (B) S. italica (4A); and (C) Z. simplex (5A)*

Table 4. *Summary of unigenes annotation of R. stricta (1A), S. italica (4A), and Z. simplex (5A)*

Samples	*R. stricta (1A)*	*S. italica (4A)*	*Z. simplex (5A)*
NR	47,940	40,957	51,456
NT	43,789	40,313	45,272
Swiss-Prot	32,126	27,431	35,073
GO	33,559	29,698	36,911
KEGG	29,503	23,739	30,619
COG	20,847	15,261	20,931
All	49,208	43,007	52,588

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NR annotation (similarity analysis)

The unigenes annotation of NR database of *R. stricta* (1A) showed: (a) The E-value distribution analysis found that 63.44% of sequences showed strong homology (E-value < 1e−45), while 36.56% of sequences showed homology between 1e−5 and 1e−45 (*Fig. 2A*); (b) The similarity distribution analysis showed that 69.54% of the unigenes have similarity higher than 60%, while 23.21% unigenes have similarity between 40% to 60% (*Fig. 2B*); (c) The species distribution revealed that 21.66% of unigenes were matched with sequences of *Solanum tuberosum* followed by *Vitis vinifera* (19.46%), *Solanum lycopersicum* (11.97%), and *Erythranthe guttata* (9.25%) (*Fig. 2C*).

The unigenes annotation of NR database of *S. italica* (4A) showed: (a) The E-value distribution analysis found that 63.13% of sequences showed strong homology (E-value < 1e−45), while 36.87% of sequences showed homology between 1e−5 and 1e−45 (*Fig. 3A*); (b) The similarity distribution analysis showed that 81.82% of the unigenes have similarity higher than 60%, while 13.63% unigenes have similarity between 40% to 60% (*Fig. 3B*); (c) The species distribution revealed that 39.83% of unigenes were matched with sequences of *Glycine max* followed by *Phaseolus vulgaris* (13.44%), and *Cicer arietinum* (12.93%) (*Fig. 3C*).

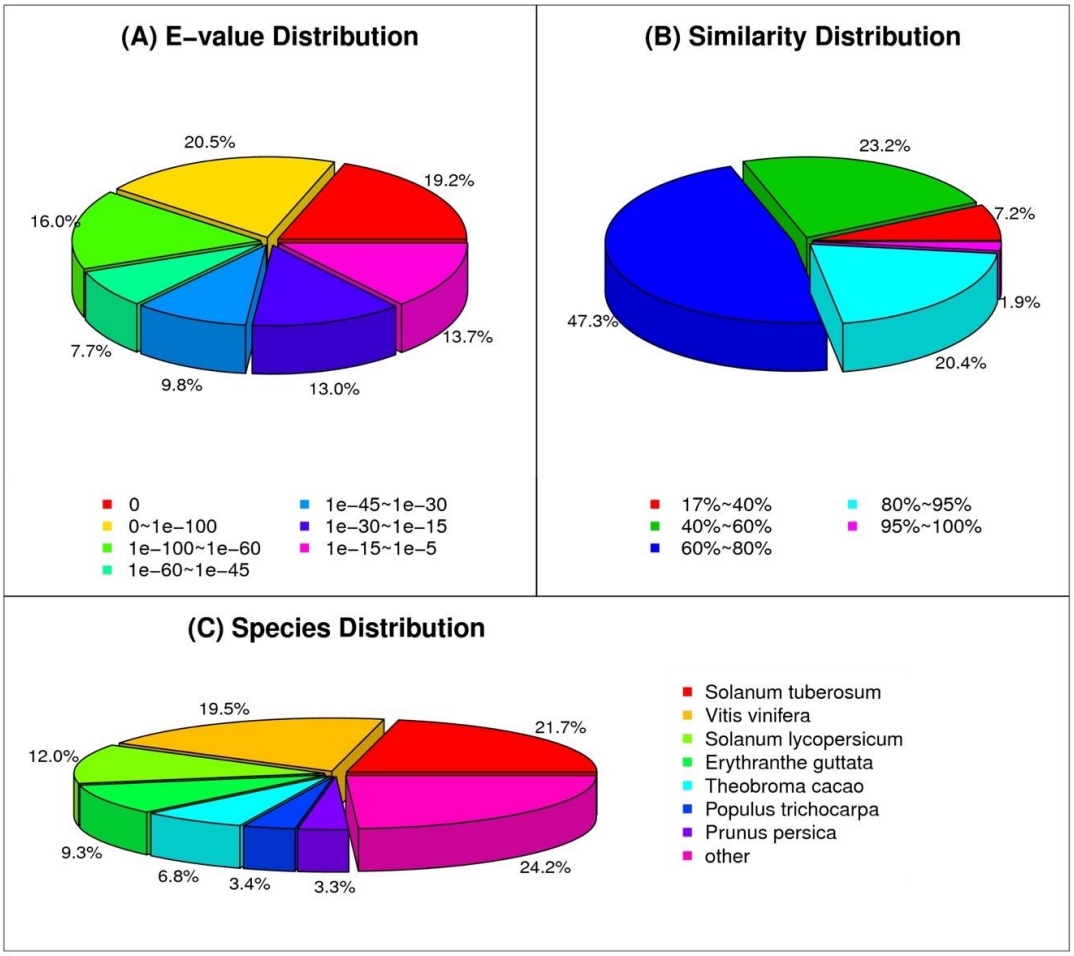

Figure 2. *Annotation results of homology search of R. stricta (1A) unigenes against NR database. (A) The E-value distribution of the result of NR annotation. (B) The similarity distribution of the result of NR annotation. (C) The species distribution of the result of NR annotation*

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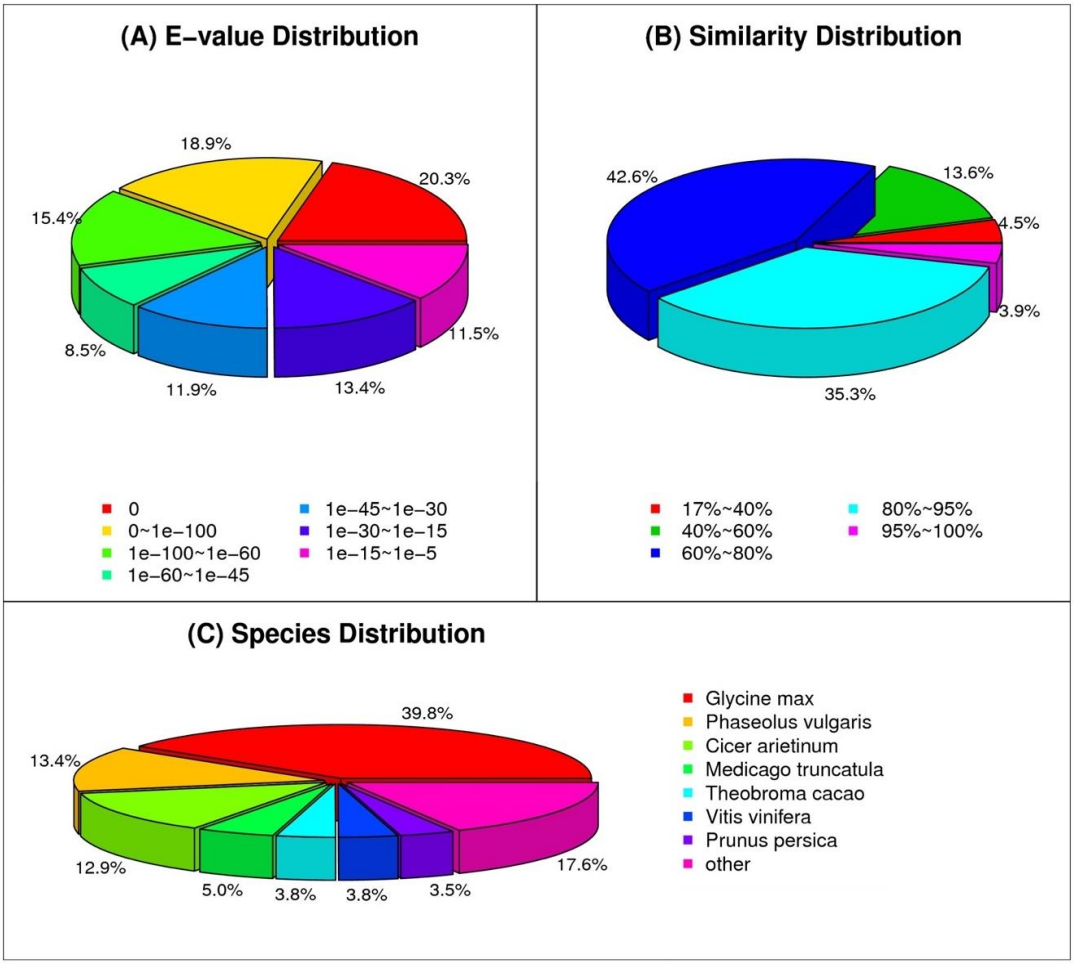

Figure 3. *Annotation results of homology search of S. italica (4A) unigenes against NR database. (A) The E-value distribution of the result of NR annotation. (B) The similarity distribution of the result of NR annotation. (C) The species distribution of the result of NR annotation*

The unigenes annotation of NR database of *Z. simplex* (5A) showed: (a) The E-value distribution analysis found that 62.11% of sequences showed strong homology (E-value < 1e−45), while 37.89% of sequences showed homology between 1e−5 and 1e−45 (*Fig. 4A*); (b) The similarity distribution analysis showed that 66.67% of the unigenes have similarity higher than 60%, while 25.85% unigenes have similarity between 40% to 60% (*Fig. 4B*); (c) The species distribution revealed that 17.05% of unigenes were matched with sequences of *Theobroma cacao* followed by *Vitis vinifera* (13.97%), and *Prunus persica* (8.53%) (*Fig. 4C*).

GO annotation

With NR annotation, the Blast2GO program was used to get GO annotation, including molecular function (MF), biological process (BP), and cellular component (CC) of unigenes of each plant. After getting GO annotation, WEGO software was used for functional classification for all unigenes to understand gene functions distribution.

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(a) In *R. stricta* (1A), there were 33,559 unigenes assigned with at least one GO term in BP, CC, or MF (*Fig. 5A*). The BP was further classified as 22 functional groups, among them "metabolic process" is the highest number of unigenes (21,297) followed by "cellular process" (18,918 unigenes), and "single-organism process" (16,448 unigenes), "response to stimulus" (7,487 unigenes) and "biological regulation" (5,719 unigenes). The CC were further classified as 17 functional groups, among them "cell", and "cell part" are the highest number of unigenes (18,711) followed by "organelle" (14,733 unigenes), and "membrane" (9,081 unigenes), "organelle part" (6,036 unigenes) and "membrane part" (4,431 unigenes). The MF was further classified as 17 functional groups, among them "catalytic activity" is the highest number of unigenes (18,293) followed by "binding" (14,695 unigenes), and "transporter activity" (2,715 unigenes), "structural molecule activity" (504 unigenes) and "molecular transducer activity" (476 unigenes).

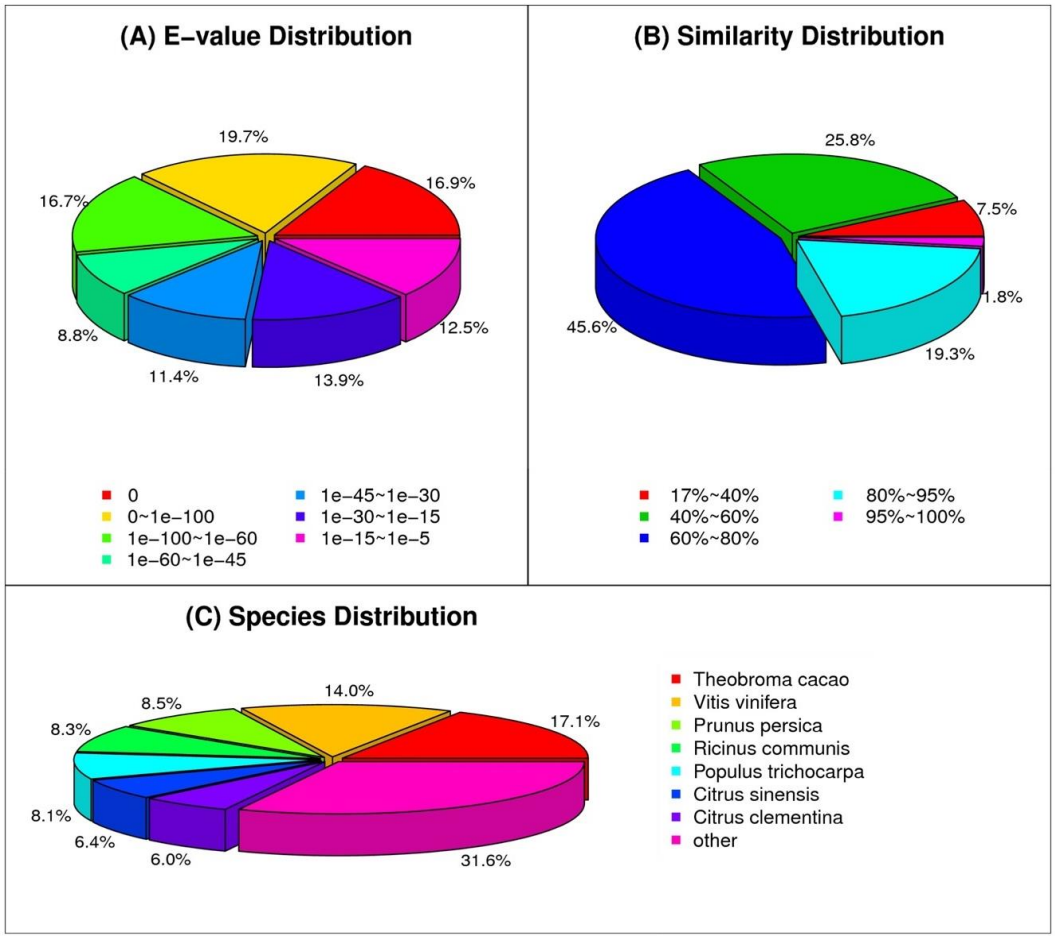

Figure 4. *Annotation results of homology search of Z. simplex (5A) unigenes against NR database. (A) The E-value distribution of the result of NR annotation. (B) The similarity distribution of the result of NR annotation. (C) The species distribution of the result of NR annotation*

(b) In *S. italica* (4A), there were 29,698 unigenes assigned with at least one GO term in BP, CC, or MF (*Fig. 5B*). The BP was further classified as 23 functional groups, among them "metabolic process" is the highest number of unigenes (19,400) followed by "cellular process" (17,471 unigenes), and "single-organism process" (14,105

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unigenes), "response to stimulus" (7,242 unigenes) and "biological regulation" (5,820 unigenes). The CC was further classified as 17 functional groups, among them "cell", and "cell part" are the highest number of unigenes (16,559) followed by organelle (12,572 unigenes), and "membrane" (7,933 unigenes), "organelle part" (5,237 unigenes) and "membrane part" (3,812 unigenes). The MF was further classified as 17 functional groups, among them "catalytic activity" is the highest number of unigenes (15,515 unigenes) followed by "binding" (14,781 unigenes), and "transporter activity" (2,004 unigenes), "structural molecule activity" (580 unigenes) and "nucleic acid binding transcription factor activity" (565 unigenes).

(c) In *Z. simplex* (5A), there were 36,911 unigenes assigned with at least one GO term in BP, CC, or MF (*Fig. 5C*). The BP was further classified as 23 functional groups, among them "metabolic process" is the highest number of unigenes (24134) followed by "cellular process" (22,005 unigenes), "single-organism process" (18,983 unigenes), "response to stimulus" (9,433 unigenes) and "biological regulation" (7,841 unigenes). The CC was further classified as 17 functional groups, among them "cell", and "cell part" are the highest number of unigenes (22,496) followed by "organelle" (17,197 unigenes), "membrane" (10,107 unigenes), "organelle part" (6,766 unigenes) and "membrane part" (4,527 unigenes). The MF was further classified as 17 functional groups, among them "catalytic activity" is the highest number of unigenes (19,140) followed by "binding" (17,208 unigenes), "transporter activity" (2,553 unigenes), "structural molecule activity" (758 unigenes) and "nucleic acid binding transcription factor activity" (642 unigenes). The analysis of GO annotation indicated that a large number of unigenes were associated with overlapping biological functions among *R. stricta* (1A), *S. italica* (4A), and *Z. simplex* (5A).

In response to the abiotic stress, the plant adjusts the metabolic process to repair the cell damage and restore cell homeostasis. We observed that a majority of unigenes from *R. stricta* (1A), *S. italica* (4A), and *Z. simplex* (5A) are annotated as "metabolic process". Previous studies showed a strong association of metabolic process in abiotic stress resistance in plants; thus, our study supports the previous findings (Pan et al., 2016; Puranik et al., 2011).

COG annotation

The unigenes were further annotated against COG database for functional prediction and classification. COG is used to annotate the newly sequenced genome into 25 functional categories.

(a) *R. stricta* (1A): 20,847 unigenes were categorized into 25 groups with the largest category was "General function prediction only" (6,669 unigenes) followed by "Replication, recombination and repair" (3,368 unigenes), "Transcription" (3,099 unigenes), "Posttranslational modification, protein turnover, chaperones" (2,523 unigenes), "Signal transduction mechanisms" (2,449 unigenes). However, only 9 and 2 unigenes are assigned to "Extracellular structures" and "Nuclear structure", respectively (*Fig. 6A*).

(b) *S. italica* (4A): 15,261 unigenes were categorized into 25 groups with the largest category was "General function prediction only" (5,318 unigenes) followed by "Transcription" (2,612 unigenes), "Replication, recombination and repair" (2,525 unigenes), "Signal transduction mechanisms" (2,311 unigenes), "Posttranslational modification, protein turnover, chaperones" (1951 unigenes). However, only 6 and 2 unigenes are assigned to "Extracellular structures" and "Nuclear structure", respectively (*Fig. 6B*).

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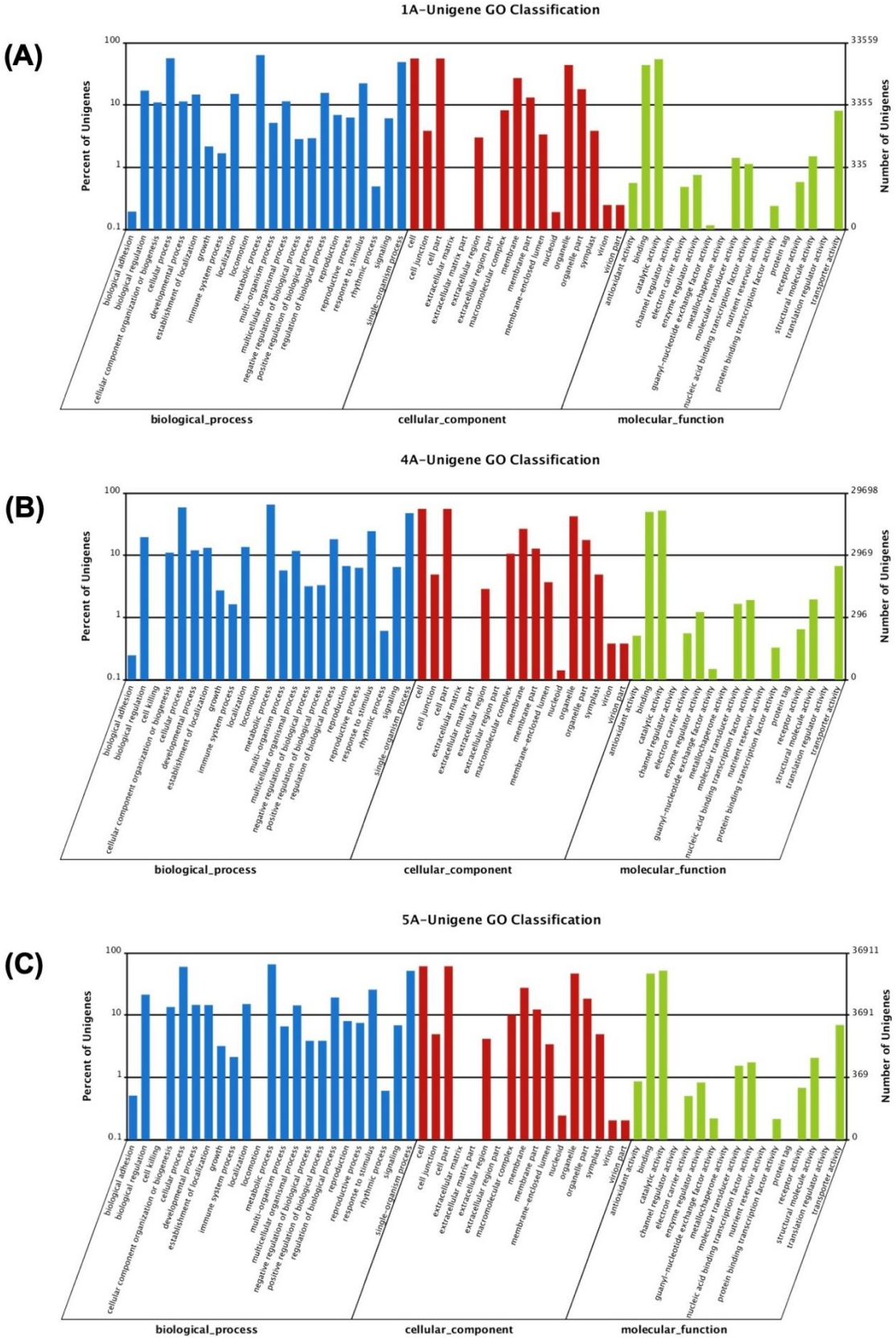

Figure 5. *Histogram of GO annotation of assembled unigenes. (A) R. stricta (1A); (B) S. italica (4A); and (C) Z. simplex (5A). GO categories are: biological process (BP), cellular component (CC), and molecular function (MF)*

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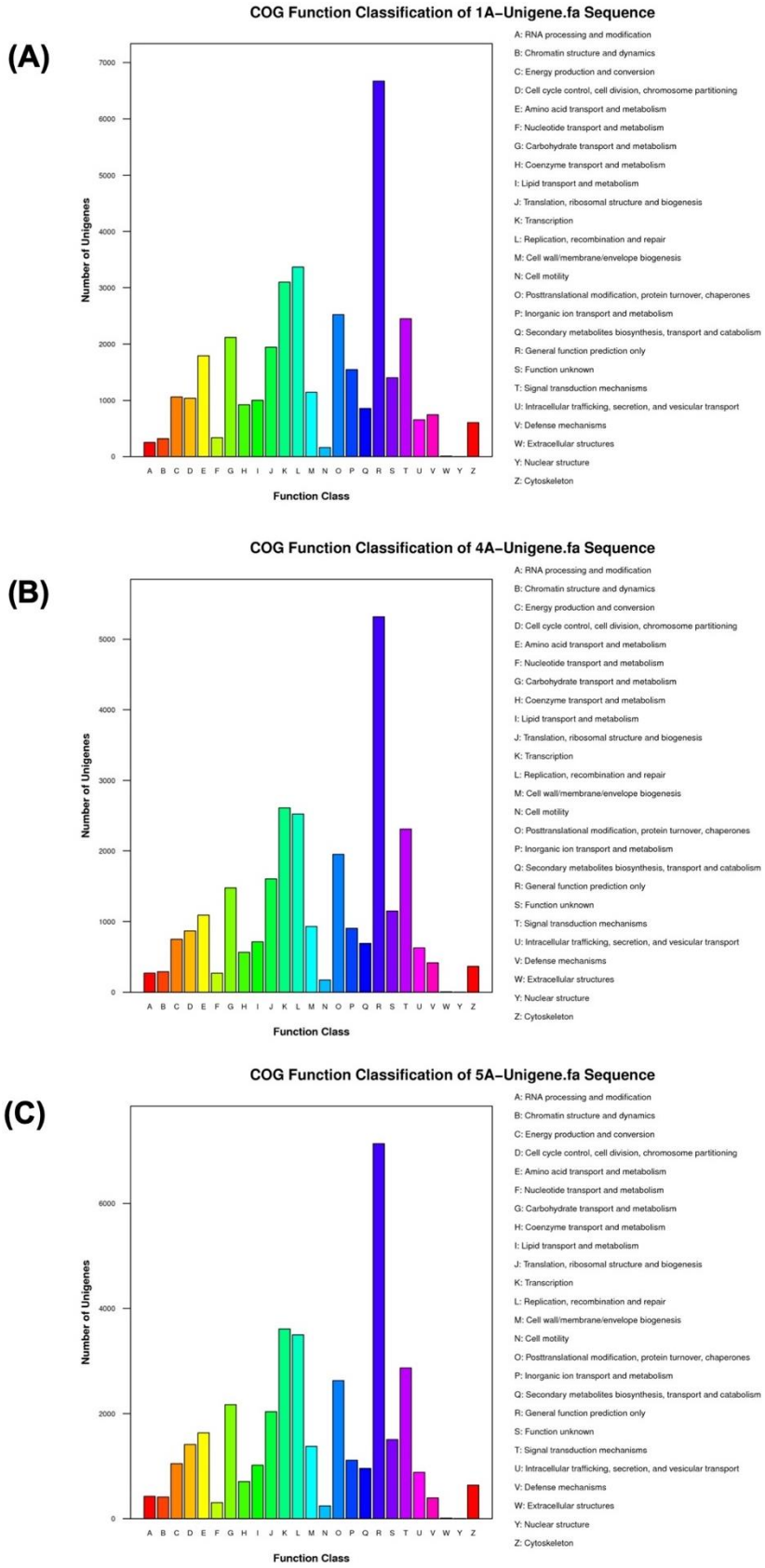

Figure 6. *Histogram of COG annotation of assembled unigenes. (A) R. stricta (1A); (B) S. italica (4A); and (C) Z. simplex (5A)*

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(c) *Z. simplex* (5A): 20,931 unigenes were categorized into 25 groups with the largest category was "General function prediction only" (7,136 unigenes) followed by "Transcription" (3,611 unigenes), "Replication, recombination and repair" (3,498 unigenes), "Signal transduction mechanisms" (2,870 unigenes), "Posttranslational modification, protein turnover, chaperones" (2629 unigenes). However, only 10 and 3 unigenes are assigned to "Extracellular structures" and "Nuclear structure", respectively (*Fig. 6C*).

In our study, a large number of unigenes from *R. stricta* (1A), *S. italica* (4A), and *Z. simplex* (5A) were assigned to "Transcription", "Replication, recombination and repair", "Signal transduction mechanisms", and "Posttranslational modification, protein turnover, chaperones". Activation of these processes is essential in modulating the gene expression and signal transduction, to repair the damage of DNA and proteins, and modification of proteins to preventing the cellular damage due to abiotic stresses and thus support previous findings (Arisha et al., 2020; Wang et al., 2004; Sarwar et al., 2019).

Functional classification by KEGG

In order to understand the biological functions, unigenes were searched against the KEGG database.

(a) *R. stricta* (1A): We found 29503 unigenes were associated with 128 KEGG pathways (*Table S7*). Among them, top 10 enriched pathways were "Metabolic pathways" (ko01100) with 6077 (20.6%) unigenes, "Biosynthesis of secondary metabolites" (ko01110) with 2970 (10.07%) unigenes, "Plant-pathogen interaction" (ko04626) with 1316 (4.46%) unigenes, "Plant hormone signal transduction" (ko04075) with 1112 (3.77%) unigenes, Spliceosome (ko03040) with 1095 (3.71%) unigenes, "RNA transport" (ko03013) with 849 (2.88%) unigenes, "Protein processing in endoplasmic reticulum" (ko04141) with 780 (2.64%) unigenes, "RNA degradation" (ko03018) with 595 (2.02%) unigenes, "Purine metabolism" (ko00230) with 560 (1.9%) unigenes, "Starch and sucrose metabolism" (ko00500) with 551 (1.87%) unigenes (*Fig. 7*).

(b) *S. italica* (4A): We found 23,739 unigenes were associated with 128 KEGG pathways (*Table S7*). Among them, top 10 enriched pathways were "Metabolic pathways" (ko01100) with 4,976 (20.96%) unigenes, "Biosynthesis of secondary metabolites" (ko01110) with 2,399 (10.11%) unigenes, "Plant hormone signal transduction" (ko04075) with 1,305 (5.5%) unigenes, "Plant-pathogen interaction" (ko04626) with 1,296 (5.46%) unigenes, Spliceosome (ko03040) with 972 (4.09%) unigenes, "RNA transport" (ko03013) with 872 (3.67%) unigenes, "mRNA surveillance pathways" (ko03015) with 618 (2.6%) unigenes, "Protein processing in endoplasmic reticulum" (ko04141) with 608 (2.56%) unigenes, "Purine metabolism" (ko00230) with 559 (2.35%) unigenes, "Starch and sucrose metabolism" (ko00500) with 541 (2.28%) unigenes (*Fig. 8*).

(c) *Z. simplex* (5A): We found 30,619 unigenes were associated with 128 KEGG pathways (*Table S7*). Among them, top 10 enriched pathways were "Metabolic pathways" (ko01100) with 6,657 (21.74%) unigenes, "Biosynthesis of secondary metabolites" (ko01110) with 2,940 (9.6%) unigenes, "Plant hormone signal transduction" (ko04075) with 1,619 (5.29%) unigenes, "Plant-pathogen interaction" (ko04626) with 1,342 (4.38%) unigenes, "Spliceosome" (ko03040) with 1079 (3.52%) unigenes, "RNA transport" (ko03013) with 1,003 (3.28%) unigenes, "Glycerophospholipid metabolism" (ko00564) with 831 (2.71%) unigenes, "Endocytosis" (ko04144) with 823 (2.69%) unigenes, "Protein processing in endoplasmic reticulum" (ko04141) with 799 (2.61%) unigenes, "Starch and sucrose metabolism" (ko00500) with 747 (2.44%) unigenes (*Fig. 9*).

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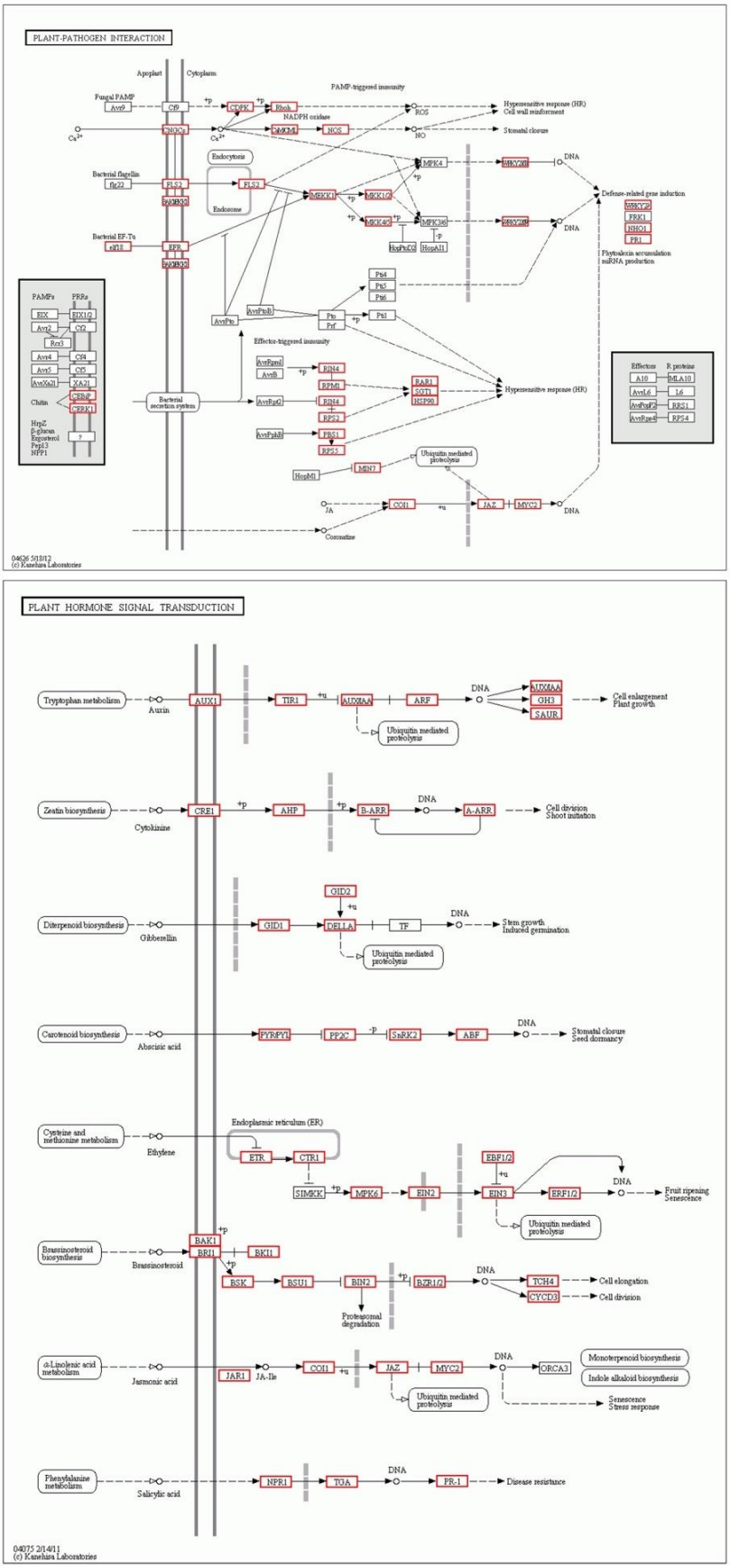

Figure 7. *Annotation of R. stricta (1A) unigenes to KEGG pathways*

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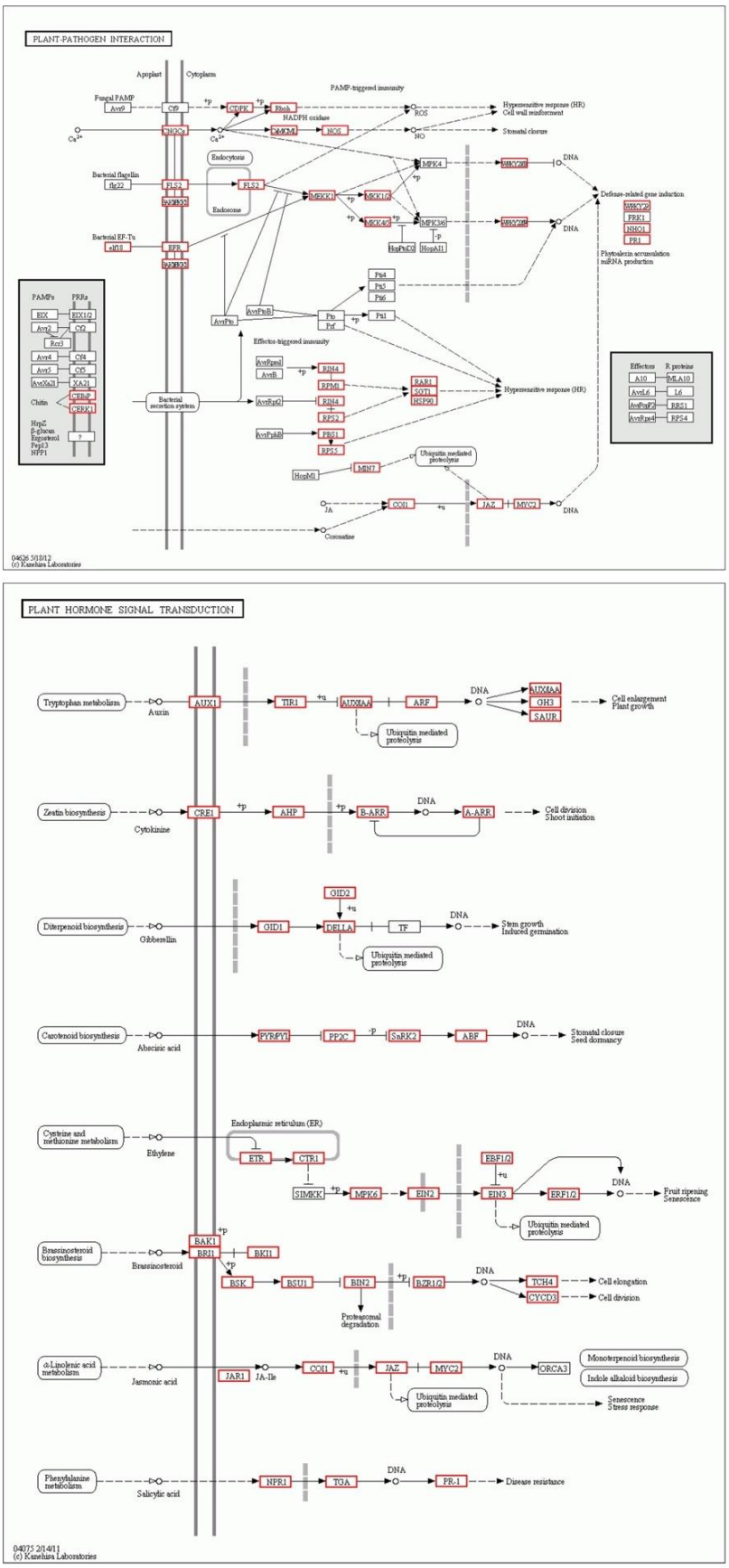

Figure 8. *Annotation of S. italica (4A) unigenes to KEGG pathways*

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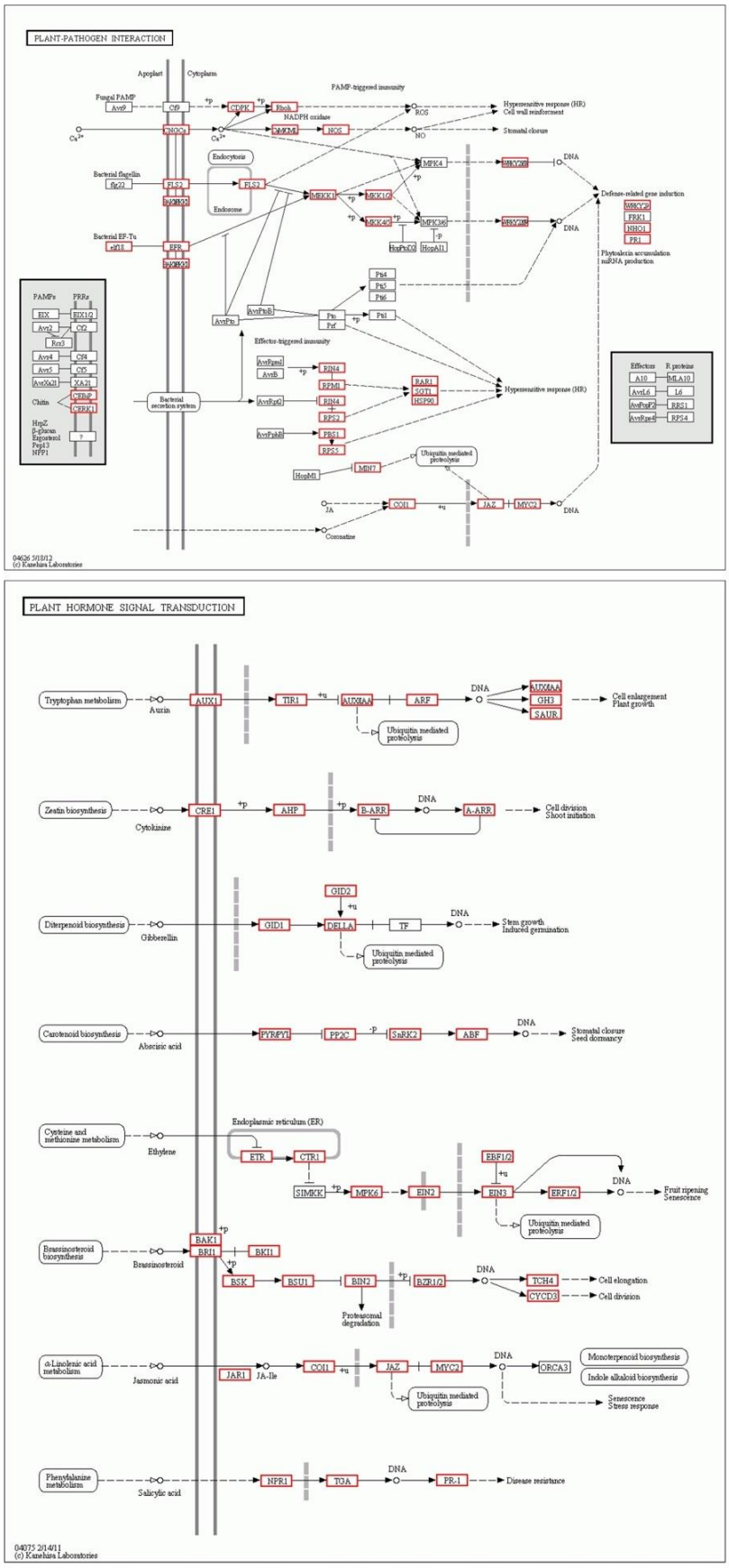

Figure 9. *Annotation of Z. simplex (5A) unigenes to KEGG pathways*

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Comparison of KEGG pathways

KEGG is a bioinformatics resource for biological interpretation of gene sequence and linking genomic information in terms of molecular networks (Kanehisa et al., 2012). Based upon sequence similarity, unigenes sequences were annotated in terms of KO (KEGG Orthology). Furthermore, the set of KOs used to construct KEGG pathways and modules for interpreting the functions (Kanehisa et al., 2012). In order to identify the most common pathways and functions activated during the abiotic stress, the distribution of unigenes and KOs assigned to 128 KEGG pathways among *R. stricta, S. italica, and Z. simplex* were analyzed. We found that the top six common pathways annotated with large number of unigenes are "Metabolic pathways" (ko01100), "Biosynthesis of secondary metabolites" (ko01110), "Plant-pathogen interaction" (ko04626), "Plant hormone signal transduction" (ko04075), "Spliceosome" (ko03040), and "RNA transport" (ko03013) (*Tables 5* and *S7*). In addition, we observed that the top six common pathways annotated with a large number of KOs are "Metabolic pathways" (ko01100), "Biosynthesis of secondary metabolites" (ko01110), "Ribosome" (ko03010), "Spliceosome" (ko03040), and "RNA transport" (ko03013) and "Purine metabolism" (ko00230) (*Tables 5* and *S7*). In order to identify the common KOs present in the KEGG pathways of three plants, we analyzed the KOs data and observed that most of the KOs are common among *R. stricta, S. italica* and *Z. simplex* (*Fig. 10*).

Table 5. *Comparative analysis of number of unigenes and KOs present in different pathways*

#Pathway	KEGG pathway ID	Unigenes count			KOs count		
		R. stricta	*S. italica*	*Z. simplex*	*R. stricta*	*S. italica*	*Z. simplex*
Metabolic pathways	ko01100	6077	4976	6657	795	796	797
Biosynthesis of secondary metabolites	ko01110	2970	2399	2940	365	365	368
Plant-pathogen interaction	ko04626	1316	1296	1342	37	37	37
Plant hormone signal transduction	ko04075	1112	1305	1619	41	41	41
Spliceosome	ko03040	1095	972	1079	101	101	101
RNA transport	ko03013	849	872	1003	97	97	97
Protein processing in endoplasmic reticulum	ko04141	780	608	799	76	76	76
RNA degradation	ko03018	595	492	692	50	49	49
Purine metabolism	ko00230	560	559	595	87	88	88
Ribosome	ko03010	405	471	539	127	124	124

A previous study used ChIP-Seq and RNA-Seq techniques and examined the histone methylation and gene expression pattern under the drought condition in rice (Zong et al., 2013). The study identified the top ten pathways up-regulated in rice under drought stress conditions (Zong et al., 2013). We compared the top ten pathways activated in *R. stricta, S. italica* and *Z. simplex* (annotated with a large number of unigenes) with the previous study. We found that the "Metabolic pathways" (ko01100), "Biosynthesis of secondary metabolites" (ko01110), "Plant-pathogen interaction" (ko04626), and "Plant hormone signal transduction" (ko04075) are commonly activated pathways during the abiotic stress condition (*Table 5*) (Zong et al., 2013). Another study revealed that in response to drought stress in grapevine, differentially expressed genes are associated with "Metabolic pathways" (ko01100), "Biosynthesis of secondary metabolites" (ko01110), and "Plant-pathogen interaction" (ko04626) (Haider et al., 2017).

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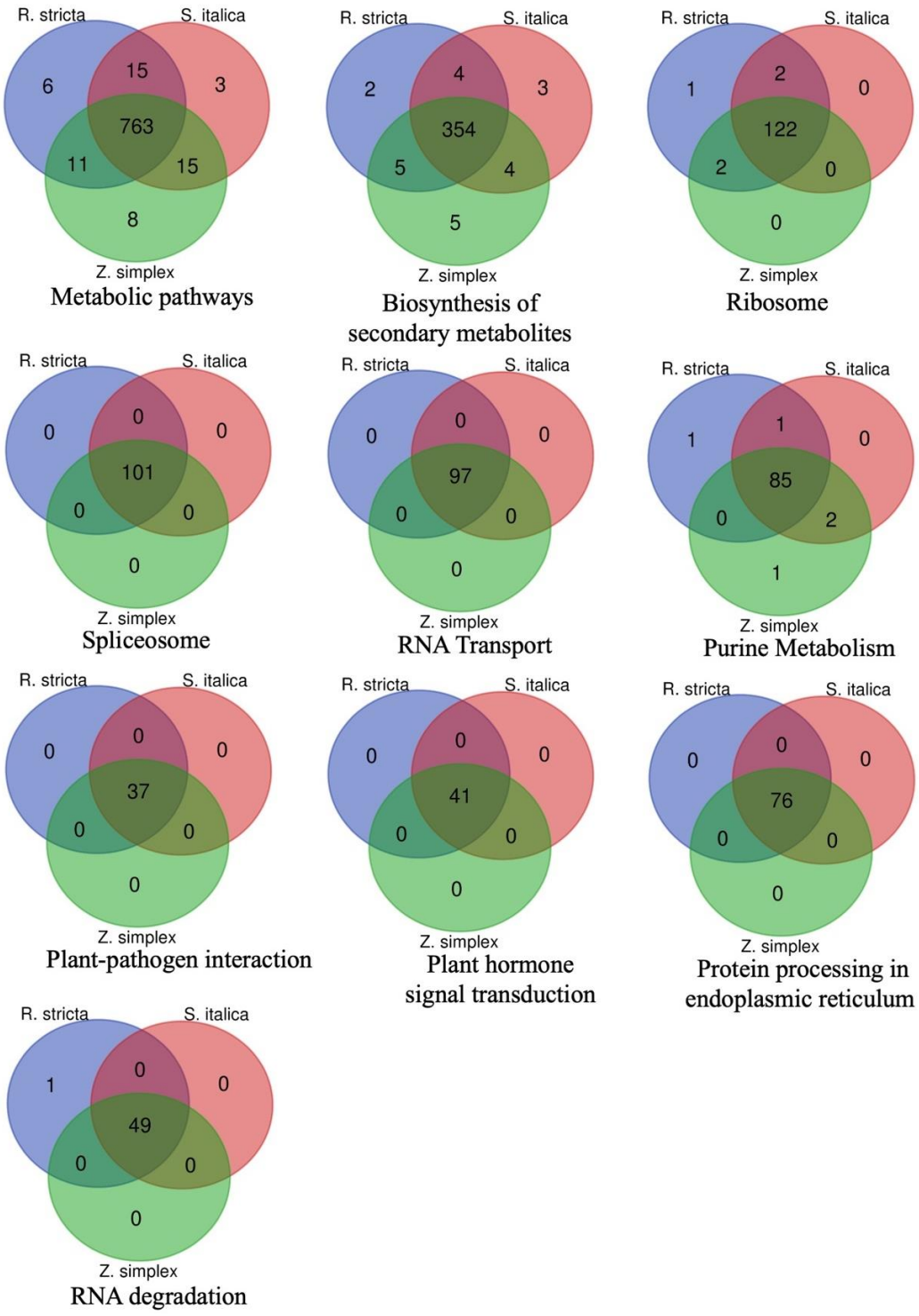

Figure 10. *Venn diagrams showing common KOs annotated in different KEGG pathways*

Furthermore, our finding also supports the previous study, which showed that under salinity stress, top ten KEGG pathways: "Metabolic pathways" (ko01100), "Biosynthesis of secondary metabolites" (ko01110), "Plant-pathogen interaction" (ko04626), "Plant hormone signal transduction" (ko04075), "Spliceosome" (ko03040), and "RNA transport" (ko03013), "Ribosome" ko03010, "Protein processing in

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endoplasmic reticulum" (ko04141), "Endocytosis" ko04144, and "Purine metabolism" (ko00230) are activated in *Chrysanthemum crassum* (Guan et al., 2017).

Our findings are aligned with previous studies and observed that simultaneously abiotic stress of drought, heat, and salinity significantly induce several common pathways in *R. stricta (1A), S. italica* (4A), and *Z. simplex* (5A). Thus, suggesting that these pathways play an essential role in maintaining the cellular homeostasis, plants' growth, and development under harsh stressed conditions (*Figs. 7, 8, 9, 10; Table S7*).

SSR discovery

SSRs are the most important molecular markers to study the evolutionary, genetics, and breeding in plants (da Costa et al., 2017). SSR markers has been widely used for the genetic linkage mapping (Hong et al., 2010), to understand the genetic diversity (Ren et al., 2014; Feng et al., 2016), and to identify of species (Shirasawa et al., 2013). Furthermore, a gene contains several functional *cis*-elements which influence the transcription and translation of mRNA and regulations of gene expression (Ahmed et al., 2011). Therefore, SSRs motifs and their variations can affect gene expression, translation, mRNA splicing, and mRNA export to the cytoplasm. A strong selective pressure would have been forced to select the SSRs on the coding region compared to the rest of the genomic region.

The unigenes were investigated for the SSR profiles and found the following results: (a) *R. stricta* (1A): There were 10,706 SSRs detected in 9,506 unigenes out of 71,116 examined unigenes (*Fig. 11A*). (b) *S. italica* (4A): There was 10,277 SSRs detected in 8,465 unigenes out of 59,274 examined unigenes (*Fig. 11B*); (c) *Z. simplex* (5A): There was 12,374 SSRs detected in 10422 unigenes out of 70,300 examined unigenes (*Fig. 11C*).

SNP discovery

In plants, SNP is the most usual type of DNA variation. SNPs could alter the structure and stability of proteins in both plants and animals (Milenkovic et al., 2018; Alzahrani et al., 2020; Bhardwaj et al., 2016). Detection of SNPs helps to understand plant species' genetic diversity, identify the complex traits including biotic and abiotic stress tolerance, and improve crop yields (Rafalski, 2002; Villordo-Pineda et al., 2015; Parida et al., 2012). Our analysis of SNPs profile found the following results:

(a) *R. stricta* (1A): There are 37,754 high-quality SNPs were detected from the unigenes including 22,845 transitions and 14,909 transversions (*Fig. 12A*). (b) *S. italica* (4A): There are 16,530 high-quality SNPs were detected from the unigenes including 10,406 transitions and 6,124 transversions (*Fig. 12B*). (c) *Z. simplex* (5A): There are 64,996 high-quality SNPs were detected from the unigenes including 39,014 transitions and 25,982 transversions. (*Fig. 12C*).

There are different classes of small non-coding RNAs, including miRNAs, siRNA, and tasiRNAs, are produced in plants for regulating gene expression at transcriptional or post-transcriptional levels (Khraiwesh et al., 2012). Under the normal physiology, abiotic, and biotic stress condition, plants produced various small non-coding RNA to modulate the gene expression for plant adaptation (Khraiwesh et al., 2012; Ahmed et al., 2014). Therefore, siRNA could be a design and expressed against specific genes or SNPs for gene silencing for better abiotic-stress tolerant plants (Ahmed et al., 2015; Ahmed and Raghava, 2011; Ahmed et al., 2020).

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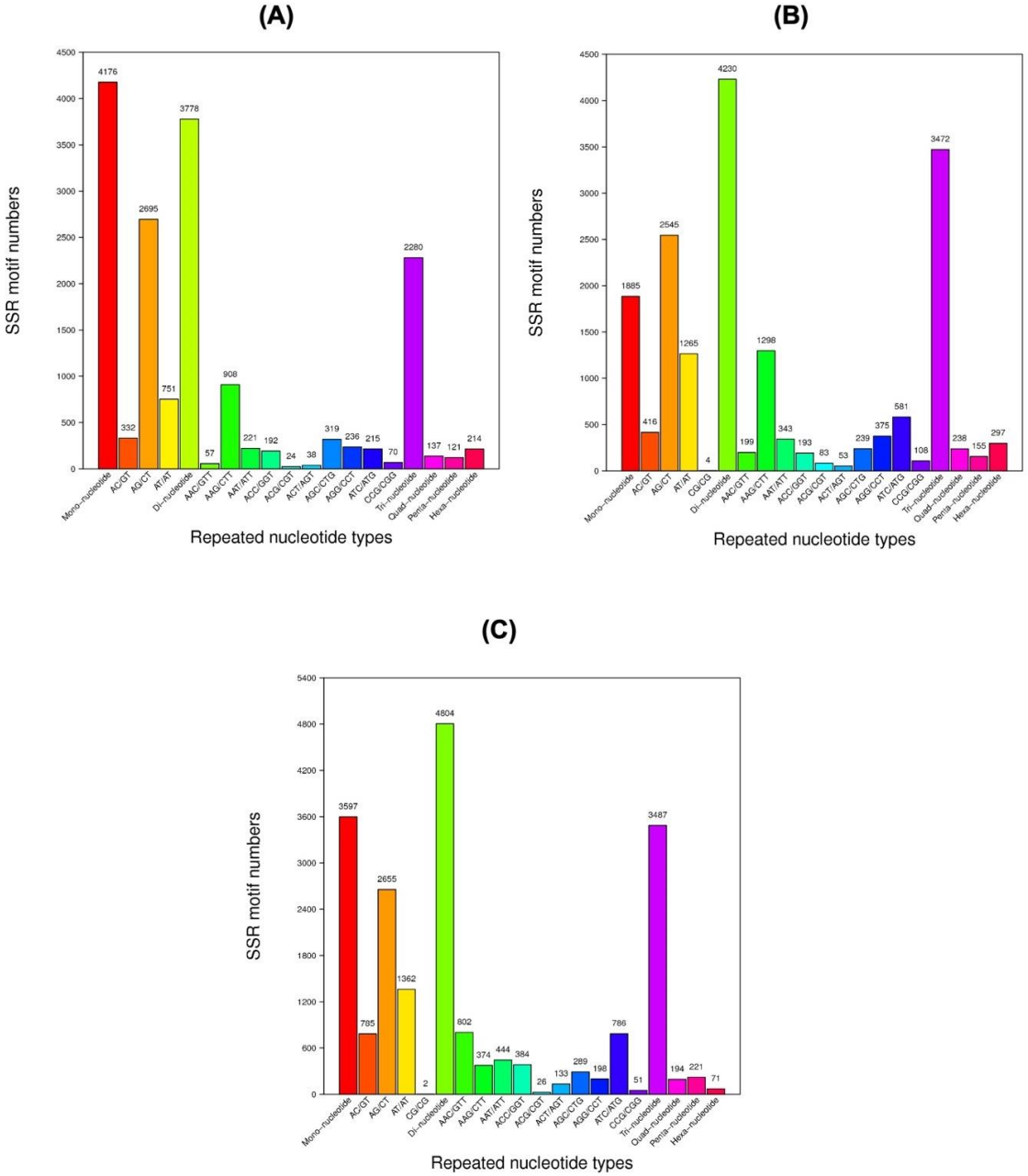

Figure 11. *SSR distribution of unigenes. (A) R. stricta (1A); (B) S. italica (4A); and (C) Z. simplex (5A)*

Protein-coding sequence prediction (CDS) and expression level analysis of Unigenes

For the analyses of CDS and predicted proteins. The length-frequency distributions of these Unigene CDSs by BLASTx and ESTscan were provided in *Figure 13* for *R. stricta*; *Figure 14* for *S. italica;* and *Figure 15* for *Z. simplex*. The complete list of CDS predicted sequence are provided in Supplementary data: (a) For *R. stricta*: *Tables S8, S9, S10,* and *S11;* (b) For *S. italica*: *Tables S12, S13, S14,* and *S15;* For *Z. simplex*: *Tables S16, S17, S18,* and *S19*. The expression level of Unigene from *R. stricta*, *S. italica* and *Z. simplex* is provided in supplementary data *Tables S20, S21,* and *S22,* respectively.

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Detailed annotation of unigenes and their statistics of *R. stricta* (1A), *S. italica* (4A), *and Z. simplex (5A)* are provided in the "Annotation_Folder" in supplementary data.

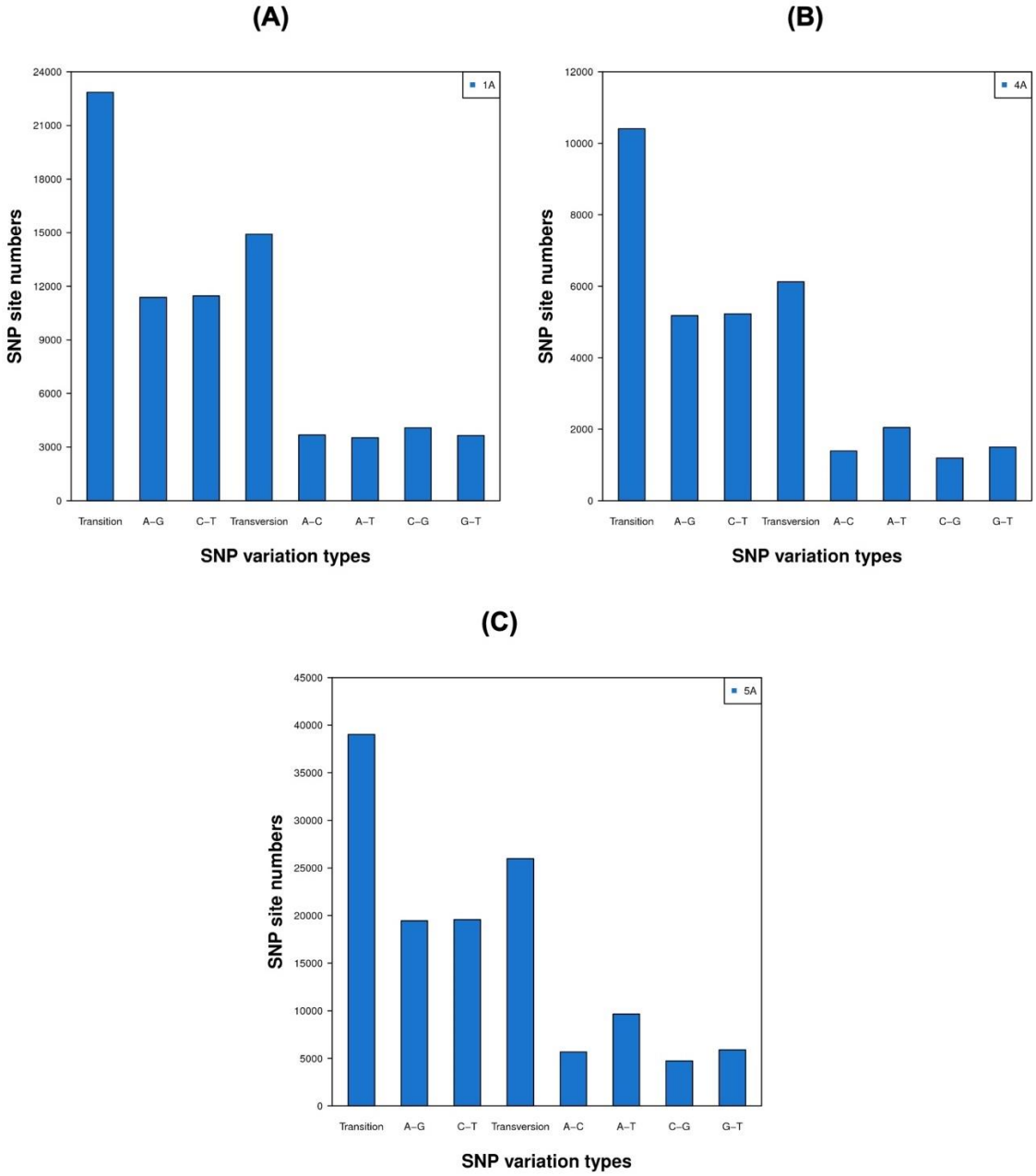

Figure 12. *SNP distribution of unigenes. (A) R. stricta (1A); (B) S. italica (4A); and (C) Z. simplex (5A)*

Transcription factors responding to combined abiotic stresses

Transcription factors (TFs) are important regulators of gene expression through binding to *cis*-acting elements of target genes. Alteration in the expression of TFs is one of the important regulatory mechanisms in plants to tolerate different abiotic stresses (REF). We analyzed all Unigenes sequences of *R. stricta* (1A), *S. italica* (4A), and *Z. simplex* (5A) for TFs prediction using PlantTFcat and found that 4426, 3895, and 5887

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Unigene sequences from *R. stricta* (*Table S23*), *S. italica* (*Table S24*) and *Z. simplex* (*Table S25*) were predicted as TFs, respectively. A comparative analysis of the TFs-family expressed in these three plants was given in *Table S26*. Furthermore, we identified the most abundant TF-families in unigenes across these plants. For this, we searched the TF-families associated with more than 100 unigenes across these plants and identified seven TFs: C2H2, C3H, CCHC(Zn), MYB-HB-like, PHD, WD40-like, and bHLH (*Table 6*).

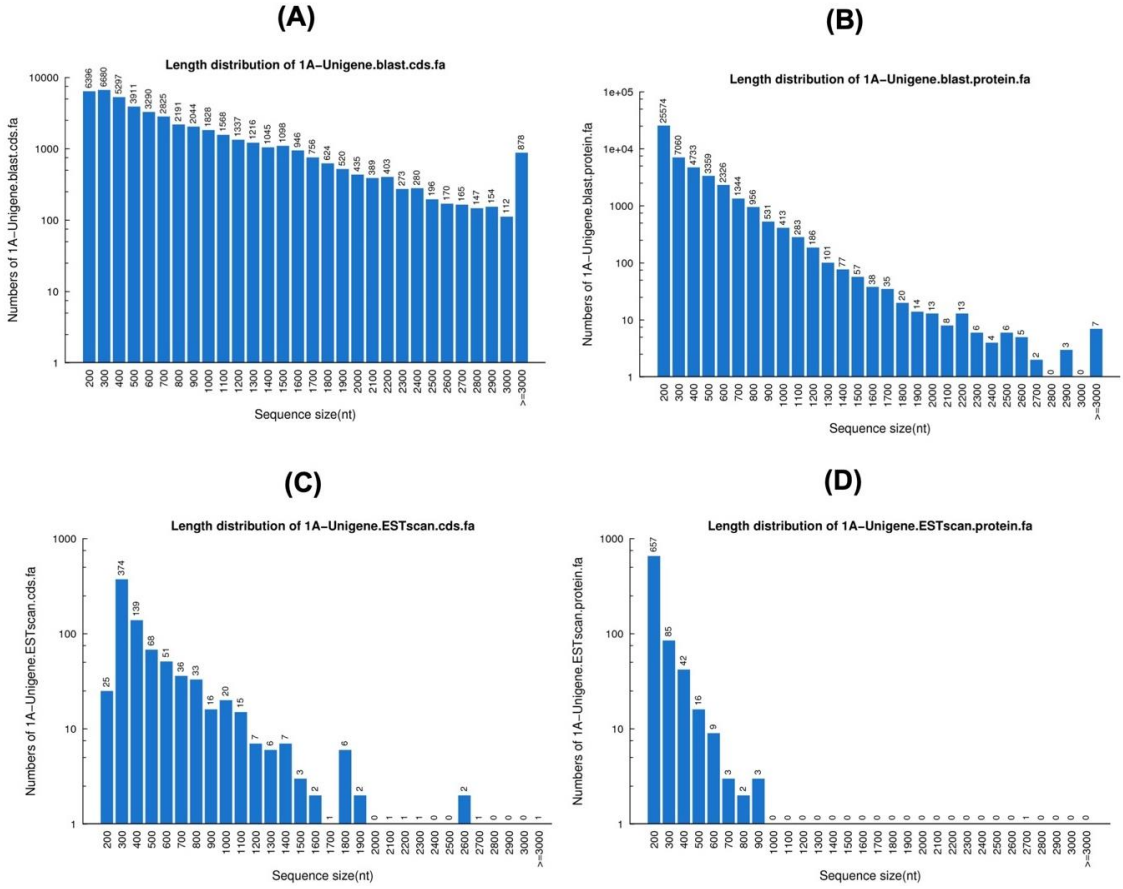

Figure 13. *Protein coding sequence (CDS) region prediction of R. stricta: (A) The length distribution of CDS nucleotide produced by searching unigenes sequences against various protein databases using BLASTx. (B) The length distribution of proteins predicted from CDS sequence using BLASTx. (C) The length distribution of CDS using ESTscan. (D) The length distribution of protein using ESTscan. The horizontal coordinates are CDS length, and the vertical coordinates are numbers of CDS*

Several studies found that the zin finger TFs families, including C2H2, C3H, and CCHC(Zn), are playing a very important role in plant growth, development, and abiotic stress such as high salt, drought, and high-temperature responses (Kielbowicz-Matuk, 2012; Han et al., 2020; Jiang et al., 2014; Lee and Kang, 2016). In addition, PHD family proteins act as epigenome readers and recruit various chromatin regulators and transcription factors that control gene expression which responds to environmental stresses (Sun et al., 2017; Sanchez and Zhou, 2011). Previous studies showed that overexpression of HbMYB1 TF in tobacco leads to enhanced resistance to UV-B stress

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and suppresses stress induces cell death (Li et al., 2015; Peng et al., 2011). A study found in wheat that WD40-like TFs are involved in abiotic stress response (Kong et al., 2015). bHLH TF family response to plant abiotic stresses such as drought, salt, and cold stress (Sun et al., 2018).

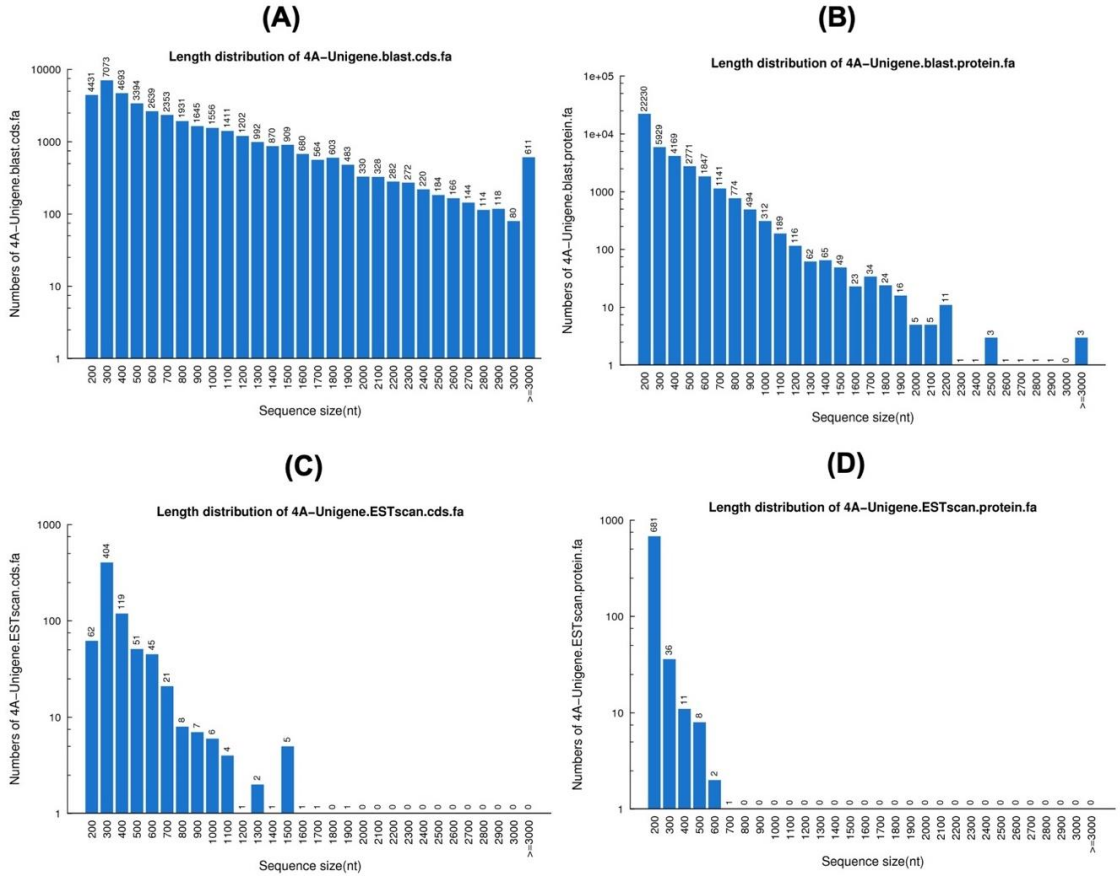

Figure 14. *Protein coding sequence (CDS) region prediction of S. italica: (A) The length distribution of CDS nucleotide produced by searching unigenes sequences against various protein databases using BLASTx. (B) The length distribution of proteins predicted from CDS sequence using BLASTx. (C) The length distribution of CDS using ESTscan. (D) The length distribution of protein using ESTscan. The horizontal coordinates are CDS length, and the vertical coordinates are numbers of CDS*

Table 6. *Comparative analysis of number of unigenes belongs to different TFs*

TFs-family	Family_type	Unigenes count		
		R. stricta	*S. italica*	*Z. simplex*
C2H2	Transcription factor	759	685	923
C3H	Transcription factor	156	152	201
CCHC(Zn)	Transcription factor interactor and regulator	213	264	353
MYB-HB-like	Transcription factor	248	225	358
PHD	Chromatin regulator	213	166	378
WD40-like	Transcription factor	868	654	890
bHLH	Transcription factor	129	131	177

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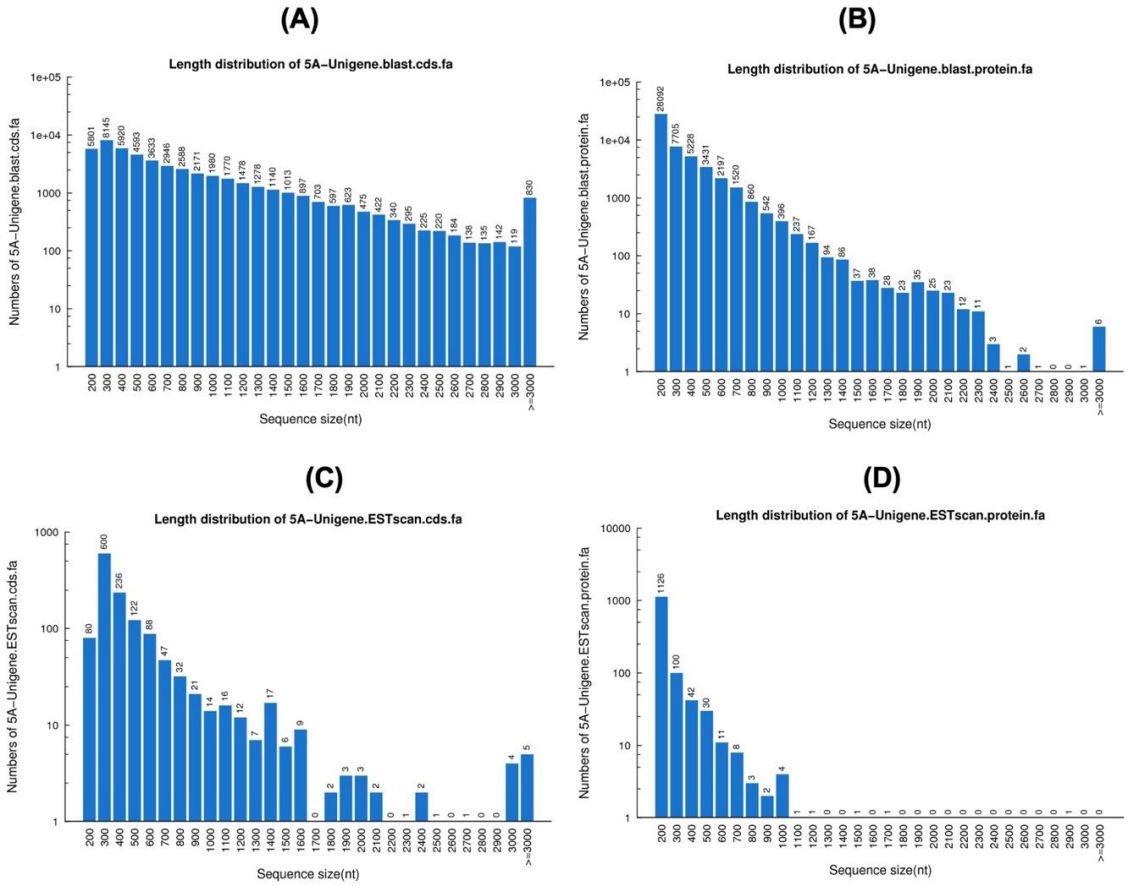

Figure 15. *Protein coding sequence (CDS) region prediction of Z. simplex: (A) The length distribution of CDS nucleotide produced by searching unigenes sequences against various protein databases using BLASTx. (B) The length distribution of proteins predicted from CDS sequence using BLASTx. (C) The length distribution of CDS using ESTscan. (D) The length distribution of protein using ESTscan. The horizontal coordinates are CDS length, and the vertical coordinates are numbers of CDS*

Abiotic stress triggers the osmotic and oxidative stresses signaling in a cell cause damage to the structure of proteins and cell membrane. However, stress tolerance plants induce the signaling which activates the transcription factors that express the stress-responsive genes to repair the damaged proteins and maintain cellular homeostasis (Wang et al., 2003). Our study could be used to understand gene regulatory networks during different stresses and their response mechanisms. Furthermore, finding TFs might be used to develop stress-tolerant crops that could increase the crop yield in the desert climate.

Conclusions

This study reported the first-time de novo transcriptome sequencing of leaf tissues of three abiotic stress plants: (a) *R. stricta,* (b) *S. italica,* and (c) *Z. simplex.* The illumine platform generated sequencing data assembled into 71,116; 59,274; 70,300 unigenes for *R. strict; S. italica;* and *Z. simplex*, respectively. Annotation and analysis of unigenes identified several candidate genes involved in abiotic stress, growth, development, and

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signal transduction pathways. Furthermore, our study identified the SSRs and SNPs in the datasets. Comparative analysis of Unigenes among all three plants identified seven transcription factor and transcriptional regulator responses to plants' abiotic stresses.

Abiotic stress triggers the osmotic and oxidative stresses signaling in a cell cause damage to the structure of proteins and cell membrane. However, stress tolerance plants induce the signaling which activates the transcription factors that express the stress-responsive genes to repair the damaged proteins and maintain cellular homeostasis (Wang et al., 2003). Our study could be used to understand how gene regulatory networks behave during different stresses and how networks induce the response mechanisms. Therefore, this study and datasets would be very useful for understanding the mechanism of abiotic stress management in plant studies in the future, and will also help improve crop productions in stressed geographical locations.

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Author contributions. Dr. Mohammed Baeshen is the PI of the project, and along with Prof. Nabih Baeshen they designed the project along with Prof. John Heulsenbeck, Dr. Baeshen and Prof Baeshen determinate the study location, collected the samples of the study, supervised the nucleic acid extraction and following up the sequencing results for further transcriptomic analysis. Dr. Firoz along with team wrote the project proposal and analyzed along with Prof. Tarek Musa the transcriptomic data. All other part of the team revised the manuscript and prepare it for publication.

Declaration of competing interests. The authors declare that they have no conflict of interests.

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ELECTRONIC APPENDIX

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SEXUAL DISCRIMINATION AND FECUNDITY OF BARBEL STEED (*HEMIBARBUS LABEO*) IN THE JINJIANG RIVER, CHINA

TUO, Y.[1,2] – LI, J.[3] – XIAO, T.[2*] – WANG, H.[2]

[1]*College of Life Science and Resources Environment, Yichun University, Yichun 336000, China*

[2]*Hunan Engineering Technology Research Center of Featured Aquatic Resources Utilization, Hunan Agricultural University, Changsha 410128, China*

[3]*College of Mathematics and Computer Science, Yichun University, Yichun 336000, China*

**Corresponding author*
e-mail: tyxiao1128@163.com

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Abstract. In order to establish a method to identify the sex of *H. labeo* by quantifying morphological characteristics and analyze their fecundity, 19 morphological characteristics and the condition factor from 180 *H. labeo* individuals (123 for modeling and 57 for verification) were collected from the Jinjiang River in China, were measured, standardized and analyzed; and 81 female individuals of *H. labeo* with stage III or IV of ovarian were randomly collected for analyzing fecundity in this study. Based on the data, six morphological characteristics were screened from the 19 total morphological characteristics by a stepwise discriminant method and used to establish discriminant equations. The correct identification rate was 93.50% and the verified accuracy rate was 91.23%. The individual absolute fecundity (*F*) of *H. labeo* in the Jinjiang River was 25945.57 ± 19519.32 eggs. The *F* of *H. labeo* positively related to body weight, net weight, gonadal weight, and age, but negatively related with the body length, body weight × body length, maturity coefficient, and body fat. These results provided important reference information for the protection of wild *H. labeo* in the Jinjiang River.
Keywords: *community ecology, heteromorphism, morphological discrimination, multivariate analysis, reproduction*

Introduction

Sexual discrimination and fecundity are two fundamental research topics in fish population ecology and aquaculture, and their results provide important information to conserve wild fish resources and carry out artificial propagation (Macinnis and Corkum, 2000; McEvoy et al., 2009). Sexes can be distinguished by the differences in body size, body color, and secondary sex characteristics for fish with obvious heteromorphism (Jiang et al., 2019a, b). However, it is very hard to distinguish the sexes of fish that do not have obvious heteromorphism, such as *Gadus morhua* (McEvoy et al., 2009), *Acrossocheilus wenchowensis* (Xu et al., 2006), *Acanthorhodeus chankaensi* (Chen et al., 2013a), and *Eryghroculter ilishaeformis* (Chen et al., 2013b). Although anatomy and observation of gonad can accurately identify sex, there are a lot of limitations in the use of anatomy and other sex identification methods, which are not conducive to protecting studied fish and implement artificial reproduction. Ultrasound has been used for determining sex in some marine fish species (Davie et al., 2003; Glebe et al., 2003; McEvoy et al., 2009). However, this method does not suit to stream fish species. In addition, accuracy rates of this method are influenced by the maturity of fish (Martin et al., 1983). Methods that identify male and female individuals by quantitative indicators of body shape characteristics have been successfully applied in many fish, such as *Ilisha*

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elongate (Ni and Chen, 2003), *Aniguilla japonica* (Guo et al., 2011), *Hemibarbus maculatus* (Tuo et al., 2020), and *Scatophagus argus* (Wu et al., 2014). Applying discriminant equation established according to fish morphological characteristics, the accuracy of sex identification can be as high as 85% (Guo et al., 2011; Wu et al., 2014).

Fecundity generally refers to the average number of mature eggs per female before spawning. Fecundity is an important biological characteristic, which reflects the adaptability of species or population to environmental changes and is related to the supplement of the population (Yatuha et al., 2018). Its change reflects the influence of the environment and adaptability of a population (Yin, 1995; Santangeli et al., 2017). Individual fecundity is not only related to genetic characteristics, environmental factors, nutritional status, and fishing pressure (Wootton, 1990; Niemuth and Klaper, 2015; Santangeli et al., 2017), but also related to biological indicators such as age, body length, and body weight (Macinnis and Corkum, 2000; He et al., 2007). The relationship between individual fecundity and its biological indexes can not only correctly evaluate the change of the fish population, but also provide a basis for the protection and management of fishery resources.

Hemibarbus labeo is a cyprinid fish that occurs all over East Asia, such as eastern mainland China, Japan, and Korea (Lin et al., 2007; Wang et al., 2016). Most individuals of the species are bottom-dwellers in streams and feed on aquatic insects (Lin et al., 2007). Unfortunately, due to habitat destruction and human over-consumption, its wild populations have been seriously threatened in the past decades (Lin et al., 2007). Although polymorphic microsatellite loci in the fish have been isolated and characterized, their population structures, especially sex composition and fecundity of their populations are still rarely investigated. One of the main reasons that block the investigation is there is no obvious difference in external morphology between male and female *H. labeo*. To provide technical support for the sex investigation of *H. labeo*, we established a technical method to identify the sex of *H. labeo* by quantifying its morphological characteristics, and we also analyzed the fecundity of *H. labeo* in the present study. Our results provided an important technical reference for the investigation and conservation of *H. labeo* population.

Materials and methods

Study area

Jinjiang River is a first-class tributary on the left bank of the lower reaches of the Ganjiang River. The basin is in the western part of Jiangxi Province. The average river width of the sampling section is 126.46 m, and the average flow velocity is 0.21 m/s. The average water depth of the nearshore is 0.77 m. The river is sand and gravel bottom, and the water quality is national class III according to the Environmental Quality Standards for Surface Water of China (GB 3838-2002).

Sample collection

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hunan Agricultural University (permit number 20171009). To sexual discrimination, a total of 123 individuals of H. labeo were collected from the Shanggao section (114°28′ - 115°10′ E, 28°02′ - 28°25′ N) of the Jinjiang River in Jiangxi Province of China from March to December 2014 for modeling. Other 57

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individuals of H. labeo were collected at the same section of the Jinjiang River from January to November 2015 for verification. To analyze fecundity, 81 female individuals of H. labeo with stage III or IV of ovarian maturity were randomly collected from the Shanggao section of the Jinjiang River from March to December 2014. The fish samples were caught by screen meshes (the mesh size is 2 cm), ground cages (the mesh size is 0.5 cm), and electrofishing techniques. The samples were put into a container with oxygen pumps for continuously oxygenation and quickly transported to the laboratory for temporary feeding. Before morphological measuring, the samples were anesthetized by anesthetics MS-222 (50 mg/L) for 5 min.

Morphological analysis

Body length (L), head length (L_{HL}), head height (L_{HH}), head width (L_{HW}), snout length (L_{SL}), postorbital length (L_{PL}), eye diameter (L_{ED}), interorbital width (L_{IW}), mouth breadth (L_{MB}), mouth length (L_{ML}), body highness (L_{BH}), caudal peduncle length (L_{CL}), caudal peduncle height (L_{CH}), the distance between snout and pelvic fin (L_{PSL}), pectoral fin length (L_{PFL}), caudal fin length (L_{CFL}), the distance between snout and dorsal fin (L_{DSL}), dorsal fin coxal length (L_{DFL}), and the distance between pelvic fin and anal fin (L_{PAFD}) of each sampled individual were measured using a ruler and Vernier calipers (*Fig. 1*). Then the fish samples were dissected and distinguished male and female through naked eye observation of their gonads. Gonads of the samples that could not be sex identified were fixed by 10% formalin solution and identified their sex through histological observation as previous studies (Li et al., 2000; Blazer, 2002). Bodyweight (W), gland weight (W_g), and net weight (W_n) were weighed by ML-T precision electronic balance with 0.01 g of the accuracy (Mettler Toledo, Switzerland). The data were accurate to two decimal places. The condition factor (K), and gender heteromorphic index (GHI) were calculated as *Equations 1* and *2:*

$$K = 100 \times \frac{W}{L^3} \quad \text{(Eq.1)}$$

$$GHI = 1 - \frac{\overline{L_{min}}}{\overline{L_{max}}} \quad \text{(Eq.2)}$$

where K was the condition factor, W was body weight, L was body length, GSI was the gonadosomatic index, $\overline{L_{min}}$ was the mean of the body length of the fish with shorter body length, and $\overline{L_{max}}$ was the mean of the body length of the fish with longer body length.

To overcome the influence of individual size differences on the local morphological characteristics, the 18 proportional morphological characteristics were calculated by dividing the measured morphological data of each fish by its body length.

Fecundity analysis

Fecundity was calculated by weight method (Ni, 2000), i.e. took the whole ovary and weighed, then counted all the eggs that begin to deposit yolk or had already deposited yolk of 0.1 -0.5 g front, middle and rear ovary, respectively. The individual absolute fecundity (F) was calculated with the average value of the three parts of the ovary, i.e. F = total number of eggs / (sample weight × whole ovary weight). The relative fecundity of body length (F_L), relative fecundity of body weight (F_W), maturity

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coefficient (*GSI*), and body fat (*K*) were calculated according to *Equations 3, 4, 5,* and *6,* respectively:

$$F_L = \frac{F}{L} \quad \text{(Eq.3)}$$

$$F_w = \frac{F}{W_n} \quad \text{(Eq.4)}$$

$$GSI = \frac{W_g}{W} \times 100 \quad \text{(Eq.5)}$$

$$K = \frac{W}{L^3} \times 100 \quad \text{(Eq.6)}$$

where F_L was the relative fecundity of body length, F was the individual absolute fecundity, L was the body length, F_w was the relative fecundity of body weight, W was the body weight, W_n was the net weight, and W_g was the gonadal weight.

Eight intact scales in the second row above the lateral line of the middle and anterior sides of the fresh fish were taken to age identification according to previous reports (Xie et al., 1988; Xu et al., 2009). After taking photos with DMBA300 microscope (Motic, China), the scale diameter and wheel diameters of scales were measured with Motic Images Advanced 3.2 software (Motic, China).

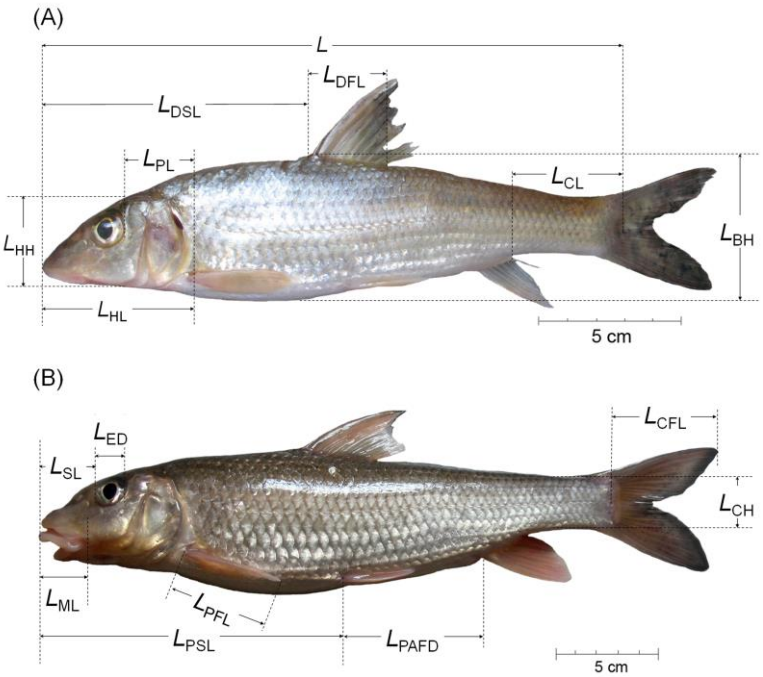

Figure 1. *External morphologic images and morphological measurement characteristics of male (A) and female (B) H. labeo. All of 19 morphological characteristics of each sample were measured. L, body length; L_{HL}, head length; L_{HH}, head height; L_{SL}, snout length; L_{PL}, postortital length; L_{ED}, eye diameter; L_{ML}, mouth length; L_{BH}, body highness; L_{CL}, caudal peduncle length; L_{CH}, caudal peduncle height; L_{PSL}, distance between snout and pelvic fin; L_{PFL}, pectoral fin length; L_{CFL}, caudal fin length; L_{DSL}, distance between snout and dorsal fin; L_{DFL}, dorsal fin coxal length; L_{PAFD}, distance between pelvic fin and anal fin*

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Data analysis

Data were expressed as mean ± standard deviation (S.D.). The comprehensive indexes with the largest eigenvalue vector were calculated and selected from the morphological characteristics. Kolmogorov-Smirnov test for normal distribution and Levene's Test for equality of variances was conducted firstly, then independent t-test was used to compare the body length, body weight and the comprehensive indexes of male and female, and One-way ANOVA with Tukey-Kramer post-hoc test was used to compare the fecundity indices among different age groups. The principal components with larger contribution rates were determined by the principal component analysis (PCA). Stepwise discriminant regression was used to further analyze and screen out the characteristics with significant differences between male and female populations, and the discriminant equations of female and male were established. Five correlation models (linear correlation, power correlation, exponential correlation, logarithmic correlation, quadratic correlation) were used to fit the relationship between individual fecundity and L, W, Wn, Wg, age, GSI, K, and L×W. The best correlation model was the one with the largest coefficient of determination R2. The multiple parameters between the individual fecundity and the biological indexes were described by multiple stepwise regression equation. All statistical analyses were completed by R (R Core Team, 2014) and SPSS 19 software. The significant level was set to $p = 0.05$.

Results and discussion

Morphological characteristics and sexual discrimination

The body lengths of the female modeling samples (50/123) were ranged from 13.00 to 29.00 cm (20.90 ± 3.39 cm), and their body weights were range from 43.10 to 432.88 g (202.07 ± 103.26 g). The body lengths of the male modeling samples (73/123) were ranged from 11.00 to 25.00 cm (17.17 ± 3.17 cm) and their body weights were ranged from 25.96 to 260.00 g (105.32 ± 63.91 g) (*Table A1* in the *Appendix*). Although there were significant differences between female and male in body length (Independent t-test, $t = 6.15$, $p < 0.001$) and body weight (Independent t-test, $t = 5.90$, $p < 0.001$), there was a large overlap between male and female modeling *H. labeo* (*Fig. 2*). The *GHI* of the modeling group was 0.18. The body lengths of the female verification samples (28/57) were ranged from 11.60 to 34.00 cm (20.75 ± 4.23 cm), and their body weights were range from 27.50 to 629.00 g (206.71 ± 136.29 g). The body lengths of the male verification samples (29/57) were ranged from 11.60 to 25.5 cm (18.15 ± 4.0 cm), and their body weights were range from 25.50 to 361.10 g (136.05 ± 91.40 g). Similarly, although there were significant differences between female and male in body length (Independent t-test, $t = 2.63$, $p = 0.011$) and body weight (Independent t-test, $t = 2.44$, $p < 0.018$), there was a large overlap between male and female verification *H. labeo* (*Fig. 2*). The *GHI* of the verification group was 0.13.

The PCA of morphological characteristics of *H. labeo* samples showed that 86.38% of the variation was explained by the first two principal components extracted (*Table 1*). For the first principal component, L, L_{HH}, L_{HW}, L_{SL}, L_{PL}, L_{IW}, L_{BH}, L_{PAFD}, L_{PFL}, and L_{DFL} exhibited large negative load factors, and K exhibited large negative load factors in the second principal component (*Table 1*). Most of the screened morphological characteristics were significantly different between female and male modeling samples. The scores of the first principal component of females and males were significantly

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different (independent *t*-test, $t = -5.47$, $P < 0.001$), but no significant difference was detected in the second principal component (independent *t*-test, $t = -1.20$, $P > 0.05$). Taking the first and second principal components as the X- and Y-axis, the male and female samples overlapped greatly, and only partial samples were distinguished (*Fig. 3A*).

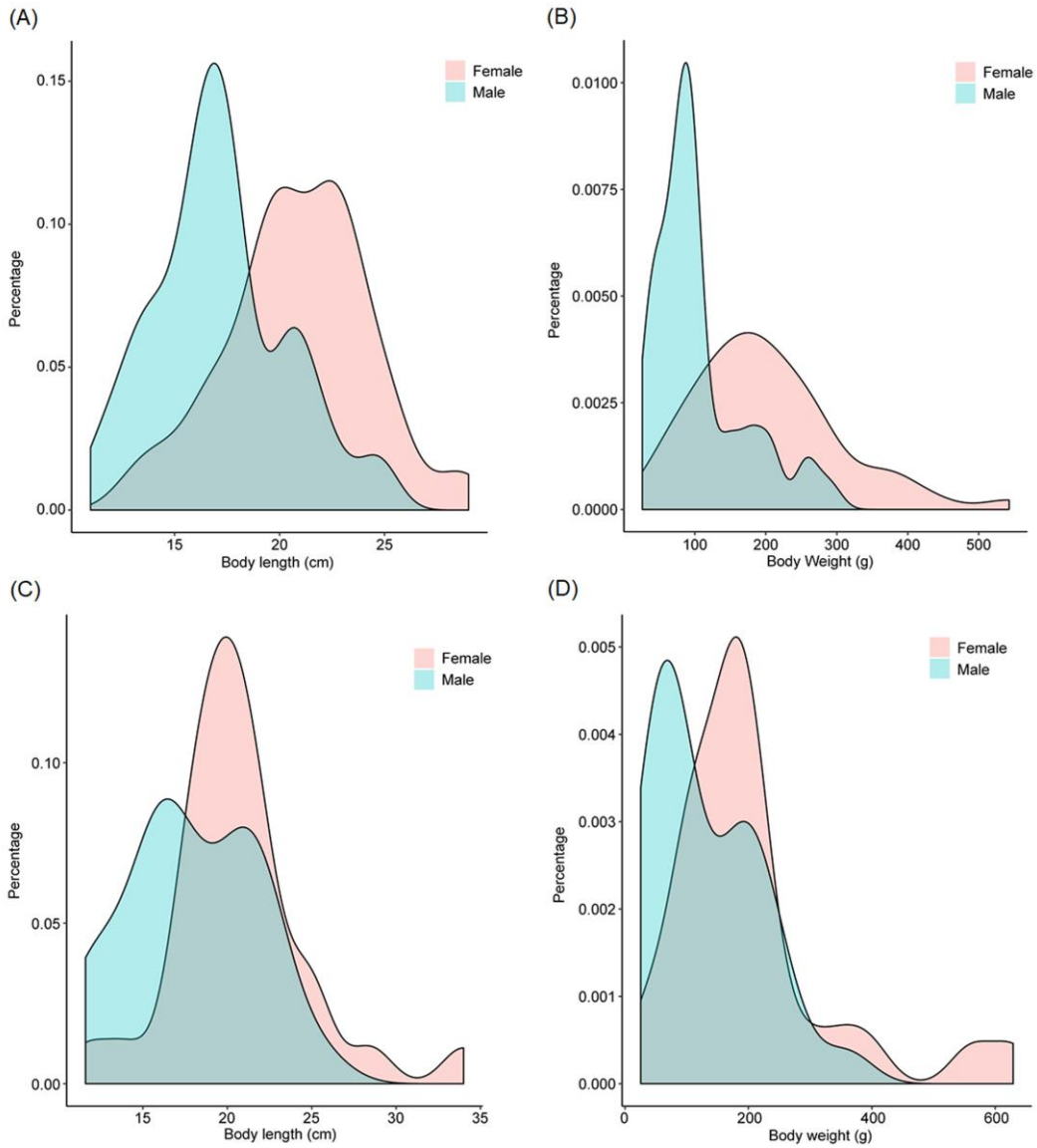

Figure 2. *Body length and body weight distributions of male and female H. labeo. (A), density distributions of body length of modeling samples; (B), density distributions of body weight of modeling samples; (C), density distributions of body length of verification samples; (D), density distributions of body weight of verification samples*

Six morphological characteristics with significant discrimination effect of sex, i.e. L_{PL}, L_{MW}, L_{BH}, L_{CH}, L_{PFL}, and L_{DFL}, were screened from the 18 proportional morphological characteristics and condition factor through backward stepwise discriminant analysis (Wilks' Lambda: 0.3494; $F(6,116) = 35.9992$, $p < 0.001$), and the discriminant formulas of *H. labeo* were established as *Equations 7* and *8*.

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$$Y_1=-327.54+1143.57L_{PL}+111.70L_{MW}+89.83L_{BH}+1047.57L_{CH}+545.30L_{PFL}+2352.36L_{DFL} \quad \text{(Eq.7)}$$

$$Y_2=-338.85+1296.42L_{PL}+3.91L_{MW}-27.26L_{BH}+1351.29L_{CH}+686.96L_{PFL}+2192.87L_{DFL} \quad \text{(Eq.8)}$$

If $Y_1 > Y_2$, the fish was female, otherwise it was male. The frequency distribution was obtained by calculating the discrimination score of each individual. It showed that the model could distinguish the sex of *H. labeo* (*Fig. 3B*). The six morphological characteristics of the 123 fish individuals were substituted into the discrimination equations, and Y_1 and Y_2 were calculated respectively for sex identification. After anatomical verification, only 8 samples were misjudged in terms of sex, with a misjudged rate of 6.5% (*Table 2*). The results of male and female discrimination of 57 *H. labeo* individuals in the verification group showed that the accuracy rate of male and female discrimination was 93.10% and 89.28%, respectively, and the comprehensive accuracy rate was 91.23% (*Table 3*), which was consistent with previous studies in other fish (Ni and Chen, 2003; Guo et al., 2011; Wu et al., 2014).

Table 1. *Loading factors of each morphological characteristics on the first two axes of principal component analysis*

Morpholopical variables	Loading factor	
	P1	**P2**
L	-0.98	0.12
L_{HL}	-0.90	0.15
L_{HH}	-0.97	-0.03
L_{HW}	-0.94	-0.08
L_{SL}	-0.93	0.09
L_{PL}	-0.94	0.03
L_{IW}	-0.95	-0.16
L_{ED}	-0.84	0.17
L_{MB}	-0.89	0.06
L_{ML}	-0.88	-0.15
L_{BH}	-0.92	-0.28
L_{CL}	-0.81	-0.13
L_{CH}	-0.97	-0.07
L_{PSL}	-0.93	0.20
L_{PFL}	-0.93	0.00
L_{CFL}	-0.91	-0.05
L_{DSL}	-0.94	0.16
L_{DFL}	-0.94	0.12
L_{PAFD}	-0.84	0.19
K	-0.34	-0.90
Variance explained	80.53%	5.85%

Morphological characteristics with the main contribution to each factor are highlighted by bold
L, body length; L_{HL}, head length; L_{HH}, head height; L_{HW}, head width; L_{SL}, snout length; L_{PL}, postortital length; L_{IW}, interorbital width; L_{ED}, eye diameter; L_{ML}, mouth length; L_{MB}, mouth breadth; L_{BH}, body highness; L_{CL}, caudal peduncle length; L_{CH}, caudal peduncle height; L_{PSL}, distance between snout and pelvic fin; L_{PFL}, pectoral fin length; L_{CFL}, caudal fin length; L_{DSL}, distance between snout and dorsal fin; L_{DFL}, dorsal fin coxal length; L_{PAFD}, distance between pelvic fin and anal fin; K, condition factor

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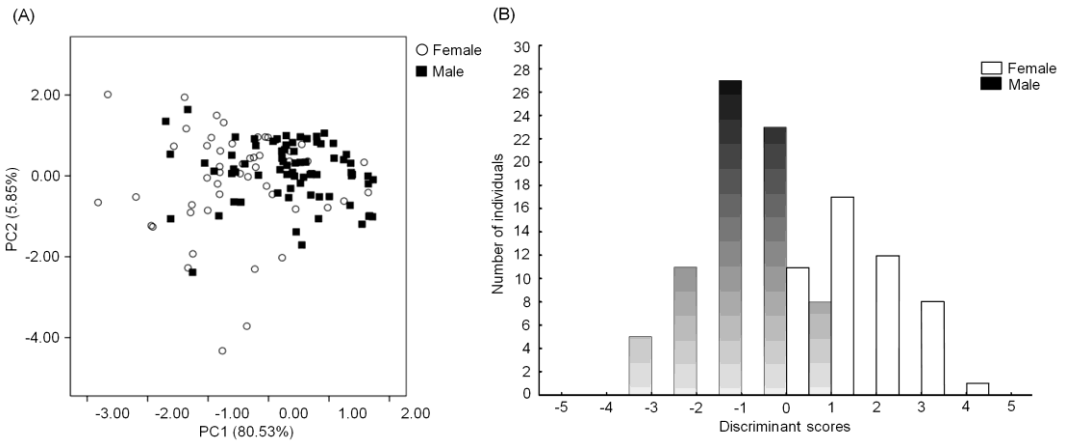

Figure 3. *Principal component analysis profile (A) and frequency distribution of discrimination values of stepwise discriminant function analysis (B)*

Table 2. *Discriminant analysis results of stepwise discriminant function analysis based on standardized morphological data of H. labeo*

Sex	Identified sex	Predicted result		Accuracy of discrimination (%)	Total accuracy of discrimination (%)
		♂	♀		
Male	73	71	2	97.26	93.50
Female	50	44	6	88	

Table 3. *Results of discriminant verification for 57 H. labeo samples*

Sex	Identified sex	Predicted result		Accuracy of discrimination (%)	Total accuracy of discrimination (%)
		Male	Female		
Male	29	27	2	93.10	91.23
Female	28	25	3	89.29	

As an important issue of fish reproductive capacity, sex ratio and sex differences in behavior have always been concerned by fish ecologists and aquaculture experts (Teixeira and Musick, 2001; Kumar et al., 2006). Identification of fish sex is an important prerequisite for calculating the sex ratio of the fish. However, for fish that does not have obvious heteromorphism, there was no suitable method to identify the sex of freshwater fish living in stream except for anatomical identification and ultrasonic identification of the sex for marine fish. Our results provided an accurate method to identify the sex of *H. labeo*. However, the data collection of fish morphology is still tedious work, which also limits the wide application of the current method of sex distinguish by quantitative morphological characteristics. In view of the development of computer technology, especially the automatic image recognition technology (Reeder et al., 2004), the development of automatic recognition and acquisition of fish morphological data technology methods and software will greatly make up for the shortcomings of the technology and contribute to the wide application of the technology. In addition, although our results showed that using six morphological characteristics with significant discrimination the effect could distinguish the sex of *H.*

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labeo, considering fish morphological parameters probably changed with the environment changes (Poulet et al., 2005; Michel et al., 2017), whether the discriminant formulas suited for other *H. labeo* living in other habitats still needs further verification.

Biological indices and individual fecundity of H. labeo

A total of 81 female *H. labeo* individuals were analyzed. The individuals were composed of six ages and were mainly 1^+ and 2^+ age (*Table A1*). The minimum age of sexual maturity was 0^+. The average body length and body weight of all female individuals were 20.82 ± 4.26 cm, and 207.11 ± 133.39 g, respectively. The F, F_L, and F_w ranged from 1142.20 to 87047.68 (25945.57 ± 19519.32) eggs, 96.80 to 3481.90 (1158.88 ± 716.48) eggs per cm, and 13.03 to 296.32 (126.29 ± 62.09) eggs per gonad. There were significant differences in the F (one-way ANOVA, $F = 12.877$, $p < 0.001$) and F_L (one-way ANOVA, $F = 7.096$, $p < 0.001$) among different age groups, and the F and F_L increased with age (*Table A1*). There was no significant difference in the F_W among different age groups (one-way ANOVA, $F = 1.063$, $P > 0.05$). The F and F_L increased with age, while the F_W fluctuated with age in a certain range (*Table A1*). The results of correlation analysis between the F and biological indexes of *H. labeo* showed that the F had a power correlation with the *GSI* and *K*, quadratic correlation with body weight and net weight, linear correlation with body length, gonad weight, body length × body weight, and age (*Fig. 4; Table A2*).

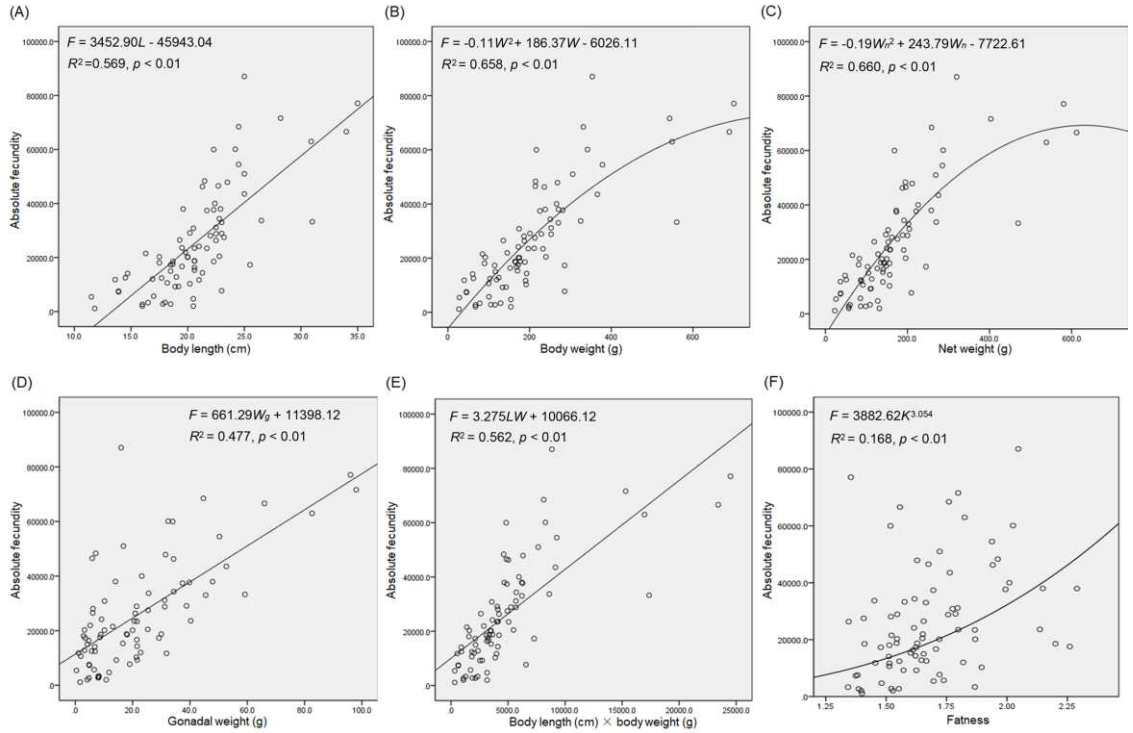

Figure 4. *Correlation between absolute fecundity and biological indices of H. labeo in the Jinjiang River. F, absolute fecundity*

The sexual maturity age of *H. labeo* was more than 4^+ years in Heilongjiang River basin (Nicholsky, 1960), and it was 2^+ years in the Yangtze River and its tributaries

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(Department of Fish Research, Hubei Institute of Hydrobiology, 1976). Our results showed that sexual maturity age of *H. labeo* in the Jinjiang River was 1^+ year, which indicated that the sexual maturity age of *H. labeo* in the Jinjiang River was younger. Comparing with other fish species in the same genus, the sexual maturity age of *H. labeo* in the Jinjiang River was the same with *H. medius* in the Beijing River in Guangdong of China (Lan et al., 2010), and *H. maculates* in the South Lake in Wuchang of China (Gong et al., 1990) and in the Yuanhe River in Jiangxi of China (Tuo, 2013), which showed that the adaptation mechanism of *Hemibarbus* in river and lake that locate at the middle reach of the Yangtze River under the current environmental pressure and fishing pressure, because of females from populations with high predation pressure mature earlier and at a smaller size (Reznick et al., 2004). Increasing the number of breeding population was conducive to the generation of more offspring, to supplement the shortage of natural population. The relative fecundity is used to reflect the reproductive strategies of fish (Yin, 1995). The higher F_W indicated that the eggs of *H. labeo* in the Jinjiang River were a small size and large amount. The lack of nutrients might lead to less yolk accumulation. Simultaneously, it reflected the compensatory adaptation of *H. labeo* in the Jinjiang River to environmental changes. This was a natural reproduction strategy formed under specific environmental conditions (Zúñiga-Vega et al., 2017). It showed the current situation of resource decline. The breeding strategy of *H. labeo* in the Jinjiang River tended to r-strategy to resist environmental pressure and ensure the continuation of the race.

Multiple parameter relationships between the individual fecundity and biological indexes of H. labeo

The relationship between the F and the L, W, W_n, W_g, age, GSI, K, and $L \times W$ was fitted by multiple regression analysis, and the regression equation was as *Equation 9* with $R^2 = 0.689$ (N = 81).

$$F=38718.49-2828.99L+312.39W+216.18W_n+53.54W_g-9.08W\times L+730.75\text{age}-126.62GSI-8093.95K \quad \text{(Eq.9)}$$

The stepwise regression equation between the F and biological indexes was as *Equation 10* with $R^2 = 0.665$ (N = 81).

$$F= -7080.098+265.282W-4.520W\times L \quad \text{(Eq.10)}$$

Equation 9 showed that the F increased with the increase of W, W_n, W_g, and *age*, but decreased with the increase of L, $W \times L$, GSI, and K. *Equation 10* showed that the F was positively correlated with the W and negatively correlated with the $W \times L$.

The individual fecundity of fish is not only related to the essential characteristics of species and environmental conditions, but also significantly related to biological indicators (Kraus et al., 2000; Macinnis and Corkum, 2000; Vrtilek and Reichard, 2016). Our results showed that the F was positively quadratic correlated with W and W_n, which was similar to *Hemibarbus maculates* (Tuo, 2013), *Pelteobagrus fulvidraco* (Liu, 1997), *Schizothorax lissolabiatus* (Xiao and Dai, 2010), and *Opsariichthy sbidens* (Li et al., 2010). The relationship between the F of *H. labeo* in the Jinjiang River and body fat was not significant, which was similar to *Coregonus ussurinsis* (Dong et al., 1997), *Pseudosciaena crocea* (Zheng and Xu, 1964), *Culter albumus* (Wang et al., 2007), and

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Xenocyprism icrolepis (Liu et al., 2010). Our results also showed that the *F* of *H. labeo* in the Jinjiang River was significantly affected by the biological index of *W*. Therefore, the relationship between body weight and fecundity could be used to predict the absolute fecundity of *H. labeo* in the Jinjiang River.

Distribution of egg diameter and spawning type of H. labeo

The frequency analysis of egg diameter distribution showed that there were two egg diameter groups in stage III ovary of *H. labeo* (*Fig. 5A*). The range of egg diameters of *H. labeo* in stage III ovary was 0.53 - 1.50 (1.18 ± 0.40) mm. The frequency distribution of egg diameter in stage IV ovary showed a three-peak pattern. The ranges from left peak to right peak were 0.50 - 1.20 mm, 1.25 - 1.60 mm, and 1.7 - 2.15 mm (*Fig. 5B*), which were the 3 phases, 4 phases, and 5 phases of ovum, respectively. According to the variation trend of the maturity coefficient and the duration of the breeding period, it could be preliminarily inferred that the annual oviposition of *H. labeo* was three times.

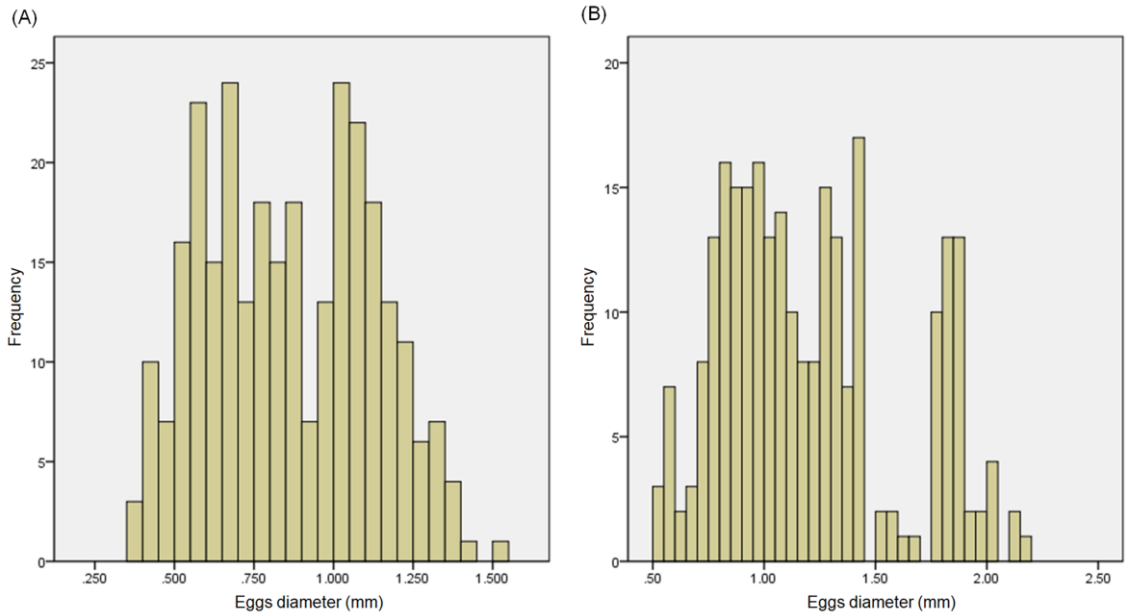

Figure 5. *Distribution of eggs diameter of H. Labeo with Ⅲ (A) and Ⅳ (B) maturity stages of ovaries*

Conclusion

Using six morphological characteristics with a significant discrimination effect could distinguish the sex of *H. labeo*, and the comprehensive accuracy rate was 91.23%. The *F*, F_L, and F_w of *H. labeo* in the Jinjiang River ranged from 1142.20 to 87047.68 (25945.57 ± 19519.32), 96.80 to 3481.90 (1158.88 ± 716.48) per cm, and 13.03 to 296.32 (126.29 ± 62.09) per g. The *F* of *H. labeo* in the Jinjiang River increased with the increase of *W*, W_n, W_g, and *age*, but decreased with the increase of *L*, *W*×*L*, *GSI*, and *K*. However, whether the discriminant formulas suited for *H. labeo* living in other habitats still needs further verification. In addition, based on the results of this study, the automatic identification of sex and the automatic evaluation of fecundity of *H. labeo* need to be further studied.

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APPENDIX

Table A1. *The biological indices and individual fecundity of H. labeo*

Biological indices		Age					
		0^+	1^+	2^+	3^+	4^+	5^+
Sample amount		2	21	33	16	4	5
L (*cm*)	Mean±S.D.	11.65±0.21	17.46±2.37	20.43±1.89	23.01±2.14	24.68±2.59	31.08±4.10
	Range	11.5-11.8	13.6-20.6	16.5-23.5	18.3-26.5	22.5-28.2	24.5-35.0
W (*g*)	Mean±S.D.	27.65±0.78	111.60±52.85	184.51±56.62	255.45±68.29	331.12±160.51	575.28±130.59
	Range	27-28	40-211	78-281	116-354	187-543	378-700
W_n (*g*)	Mean±S.D.	24.40±1.98	93.84±43.21	149.05±42.83	215.29±60.44	255.00±111.39	497.10±130.01
	Range	23.0-25.8	36.6-158.4	60.3-225.9	104.8-319.8	153.2-403.2	285.0-612
W_g (*g*)	Mean±S.D.	1.05±0.92	9.48±9.46	20.76±13.01	21.79±11.14	48.25±36.35	70.82±18.39
	Range	0.4-1.7	1.1-40.3	3.2-47.9	4.2-44.7	20.9-98.0	50.3-96.0
GSI	Mean±S.D.	4.47±4.13	9.79±6.27	13.43±7.00	10.44±5.51	17.11±5.80	14.58±2.83
	Range	1.55-7.39	2.12-25.44	2.83-27.78	3.01-20.20	11.38-24.31	10.78-17.65

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K	Mean±S.D.	1.55±0.21	1.63±6.26	1.70±0.19	1.73±0.22	1.61±0.22	1.65±0.23
	Range	1.40-1.70	1.37-2.26	1.34-2.29	1.41-2.15	1.35-1.80	1.35-1.94
F	Mean±S.D.	3285.38± 3030.92	13409.47± 7087.29	21989.13± 12873.63	38930.96± 22665.99	42605.51± 20767.20	58892.63± 16459.62
	Range	1142.20-5428.57	2009.23-23615.80	2785.44-47876.85	7717.99-87047.68	26348.63-71608.60	33276.32-77107.20
F_L (amount/*cm*)	Mean±S.D.	284.42±265.34	762.48±374.30	1043.54±570.67	1665.24±912.92	1677.38±627.19	1899.57±475.03
	Range	96.80-472.05	98.01-1319.64	156.49-2172.33	335.56-3481.91	1171.05-2539.31	1073.43-2223.88
F_W (amount/*g*)	Mean±S.D.	117.34±106.32	136.03±77.99	119.90±48.72	150.15±70.73	129.56±9.24	105.00±30.79
	Range	42.15-192.50	13.03-296.32	25.99-195.98	27.00-276.94	118.99-140.92	59.40-144.03

L, body length; W, body weight; W_n, net weight; W_g, gonadal weight; GSI, maturity coefficient; K, body fat

Table A2. *Regression equation between individual fecundity and single biological indices of H. Labeo in the Jinjiang River*

Biological indices	**Individual fecundity**		
	F_W	F	F_L
L (*cm*)	$P > 0.05$	$F = -45943.04 + 3452.90L$	$F = -2.25L^2 + 202.04L - 2031.38$
		$R^2 = 0.569$, $P < 0.01$	$R^2 = 0.374$, $P < 0.01$
W (*g*)	$P > 0.05$	$F = -0.11W^2 + 186.37W - 6026.11$	$F = -0.007W^2 + 7.915W - 54.598$
		$R^2 = 0.658$, $P < 0.01$	$R^2 = 0.486$, $P < 0.01$
W_n (*g*)	$P > 0.05$	$F = -0.19W_n^2 + 243.79W_n - 7722.61$	$F = -0.01W_n^2 + 10.02W_n - 102.38$
		$R^2 = 0.660$, $P < 0.01$	$R^2 = 0.498$, $P < 0.01$
Age	$P > 0.05$	$F = 11480.47t - 10580.13$	$F = -37.035t^2 + 0.27t - 348.51$
		$R^2 = 0.448$, $P < 0.01$	$R^2 = 0.299$, $P < 0.01$
W_g (*g*)	$P > 0.05$	$F = 661.29W_g + 11398.12$	$F = -0.127W_g^2 + 29.69W_g + 619.56$
		$R^2 = 0.477$, $P < 0.01$	$R^2 = 0.325$, $P < 0.01$
L×*W* (*cm***g*)	$P > 0.05$	$F = 3.275LW + 10066.12$	$F = -8.35E + 0.27LW + 224.53$
		$R^2 = 0.562$, $P < 0.01$	$R^2 = 0.482$, $P < 0.01$
GSI	$F = 0.58GSI^2 - 2.58GSI + 7.58$	$F = 11506GSI^{0.039}$	$F = 618.09GSI^{0.032}$
	$R^2 = 0.08$, $P < 0.05$	$R^2 = 0.075$, $P < 0.05$	$R^2 = 0.066$, $P < 0.01$
K	$P > 0.05$	$F = 3882.62K^{3.054}$	$F = -1148.851K^2 + 5540.21 - 4680.51$
		$R^2 = 0.168$, $P < 0.01$	$R^2 = 0.190$, $P < 0.01$

L, body length; W, body weight; W_n, net weight; W_g, gonadal weight; GSI, maturity coefficient; K, body fat

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THE RESPONSE OF SPINACH (*SPINACIA OLERACIA* L.) PHYSIOLOGICAL CHARACTERSTICS TO DIFFERENT BIOCHAR TREATMENTS UNDER SALINE CONDITION

SOOTHAR, M. K.[1,2] – ABDOUL KADER, M. H.[1] – SOOTAHAR, M. K.[2,3] – YANG, G.[1] – ZAIN, M.[1] – SUN, J.[1*] – PANHWAR, A. A.[2,4]

[1]*Key Laboratory for Crop Water Requirement and Regulation of Ministry of Agriculture, Farmland Irrigation Research Institute, CAAS, Xinxiang, Henan 453003, China*

[2]*Department of Soil Science, Sindh Agriculture University, Tandojam 70060, Pakistan*

[3]*Institute of Environment and Sustainable Development in Agriculture, CAAS, Beijing 100081, China*

[4]*National Key Laboratory of Crop Genetic Improvement Microelement Research Center, Huazhong Agricultural University, Wuhan, China*

[*]*Corresponding author*
e-mail: jshsun623@163.com

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Abstract. Biochar is considered beneficial for plant growth and soil properties under saline condition. A pot experiment was conducted to investigate the effect of acidic biochar on physiological and growth parameters, as well as nutrient concentration of spinach. Five treatments were arranged; Control (CK) without biochar and salt application, Wood Biochar (WB), WB0, WB1, WB2 and WB3 with 0, 15, 30 and 45 g kg^{-1} biochar, respectively, + 1% salt. Physiological characteristics and growth parameters and total nitrogen (TN) and phosphorus (TP) were determined on 48 and 50 days after sowing respectively. The results showed that the number of leaves, plant height and fresh and dry weights decreased by 58.1, 53.1 and 82.7 and 81.3%, respectively, in WB3 compared to CK. Physiological characteristics of spinach plants were negatively affected by addition of biochar. TN in spinach was lower in WB3 than CK. In contrast, TP uptake was enhanced by 93.8% in WB3 compared to CK. Moreover, biochar addition decreased soil pH and increased soil electrical conductivity (EC), TP and TN increased by 317.4 and 34.5%, respectively in biochar treated soil. We conclude that further studies should be conducted to evaluate the effect of acidic biochar on other crops and soil properties.
Keywords: *NP concentration, photosynthesis, saline condition, spinach growth, soil properties*

Introduction

Accumulation of salts, (Na, Ca, K, Cl, SO_4, Mg) in soil through application of low-quality irrigation water, anthropogenic activities, and climatic changes causes salinity in soil. Soil salinity adversely affects plants height, fresh and dry weight, growth and yield because of the total concentration of salts in the soil solution (Xu and Mou, 2016; Alagoz and Toorchi, 2018) which directly affects plants' physiological and biochemical characteristics, including the growth of root and shoot (Zhang et al., 2013), photosynthesis and respiration (Yang et al., 2020), soil microbes (Yan et al., 2015) and leads to wilting, or ultimately the death of the plant (Hamouda et al., 2015; Ahmad et al., 2016). Na and Cl at high concentrations are toxic to plants, especially when they increase in cytosol (Zorb et al., 2018). However, with the passage of time salt contents increase gradually in soil irrigated with salt water (Min et al., 2016). Final outcome as response to impure irrigation water on the metabolism of vegetables depends on the

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nature of the substance, the time of exposure and the ability of plants to adapt (Maksimovic and Ilin, 2012).

Vegetables are generally eaten as fresh or after cooking processes, are rich in phytochemicals, such as phenolics, vitamins, glucosinolates and anthocyanin with a beneficial impact on human health (Caparrotta et al., 2019). Spinach (*Spinacia oleracia* L.) is one of the important leafy vegetables which contain high amount of beta carotene, folate, vitamin C, calcium, iron and oxygen radical absorbance capacity (Dicoteau, 2000). Spinach is considered as salt tolerant leafy vegetable, which can survive at ECe > 4.2 dS m^{-1} (Ors and Suarez, 2016). However, some studies reported that salinity (mainly Na and Cl) depressed chlorophyll *a*, chlorophyll *b* and protein (Ratnakar and Rai, 2013), increased osmotic and oxidative stress, nutritional imbalance and reduction in cell division (Zhu, 2003) which directly affect water consumption and fresh yield of spinach (Unlukara et al., 2017).

Many research studies have reported different strategies including organic and inorganic sources, have been applied to combat with salinity (Amini et al., 2016). Leaching of salts, replacement of Na, use of gypsum, phytoremediation and organic matter application are most commonly methods of reclaiming these soils (Ding et al., 2010; Filho et al., 2019; Wichern et al., 2020). But those strategies did not remain effective. Biochar is recently one of the products received by pyrolysis of different organic materials (crop residue, animal or poultry waste) by heating at various temperatures. Biochar is highly recalcitrant due its high aromaticity and is able to sequester carbon for long time (Lehman and Joseph, 2009). Therefore, it has been used in agriculture on large scale since long time for various purposes, for example; it improves soil physical and chemical properties (Glab et al., 2016; Piash et al., 2019), increase plant available water storage capacity (Duarte et al., 2019), soil water holding capacity, permeability and aeration (Gavili et al., 2018; Huang et al., 2019; Danish et al., 2020), reclamation of saline soils (Amini et al., 2016) and increase yield of crops in saline soils (Lashari et al., 2013; Akhtar et al., 2015). It is reported in field and greenhouse experiments that biochar has positive impact on soil properties, field crops and vegetables under saline irrigation and drought conditions (Younis et al., 2015; Agbna et al., 2017; Huang et al., 2019). Furthermore, biochar is capable to improve soil organic matter and mineral NPK (Huang et al., 2019). A very little is known about the application of acidic biochar on vegetables. Because, generally alkaline pH biochars are used in the experiments (Lashari et al., 2013; Akhtar et al., 2015) and low pH (acidic) biochars are neglected. Thus, acidic biochar application could be a possible approach to increase spinach production under salinity stress. However, no study is reported in saline conditions with low pH biochar for spinach. The study aimed (i) to evaluate the effect of wood biochar on spinach physiological characters under saline condition, and (ii) to assess the effects of wood biochar application on soil properties and nutrient content under saline condition.

Materials and methods

Experimental soil and salt analysis

Surface soil layer (0-20 cm) used in the current experiment was taken from barren land of Alar city, Xinjiang province of China (80^{O}, 50′ 19″ E and 40^{O}, 27′ 17″ N,). The soil was ground and passed through 2 mm sieve in order to remove gravels. Physico-chemical properties of soil were analyzed before starting the experiment as

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given in *Table 1*. Soil pH and electrical conductivity (EC) were measured in 1:5 w/v distilled water extract by using pH and EC meter (Fisher scientific, USA). For soil texture, hydrometer method was used (Bouyoucos, 1962); cation exchange capacity of soil was determined by ammonium acetate method (Rayment and Higginson, 1992), and total N and P were analyzed by elemental analyzer. The local salts collected from the same locality was analyzed for chemical properties; chloride, exchangeable potassium, sodium, calcium and magnesium before using for the experiment.

Table 1. *Physio-chemical properties of soil and biochar used in the experiment*

Parameters	Soil	Biochar	Salt
Texture	Silty clay loam	-	-
Ph	8.51	2.52	-
EC mS cm^{-1}	3.31	-	-
CEC cmol (+) kg^{-1}	3.86	23.7	-
Total Nitrogen (mg g^{-1})	0.50	2.8	-
Total Phosphorus (mg g^{-1})	0.66	64.9	-
Total Potassium (mg g^{-1})	-	5.6	-
Chloride (g kg^{-1})	7.10	0.03	70.0
Exchangeable K (g kg^{-1})	-	-	0.02
Exchangeable Na (g kg^{-1})	0.18	0.50	4.46
Exchangeable Ca (g kg^{-1})	50.43	-	0.04
Exchangeable Mg (g kg^{-1})	18.33	-	1.94

n = 3, EC = Electrical conductivity, CEC = Cation exchange capacity

Biochar and its characterization

Wood biochar (WB) used in this experiment was prepared by pyrolysis of wood at approximately 250-300 °C in oxygen free kiln (Shangqiu SanLi Company, Henan province, China). The method proposed by Li et al. (2016) was used for the determination of pH of biochar in water extract with pH meter (1:10 w/v). Cation exchange capacity was determined by ammonium acetate method (Thomas et al., 1982) and total nitrogen (TN) and phosphorus (TP) were measured by acid digestion method (Gao et al., 2015) and the extract samples were run on Continuous Flow-Analyzer (Bran and Luebbe AA3, Norderstedt, Germany). The biochar characteristics are presented in *Table 1*.

Experimental design

Pot experiment was conducted between October - November 2019 (50 days) in a controlled greenhouse of Farmland Irrigation Research Institute, CAAS China. The experiment consisted of 5 treatments with 3 replications. There were two control treatments, without biochar and salt (CK), the other one with no biochar and 1% (5 g NaCl) salt stress (WB0: 0+1%), while three levels of WB 15, 30, and 45 g kg^{-1} referred as WB1, WB2, and WB3, respectively, were used against 1% (5 g) salt stress. The 1% of salt (w/w, 5 g/5 kg of soil) was used which is equal to 11.3 dS m^{-1}. The 5 kg soil was

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thoroughly mixed with the aforementioned levels of biochar and filled in plastic pots having the size 15 cm width and 20 cm height. The pots were arranged in randomized design in controlled greenhouse maintained at 25/17 °C day/night temperature (Ferreira et al., 2018) under natural illumination for 14/10 h. Initially soil was irrigated once with salt water and left for field capacity. The seeds of local spinach variety Chunqiu were disinfected with 30% H_2O_2 for ten minutes (Zama et al., 2018) and five seeds were sown in each pot. Thinning was done one week after germination to keep one spinach plant in each pot for further experiment. The recommended dose of NPK fertilizers, 100-75-75 was applied in the form of urea, single superphosphate, and sulphate of potash as basal. The pots were maintained at 60% water holding capacity throughout experiment.

Observations before harvest

Two days before harvest (48 days after sowing), plant physiological parameters such as photosynthesis rate (*Pn*), stomatal conductance (*Gs*), transpiration rate (*Tr*), and concentration of intracellular CO_2 (*Ci*) were measured from fully expanded flag leaf by using portable photosynthesis system (Li-Cor-6400 LincoIn, NE, USA) between 9:00 am to 12:00 pm. During measurements, reference CO_2 concentration was equilibrated to 400 µmol mol^{-1} with a CO_2 mixture and the light adjusted at a PAR of 1200 µmol m^{-2} s^{-2}. The block temperature was fixed at 25 °C, the leaf-to-air vapour pressure deficit (VPD) was equilibrated between 1.5 and 2.0 kPa, and the flow was fixed at 300 µmol s^{-1}. Chlorophyll fluorescence was measured on the same leaves and same day by fluoremeter (MINI-PAM-II; Heinz Walz, Effeltrich, Germany). The maximal photochemical efficiency of photosystem (Fv/Fm) was measured after leaves adapted in dark for 30 minutes.

Plant growth attributes

Plants were harvested 50 days after sowing with sharp pair of scissors from the bottom and rinsed with distilled water. Immediately, fresh weight of above ground part was measured on digital weight balance, number of leaves per plant were counted manually and plant height was recorded by ruler. For dry weight, plants were placed in oven at 70 °C for 72 hours and re-weighed by using weight balance. The oven-dried mass was ground in pestle and mortar. The samples were analyzed for total NP using acid digestion method (Gao et al., 2015). In brief, for total NP, 15 mg sample was digested using 5 ml of H_2SO_4 for 3 hours until white fumes appeared. The suspension was filtered through filter paper and run on Continuous-Flow Analyzer (AA3, Bran and Luebbe, Norderstedt, Germany).

Statistical analysis

Statistical tests were performed using SPSS 23.0 (IBM Corporation, New York, NY, USA). One-way analysis of variance (ANOVA) was applied on spinach growth parameters, nutrient contents in its tissues and soil properties. The data are expressed as the mean (n=3) ± SE (standard errors) and multiple comparison tests (Least Significant Difference, Tukey and Duncan) were performed at significance level < 0.05.

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Results

Effect of biochar on plant growth parameters

Number of leaves in spinach were significantly affected by the addition of biochar under salinity stress (*Table 2*). A constant decrease in number of leaves was observed with an increasing level of biochar under saline condition. Biochar applied at 45 g kg^{-1} rate decreased number of leaves by 58.1% in WB3 compared to that of the CK.

Table 2. *Effect of biochar on number of leaves (plant^{-1}), plant height (cm), fresh and dry weight (g plant^{-1}) under saline condition. Data are mean ± standard error. Different alphabets represent significant differences ($p<0.05$)*

Biochar rate	Number of leaves (plant^{-1})	Plant height (cm)	Fresh weight (g plant^{-1})	Dry Weight (g plant^{-1})
CK	10.3±0.9 a	21.3±1.2 a	9.27±0.54 a	0.87±0.02 a
WB0	6.7±0.3 b	12.7±1.7 bc	1.83±0.35 b	0.17±0.01 c
WB1	6.3±0.3 bc	15.7±1.2 b	2.87±0.69 b	0.28±0.03 b
WB2	6.7±0.7 b	14.0±2.1 bc	2.37±0.32 b	0.22±0.02 bc
WB3	4.3±0.9 c	10.0±1.2 c	1.60±0.29 b	0.16±0.03 bc

n = 3

Plant height was significantly influenced by the application of biochar under saline condition (*Table 2*). Increasing the rate of biochar decreased plant height as compared to CK. Plant height decreased by 113.3% in WB3 with the addition of biochar as compared to CK. Statistically no significant differences were observed among the biochar treated plants. Among biochar treated plants, WB1 showed the highest plant height (15.7 cm) followed by WB2 (14.0 cm) and WB0 (12.7 cm) when compared to salt treated plants (WB0).

Spinach fresh and dry weights were significantly affected by biochar and salt stress (*Table 2*). The increasing rate of biochar decreased 2.87, 2.37 and 1.60 g plant^{-1} fresh weight of spinach in WB1, WB2, and WB3 treatments, respectively compared to CK. FW weight decreased by 82.7% in WB3 when compared to CK. Whereas, similar trend was observed for dry weight. Biochar application decreased DW of spinach by 81.3% in WB3 compared with CK. No significant difference was noticed for fresh and dry weight among biochar and salt treated plants.

Effect of biochar on gas exchange attributes

The results regarding *Pn*, *Gs*, *Ci* and *Tr* are presented in *Figure 1*. Biochar application negatively affected *Pn*, *Gs*, *Ci*, and *Tr* under saline condition over control. The *Pn*, *Gs*, *Ci*, *Tr,* and leaf fluorescence increased by 77.2, 86.8, 27.9, and 27.3% in CK respectively over all biochar treated plants. The statistical data showed no difference among all the treatments including CK for leaf fluorescence (*Fig. 2*). The maximum reduction (11.3%) for leaf fluorescence was observed in WB3.

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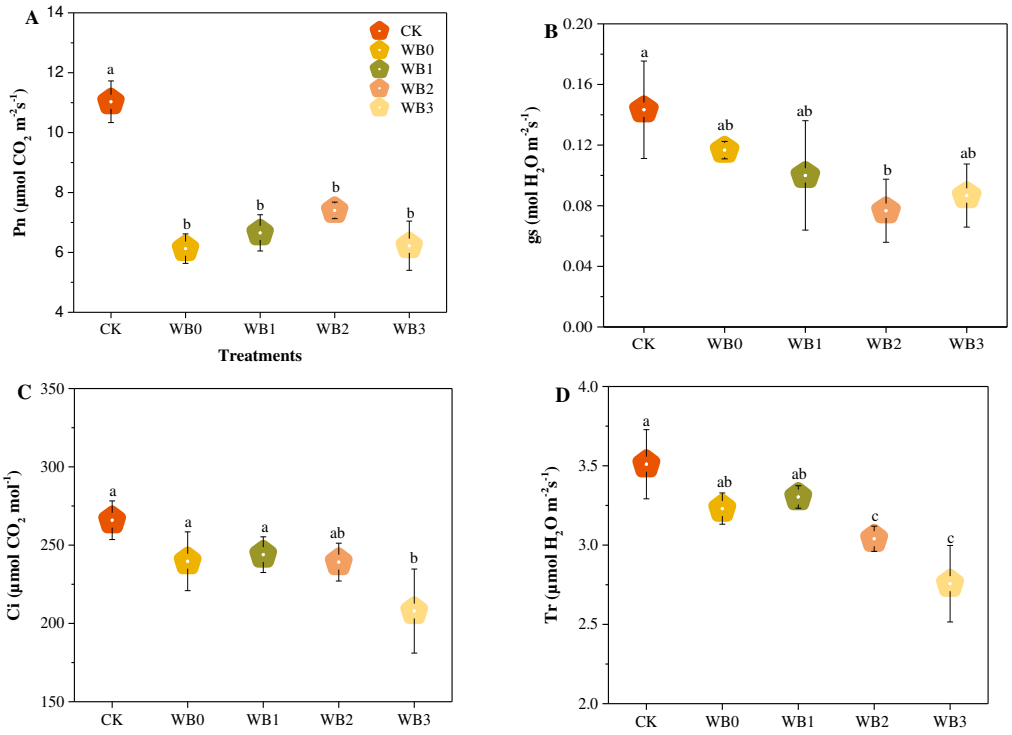

Figure 1. *Impact of biochar on Pn, gs, Tr, and Ci under saline condition. A, B, C, and D respectively. Data are mean ± standard error. Different alphabets on top of error bars represent significant differences ($p<0.05$)*

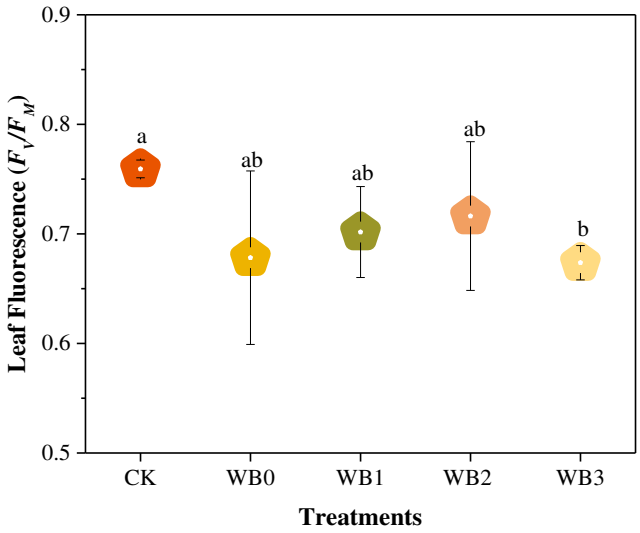

Figure 2. *Impact of biochar on leaf fluorescence under saline condition. Data are mean ± standard error. Different alphabets on top of error bars represent significant differences ($p<0.05$)*

Effect of biochar on total nitrogen (TN) and phosphorus (TP) in spinach tissue

The effect of biochar and salt stress on total N concentration in spinach tissues is shown in *Figure 3*. The results indicated that the increasing rate of biochar decreased

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TN concentration by 23.3, 20.4, and 15.6% in WB3, WB2, and WB1, respectively, compared to CK. Statistically, no significant difference was noticed for N concentration among the biochar treated and salt treated plants.

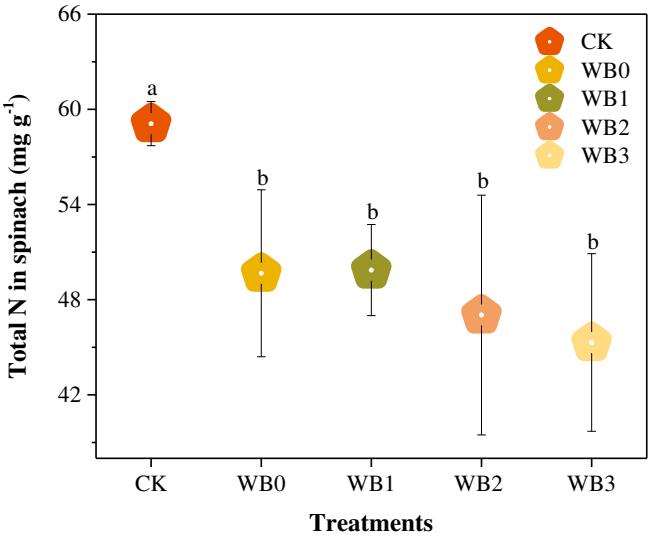

Figure 3. *Impact of biochar on total nitrogen (mg g^{-1}) in spinach tissue under saline condition. Data are mean ± standard error. Different alphabets on top of error bars represent significant differences ($p<0.05$)*

The observed values for TP concentration were different than other parameters as given in *Figure 4*. TP concentration in spinach tissues increased with increasing biochar application. TP concentration increased by 93.8% in WB3 followed by 66.2% and 27.1% in WB2 and WB1, respectively, when compared to CK.

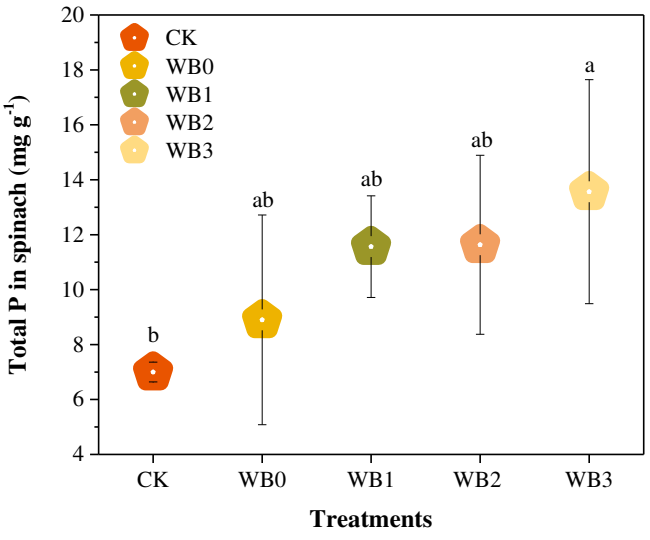

Figure 4. *Impact of biochar on total phosphorus (mg g^{-1}) in spinach tissue under saline condition. Data are mean ± standard error. Different alphabets on top of error bars represent significant differences ($p<0.05$).*

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Effect of biochar on soil properties

Soil pH significantly influenced by addition of different biochar levels under saline condition (*Table 3*). Increasing the rate of biochar decreased soil pH. Soil pH was decreased (7.63) at highest rate of biochar in WB3 compared to CK (8.51), followed by 8.05, 8.30 and 8.49 in WB2, WB1, and WB0, respectively.

Table 3. *Effect of biochar on soil pH, electrical conductivity (EC, mS cm^{-1}), total nitrogen (TN) and total phosphorus (TP) after harvest. Data are mean ± standard error. Different alphabets represent significant differences ($p<0.05$)*

Biochar rate	pH	EC (mS cm^{-1})	TN (mg g^{-1})	TP (mg g^{-1})
CK	8.51±0.01 a	1.35±0.03 a	0.46±0.08 b	0.63±0.02 d
WB0	8.49±0.01 a	1.51±0.01 a	0.42±0.01 b	0.67±0.05 d
WB1	8.30±0.01 b	1.34±0.02 b	0.47±0.01 b	1.49±0.09 c
WB2	8.05±0.02 c	1.37±0.01 b	0.51±0.04 ab	2.11±0.08 b
WB3	7.63±0.03 d	1.54±0.02 b	0.62±0.03 a	2.64±0.02 a

n = 3

Soil EC was affected by biochar is shown in *Table 3*. The application of biochar increased soil EC (1.54 mS cm^{-1}) in WB3 compared to CK (1.35 mS cm^{-1}). Soil EC increased with increasing rate of biochar (1.34, 137, and 1.54 mS cm^{-1} in WB1, WB2, and WB3), respectively.

Total N significantly increased in the soil where biochar was applied (*Table 3*). TN content increased by 34.5, 10.1, and 1.5% in WB3, WB2, and WB1 treatments respectively compared to CK.

Soil TP was significantly influenced by biochar (*Table 3*) under saline condition. The results showed that TP in soil significantly increased by 317.4, 232.6, and 135.3% in WB3, WB2, and WB1 respectively, over CK.

Discussion

Influence of biochar on spinach growth parameters

Our experimental findings are different from the results of other studies that revealed positive effects of biochar on both plant growths under saline condition (Lashari et al., 2013; Gavili et al., 2018; Yu et al., 2018; Rezaei and Razzaghi, 2018; Huang et al., 2019). In contrast, negative effects of biochar have also been reported in various studies (Deenik et al., 2010; Hol et al., 2017; Kong et al., 2019). Number of leaves (plant^{-1}), plant height (cm) and plant fresh and dry weight were notably decreased by biochar under saline condition. The reduction in growth parameters might be due to the accumulation of excess salts (Na and Cl) in root zone which causes toxicity for plants and/or the low pH of biochar caused reduction in physiological parameters. High concentration of Na^+ which disturbs physiological and biochemical processes in cells, results in growth reduction (Munns, 2002). A significant decrease in plant height, shoot and root biomass and nutrient uptake in different crops for example lettuce, sweet corn, wheat, peas under the application of biochar and salt in greenhouse and field

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experiments were also reported by Deenik et al. (2010). Decrease in plant fresh biomass due to the high concentration of salt which causes osmotic pressure and the availability of water is affected. Our findings are in agreement with the results of Hol et al. (2017), who conducted three greenhouse experiments, by using mown biochar. Their results indicated that biochar could not increase plant shoot and root biomass due to the soil biota. Water availability and sodium toxicity to plants cause reduction in shoot and dry mass (Farhangi-Arbiz, 2018). Low pH of biochar and salt stress may cause the lowering of water potential in the growth medium that reduces cell turgidity that retards cell division, expansion, elongation, and differentiation which ultimately reduces plant biomass (Mazher et al., 2007; Riffat and Ahmed, 2020). Another greenhouse study revealed that pine forest waste biochar initially decreased lettuce growth by increasing biochar rate (2-4%). Gonzaga et al. (2018) conducted a greenhouse experiment on maize and observed that pinewood biochar did not increase shoot and root growth.

Effect of biochar on gas exchange attributes

Gas exchange parameters including *Pn*, *Gs*, *Ci,* and *Tr* were affected by different biochar levels under salinity stress. The *Pn* is a biological process which enables plants to gain energy from sunlight (Yahia and Carrilo-Lopez, 2017). This energy is utilized for plant growth, development and adaptive responses to environmental conditions such as salt stress. Several studies have reported positive effect of biochar on *Pn*, *Gs*, *Ci,* and *Tr* under saline condition (Akhtar et al., 2015; Huang et al., 2019). But the results of our study are opposite. Statistically, *Pn*, *Gs*, *Ci,* and *Tr* were negatively affected by biochar under saline condition. Biochar and salt stress have negatively affected exchange parameters in all treatments. Because when plants uptake excess Na, cells start to be plasmolyzed. Salt stress may affect the gas exchange in the plants. Salinity causes plasmolysis in plants and due to which transpiration rate decreases, which directly affects the exchange of CO_2 and photosynthesis (Najar et al., 2018). Furthermore, our results are in confirmatory with Thomas et al. (2013) who reported in their glasshouse experiment and revealed that plants did not show a significant response for net photosynthesis to sawdust biochar under saline condition.

Leaf fluorescence is affected by accumulation of salts in root zone. The 20% biochar markedly decreased leaf fluorescence in bean seedling under saline condition (Farhangi-Arbiz et al., 2018). Similar results were observed in present study for leaf fluorescence among all the treatments. The leaf fluorescence was directly affected by the accumulation of salts in root zone and low uptake of nutrients.

Effect of biochar on TN and TP in plant tissues

Nitrogen and phosphorus are key elements for plant growth and development. Increasing rate of biochar caused a reduction in N concentration in spinach tissue. It directly affected plant growth parameters. But statistically no difference was noticed among other treatments where no biochar and salt were applied. The application of biochar along with salt may result in low nutrient supply and/or due to acidic nature of biochar which may cause low availability of N because of denitrification. It is reported in a study that the availability of N can greatly be influenced by low pH because the activity of bacteria is decreased at low pH (Ruan et al., 2007; Gonzaga et al., 2018; Neina, 2019). Deenik et al. (2010) reported in their greenhouse experiment on that increasing the quantity of nut shell charcoal decreased uptake of N in lettuce in a greenhouse experiment.

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In contrast, enhanced P was observed in tissues with increasing biochar level under saline condition. It is speculated that the possible reason for high concentration of P was observed in plant tissue is may be high concentration of P was recorded in biochar samples. Biochar is responsible for desorption which positively influences P uptake (Han et al., 2018). The acidic nature of biochar or soil may have played role for high uptake of P because different studies reported that P is mostly available to plants at low pH as well as P fixation is reduced at low pH because organic amendments have a high affinity for Al and Fe (Qi et al., 2017; Gonzaga et al., 2018). The results of our study also agreed with the results of Gonzaga et al. (2018), who noticed in their greenhouse experiment on maize that pinewood biochar increased P in maize plant tissue due to release of P under acidic soil.

Effect of biochar on soil properties

Soil pH and EC are two main chemical properties of soil and nutrients availability is also depending upon soil pH. Soil pH was significantly decreased with the addition of increased biochar. This decrease in soil pH is may be the result of strongly acidic wood biochar (pH 2.52). The release of acidic functional groups during the oxidation of functional groups of biochar can cause low soil pH (Cheng et al., 2006). Similar results were obtained by Ippolito et al. (2016), who applied shaving wood biochar having pH 5.8, decreased pH of calcareous soil in a pot experiment.

The EC of soil was increased with the increase of salt and biochar as compared to control. The possible reason for high EC in soil is may be the soil treated with high salt concentration. The results of our study confirm the findings of Novak et al. (2018), who discovered increase in soil EC due to the addition of lime along with miscanthus biochar.

Nitrogen is major element necessary for plant growth and yield. Comparing biochar with the soil, used in experiment, biochar had 4.6 times more N than soil. We noticed that soil N increased in the spots where higher rate of biochar was applied. The reason for high N content in soil may be the low availability of N to plants from biochar. Biochar contains high nutrients because of its organic origin and its addition to soil enhances in soil N (Duarte et al., 2019) because this increase can be associated with specific surface area that can contribute to the nitrogen amount in the soil. The findings of our study are in agreement with the results of Deenik et al. (2010), who observed that biochar addition increased soil N as compared to control.

Total phosphorus enhanced in soil with increasing rate of biochar as compared to the biochar untreated soils. The reason for high biochar in soil is the high concentration of P in biochar and easy release due to acidic nature of biochar. Biochar addition to acidic soil is expected to reduce P fixation because organic amendments have affinity for Al and Fe (Gonzaga et al., 2018).

Conclusion

The present study clearly indicated that none of the biochar levels was effective on spinach growth indicators (*Pn*, *Gs*, *Ci,* and *Tr* and plant height, number of leaves, fresh and dry weight and nitrogen concentration) under saline condition. In addition, it was noticed that P concentration was more in biochar treated plants than other treatments. Along with negative effects of biochar on spinach growth at the same time it improved soil properties. For example, soil pH was significantly decreased when biochar was

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applied at the rate of 45 g kg^{-1}, soil EC was slightly increased and soil nutritional properties (TN and TP) were significantly improved by increasing the rate of biochar application in soil. TN and TP increased by 34.5 and 317.4% in soil with the maximum level of applied biochar. Hence, we suggest that further studies should be conducted to evaluate the effect of acidic biohcar on field crops and soil properties.

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IN VITRO SELECTION OF DROUGHT TOLERANT REGENERANTS IN DURUM WHEAT (*TRITICUM DURUM* DESF.)

BENLIOGLU, B.* – OZGEN, M.

Department of Field Crops, Agriculture Faculty, Ankara University, 06110 Diskapi, Ankara, Turkey (phone: +90-312-596-1325; fax: +90-312-596-1395)

**Corresponding author*
e-mail: benlioglu@ankara.edu.tr; phone: +90-312-596-1881

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Abstract. In this study, a total of 12 genotypes were used, including 1 hulled emmer and 11 registered ones which are important as genetic resources and for durum wheat cultivation. The responses of these genotypes to different drought stress levels were determined in vitro. In this research, to create drought stress, high molecular weight polyethylene glycol (PEG 6000) was used. In the trials, 5 different PEG 6000 doses were administered to induce drought stress with rates of 0 (control), -0.50 bar, -1.48 bar, -2.95 bar and -4.91 bar. During the experiments, after the callus was formed with the endosperm supported mature embryo culture, regeneration capacity and plantlets they formed were evaluated by applying different levels of drought stress to the callus. Using these parameters, stress tolerance index, stress sensitivity index and tolerance index values of durum wheat genotypes were calculated. According to the results, there was a significant decrease in all parameters examined with the increase of drought stress. While Artuklu and Sarıçanak-98 cultivars had the highest drought tolerance, Çakmak-79, Çeşit-1252, Eminbey and Kunduru-1149 cultivars were sensitive to drought and *Triticum dicoccum*, which is an important gene source, was also sensitive.
Keywords: *drought stress, polyethylene glycol, endosperm-supported embryo culture, Triticum durum, Triticum dicoccum*

Introduction

To increase plant production under conditions of global warming and accompanying climate change, it is necessary to use cultivated plants and plant gene resources much more effectively (Ozgen et al., 2015). Today, the severity of global warming and environmental stress factors (drought, salinity, high and low temperatures, heavy metals, etc.) are gradually increasing. Among the environmental stressors, drought stress is the most prominent stress factor in both cultivated plants and wild forms globally. Climate change is predicted to create increasingly severe and prolonged drought periods in the next 30-90 years, as weather temperatures increase, affecting more than a third of the world, including the world's top food-producing areas (Dai, 2011, 2013; Cook et al., 2014; Hasanuzzaman et al., 2017).

The world population is estimated to reach 9.74 billion in 2050 (Desa, 2019) and the nutritional needs of the growing population are also increasing. One big challenge is that the agricultural areas that reach their natural limits are shrinking for various reasons and the pressure of stress factors on plants increases due to climate changes. Under these conditions, it is necessary to obtain maximum product per unit area. For this, new varieties with high yield and tolerance to biotic and abiotic stress factors should be developed (Ozgen et al., 2017).

Wheat (*Triticum* sp.) is the most widely cultivated crop worldwide with 214 million ha of cultivation area. Wheat forms the basis of human nutrition and provides an average of 531 kcal of energy per individual per day (Anonymous, 2020). Bread wheat

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is spread over a wider area than durum wheat. The tolerance of common wheat against environmental stress factors is higher than durum wheat. For high yield and quality product, durum wheat is selective in terms of climate and soil requirements. Durum wheat will be one of the types that the increasing environmental stress factors, especially the drought pressure, will affect the most among cereals.

To develop new varieties with high drought tolerance, it is important to determine the tolerance levels of existing genotypes. For this purpose, selection at the cell and tissue level under fast and highly controlled in vitro conditions can give more reliable results than studies under outdoor conditions (Mohamed et al., 2000; Rai et al., 2010).

Mannitol, sorbitol, NaCl and polyethylene glycol (PEG) are used to create drought stress in vitro. Polyethylene glycols with high molecular weight are the most widely used stress agents in tissue culture due to their water-soluble polymer structure, non-toxicity, non-metabolism and not being absorbed by plant cells (Hassan et al., 2004; Caruso et al., 2008; Chen et al., 2010).

The aim of this study is to determine rapidly and consistently the tolerance of the durum wheat cultivars which are grown widely in Turkey as well as using as a genitor in wheat breeding programs and an emmer which is grown locally as well as resistant to several stresses against drought stress under in vitro conditions.

Materials and methods

This research was carried out at the Ankara University, Faculty of Agriculture, Department of Field Crops Biotechnology Laboratory. In the research, 11 registered cultivars which are used extensively in durum wheat cultivation in Turkey and Local hulled wheat genotype emmer (*Triticum dicoccum*) obtained from Kars province of Turkey were used as material (*Table 1*).

Table 1. *Durum wheat genotypes used in the research*

Genotypes	Type	Breeding company
Altın-40/98	Cultivar	Field Crops Central Research Institute
Artuklu	Cultivar	GAP International Agricultural Research and Training Center
Çakmak-79	Cultivar	Field Crops Central Research Institute
Çeşit-1252	Cultivar	Field Crops Central Research Institute
Eminbey	Cultivar	Field Crops Central Research Institute
Kızıltan-91	Cultivar	Field Crops Central Research Institute
Kunduru-1149	Cultivar	Field Crops Central Research Institute
Meram-2002	Cultivar	Bahri Dagdas International Agricultural Research Institute
Mirzabey-2000	Cultivar	Field Crops Central Research Institute
Sarıçanak 98	Cultivar	GAP International Agricultural Research and Training Center
Selçuklu-97	Cultivar	Bahri Dagdas International Agricultural Research Institute
T. dicoccum (Emmer)	Landrace	Collected from Kars province -Turkey

High molecular weight polyethylene glycol (PEG 6000) was used in the experiments to create drought stress. For the severity of drought stress, at the levels indicated as appropriate by previous researchers; It is set to 0, -0.50 bar (5% w/v), -1.48 bar (10% w/v), -2.95 bar (15% w/v), and -4.91 bar (20% w/v) (Abdel-Haddy and El-Naggar,

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2007; Soni et al., 2011; Farshadfar et al., 2012a; El-Rawy and Hassan, 2014; Kacem et al., 2017).

In order to determine the drought responses of genotypes by in vitro methods, the most realistic environment was tried to be prepared. For this:

- embryos of mature seeds were used as an explant source,
- drought stress applied to the developed calluses, which can represent a complete plant model and
- growth regulator was used only during callus formation phase, not during the stress phase.

For surface sterilization, mature seeds were treated with 70% (v/v) ethanol for 5 min, washed 2-3 times with sterile distilled water, sterilized for 25 min with Sodium Hypochlorite (NaClO), and washed several times with sterile distilled water. The seeds were then soaked in sterile distilled water for 2 h at 33 °C.

Endosperm supported mature embryo culture was applied to create callus from mature seeds (Ozgen et al., 1998). The embryos were gently separated without disconnecting the endosperm, allowing them to form callus in darkness for 11 days in medium containing only 8 mg^{-1} 2,4-D (Merck, Germany). The calli were then grown in MS (Murashige and Skoog, 1962) nutrient medium containing sucrose (20 g^{-1}), glycine (2 mg^{-1}) and Agar (7 g^{-1}) in darkness for 21 days. The obtained calli were transferred onto regeneration medium containing MS mineral salts, sucrose (20 g^{-1}), Agar (7 g^{-1}) and different doses of PEG-6000 (0, -0.50 bar (5% w/v), -1.48 bar (10% w/v), -2.95 bar (%15 w/v) and -4.91 bar (20% w/v)) in the plates. The transferred calli were incubated at 25 °C for 5-6 weeks under 16 h/8 h (light/dark) photoperiod at 25 °C.

Calli with green spots on them were considered to be regenerated, and the "regeneration capacity" was determined by proportioning the regenerated calli to the callus formed (Ozgen et al., 2017). Regenerants that did not remain in the form of shoot primordial and were graded at least 30-40 mm were accepted as plantlets and the "plantlet formation capacity" was determined by number of plantlets proportioning by the total number of calli (Kacem et al., 2017).

Stress tolerance index (STI) (Fernandez, 1992), stress sensitive index (SSI) (Fischer and Maurer, 1978) and tolerance index (TOL) (Rosielle and Hamblin, 1981) to better understand the responses of genotypes grown under stress conditions to these stresses calculated. Stress tolerance index is used to determine the varieties that show high value in terms of the properties examined both under stressful conditions and under normal conditions. Stress sensitive index, on the other hand, is used to identify varieties that have low value in terms of the trait examined but show high value under stress.

- Stress tolerance index (STI) $= \frac{(Ypi \times Ysi)}{Yp^2}$
- Stress sensitive index (SSI) $= \frac{1-(Ysi\ /\ Ypi)}{SI}$, where $SI = 1 - \frac{Ys}{Yp}$
- Tolerance index (TOL) $= \frac{(Ypi + Ysi)}{2}$

Ypi = The trait value of each type under stress-free conditions (control)
Ysi = The value of the traits of each type under stress
Yp = The average of the traits examined of the cultivars under stress-free conditions (control)
Ys = The average of the traits examined of the cultivars under stressed conditions (control)

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Statistical analysis

A completely randomized design with three replications per species and per stress level was used. Petri dishes containing 25 seeds were considered the units of replication in callus induction and callus development stage.

Statistical analysis of the data obtained was made with MSTAT-C (Russel, 1994) and JMP-12 (SAS, Institute Inc., 2015). The effects of genotype and stress on culture responses were determined by analysis of variance and Duncan tests (Steel et al., 1980).

Results

First of all, we determined to the responses of durum wheat genotypes used in the experiments to tissue culture parameters with endosperm supported mature embryo culture. With the analysis of variance, it determined that the difference among the genotypes was statistically significant at $p < 0.01$ for all parameters examined (*Table 2*). The means of the genotypes in the parameters examined were shown in *Table 3*.

Table 2. *Analysis of variance for parameters obtained by endosperm-supported embryo culture in durum wheat genotypes*

V.R.	dF	Mean square			
		Callus induction	Callus weight	Regeneration capacity	Plantlet formation capacity
Genotype	11	137.2**	0.62**	205.2*	1039.1**
Error	24	15.7	0.08	17.9	42.2
Total	35	53.2	0.25	75.9	362.8

**Significantly different from zero at 0.01 probability

Table 3. *Response of durum wheat genotypes to tissue culture parameters*

Genotypes	Callus induction (%)	Callus weight (g)	Regeneration capacity (%)	Plantlet formation capacity (%)
Altın-40/98	89.3 a-c	3.132 ab	100.0 a	26.7 cd
Artuklu	96.0 a	2.595 b-d	100.0 a	30.0 bc
Çakmak-79	84.0 b-d	2.347 c-e	100.0 a	6.7 g
Çeşit-1252	90.7 ab	2.831 a-c	73.3 d	10.0 fg
Eminbey	94.7 a	3.279 a	83.3 c	40.0 b
Kızıltan-91	82.7 cd	2.687 b-d	100.0 a	13.3 ef
Kunduru-1149	77.3 d	2.623 b-d	93.3 ab	10.0 f
Meram-2002	82.7 cd	1.998 ef	96.7 ab	33.3 bc
Mirzabey-2000	77.3 d	1.687 f	93.3 ab	23.3 c-e
Sarıçanak 98	94.7 a	2.633 b-d	100.0 a	56.7 a
Selçuklu-97	80.0 d	2.140 d-f	96.7 ab	53.3 a
T. dicoccum	89.3 a-c	2.740 bc	90.0 bc	16.7 d-f
Mean	86.6	2.558	93.9	26.7

Means followed by the different letters are significantly different at the 0.05 probability

Among the durum wheat genotypes, the variety with the highest callus induction was Artuklu with 96%. This was followed by Eminbey and Sarıçanak-98. Kunduru-1149 has

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the least callus induction. The varieties with the highest callus weight were Eminbey and Altın 40/98 respectively. All callus of Altın-40/98, Artuklu, Çakmak-79, Kızıltan-91 and Sarıçanak-98 cultivars regenerated and their regeneration capacity was calculated as 100%. In terms of the plantlet formation capacity obtained by counting the explants that had shoot elongation of at least 30-40 mm from the regenerated calli, Sarıçanak-98, Selçuklu-97 and Eminbey had the highest values, respectively (*Table 3*).

Calli obtained by endosperm supported mature embryo culture of durum wheat genotypes (*Fig. 1*); regeneration capabilities determined by transferring MS media containing different severity of drought stress (0, -0.50, -1.48, -2.95 and -4.91 bar) (*Fig. 2*). As a result of the analysis of variance, it was seen that the difference between genotypes, stress levels and genotype x stress level interaction $p < 0.01$ level was statistically significant (*Table 4*). The "regeneration capacity" and the "plantlet formation capacity" of the genotypes, measured under different drought severity, are shown in *Table 5*.

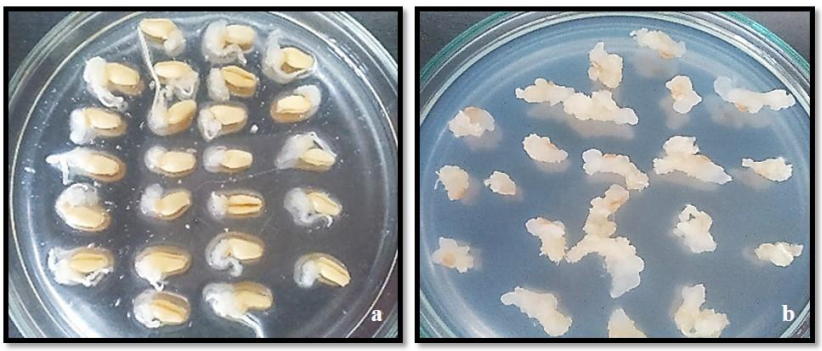

Figure 1. *Callus induction (a) and callus development stage (b)*

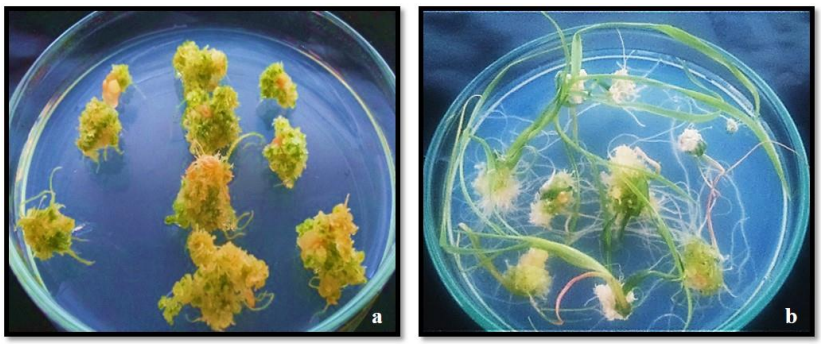

Figure 2. *Regeneration phase. (a) Callus remaining in the form of shoot primordian; (b) callus forming plantlets*

The regeneration capacity of Artuklu, Kızıltan-91, Kunduru-1149 and Sarıçanak-98 was measured 100% at the level of -0.50 bar drought stress. The genotype with the lowest regeneration capacity at this stress level was Selçuklu-97 (76.7%). The -0.50 bar stress level did not have a great effect on the regeneration capacity of the genotypes, and there was no stress effect in Artuklu, Eminbey, Kızıltan-91, Sarıçanak-98 and *T. dicoccum*. The varieties that created the highest plantlets at this stress level were Sarıçanak-98 (43.3%) and Artuklu (36.7%), respectively. Kunduru-1149 did not form

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plantlets at this stress level. Mirzabey-2000 and *T. dicoccum* were the genotypes that produced the least plantlets with 6.7%. The varieties most affected by this drought level were Kunduru-1149 and Mirzabey-2000, with 100% and 71.2% stress-strain, respectively (*Table 5*).

Table 4. *Analysis of variance for the regeneration capacity and the plantlet formation capacity of durum wheat genotypes under different drought stress*

V.R.	dF	Mean square	
		Regeneration capacity	**Plantlet formation capacity**
Genotype (G)	11	5.201**	1.867**
Stress level (S)	4	10.486 **	2.504**
G x S	44	583**	180**
Eror	120	60	54
Total	179	737	251

**Significantly different from zero at 0.01 probability

Regeneration capacity at -1.48 bar drought stress level it was measured 100% in Artuklu, Kızıltan-91 and Sarıçanak-98 varieties. Genotypes with the lowest regeneration capacity at -1.48 bar; *T. dicoccum* (36.7%) and Çeşit-1252 (46.7%). When the changes in the regeneration capacity of the genotypes between the control group and the drought level of -1.48 bar were examined, the most stress-strain occurred in *T. dicoccum* (59.2%) and Selçuklu-97 (48.3%). The regeneration capacity of Artuklu, Kızıltan-91 and Sarıçanak-98 did not change according to the control group. At this stress level, Sarıçanak-98 obtained the highest plantlet formation capacity with 36.7%. Artuklu ranked second with 26.7% plantlets. Çakmak-79 and Eminbey were determined as the lowest (0%) genotypes of the plantlet formation capacity at -1.48 bar stress level. At this drought level, the stress-strain was at least Artuklu (11%) and Kızıltan-91 (24.8%) (*Table 5*).

Genotypes with the highest regeneration capacity at -2.95 bar drought stress level, were Sarıçanak 98 (100%), Altın-40/98 (96.7%), Artuklu (96.7%) and Çakmak-79 (96.7%), respectively. *T. dicoccum* showed the lowest regeneration capacity at this stress level (10%). Between the control group and -2.95 bar stress level of genotypes, the highest stress-strain in terms of regeneration capacity occurred at 88.9% and 55.2% in *T. dicoccum* and Selçuklu-97, respectively. At this drought level, Sarıçanak-98 (43.3%) and Artuklu (23.3%) achieved the highest plantlet formation capacity however, Çakmak-79, Eminbey, Kunduru-1149 and Selçuklu-97 could not form plantlets. At this stress level, in terms of regeneration capacity and plantlet formation capacity, the stress-strain was the least in Sarıçanak-98 and Artuklu.

The varieties with the highest regeneration capacity at -4.91 bar stress level, which is the maximum drought severity in the study, were Sarıçanak-98 (100%) and Kızıltan-91 (90%). *T. dicoccum* could not regenerate at this stress level. Eminbey regeneration capacity was calculated as 6.7% and stress-strain as 92%. At this drought level, the highest plantlet formation capacity was also formed by Sarıçanak-98 (26.7%) and Artuklu (13.3%). The varieties with the least stress-strain in terms of regeneration capacity and plantlet formation capacity were Sarıçanak-98 and Artuklu.

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Table 5. *Regeneration capacity and plantlet formation capacity of durum wheat genotypes under different drought stress levels*

Genotypes	Different drought stress levels (bar)					Mean
	0	**-0.50**	**-1.48**	**-2.95**	**-4.91**	
	Regeneration capacity (%)					
Altın-40/98	100.0 a	93.3 a-c	93.3 a-c	96.7 ab	60.0 h-j	88.7 C
Artuklu	100.0 a	100.0 a	100.0 a	96.7 ab	86.7 b-e	96.7 AB
Çakmak-79	100.0 a	96.7 ab	93.3 a-c	96.7 ab	80.0 d-f	93.3 BC
Çeşit-1252	70.0 f-h	83.3 c-e	46.7 k-m	46.7 k-m	33.3 n	56.0 F
Eminbey	83.3 c-e	83.3 c-e	66.7 g-i	56.7 i-k	6.7 o	59.3 F
Kızıltan-91	100.0 a	100.0 a	100.0 a	93.3 a-c	90.0 a-d	96.7 AB
Kunduru-1149	93.3 a-c	100.0 a	80.0 d-f	70.0 f-h	53.3 j-l	79.3 D
Meram-2002	96.7 ab	93.3 a-c	83.3 c-e	60.0 h-j	53.3 j-l	77.3 D
Mirzabey 2000	93.3 a-c	86.7 b-e	76.7 e-g	56.7 i-k	33.3 n	69.3 E
Sarıçanak 98	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 A
Selçuklu-97	96.7 ab	76.7 e-g	50.0 j-l	43.3 l-n	33.3 n	60.0 F
T. dicoccum	90.0 a-d	90.0 a-d	36.7 mn	10.0 o	0.0 o	45.0 G
Mean	93.6 A	91.9 A	77.2 B	68.9 C	52.5 D	76.8
	Plantlet formation capacity (%)					
Altın-40/98	26.7 e-h	20.0 g-j	6.7 k-m	10.0 j-m	3.3 lm	13.3 CD
Artuklu	30.0 d-g	36.7 c-e	26.7 e-h	23.3 f-i	13.3 i-l	26.0 B
Çakmak-79	6.7 k-m	10.0 j-m	0.0 m	0.0 m	0.0 m	3.3 G
Çeşit-1252	10.0 j-m	10.0 j-m	6.7 k-m	3.3 lm	3.3 lm	6.7 FG
Eminbey	40.0 cd	16.7 h-k	0.0 m	0.0 m	0.0 m	11.3 CDE
Kızıltan-91	13.3 i-l	13.3 i-l	10.0 j-m	6.7 k-m	0.0 m	8.7 EF
Kunduru-1149	10.0 j-m	0.0 m	6.7 k-m	0.0 m	0.0 m	3.3 G
Meram-2002	33.3 c-f	26.7 e-g	13.3 i-l	3.3 lm	0.0 m	15.3 C
Mirzabey 2000	23.3 f-i	6.7 k-m	10.0 j-m	10.0 j-m	0.0 m	10.0 DEF
Sarıçanak 98	56.7 a	43.3 bc	36.7 c-e	43.3 bc	26.7 e-h	41.3 A
Selçuklu-97	53.3 ab	16.7 h-k	6.7 k-m	0.0 m	0.0 m	15.3 C
T. dicoccum	16.7 h-k	6.7 k-m	3.3 lm	3.3 lm	0.0 m	5.9 FG
Mean	26.7 A	17.2 B	10.6 C	8.6 C	3.9 D	13.4

Means followed by the different letters are significantly different at the 0.05 probability

Regeneration capacity and plantlet formation capacity averages of genotypes at all drought stress levels were calculated as 76.8% and 13.3%, respectively (*Table 5*). Among the genotypes used in our study, the regeneration capacity and the plantlet formation capacity the average values at different stress levels of Artuklu and Sarıçanak-98 cultivars were found to be higher than these values (*Figs. 3* and *4*).

Data belonging to stress tolerance, stress sensitive and tolerance indexes made with the data obtained from the parameters examined are shown in *Table 6*. In terms of stress tolerance index; Sarıçanak-98 formed the best scores in all parameters examined in all stress levels. Artuklu had the second-best scores. According to the stress-sensitive index, the most sensitive genotypes were determined as Eminbey, Selçuklu-97, Kunduru-1149 and *T. dicoccum*. The genotype with the highest tolerance index value was Sarıçanak-98 (*Table 6*).

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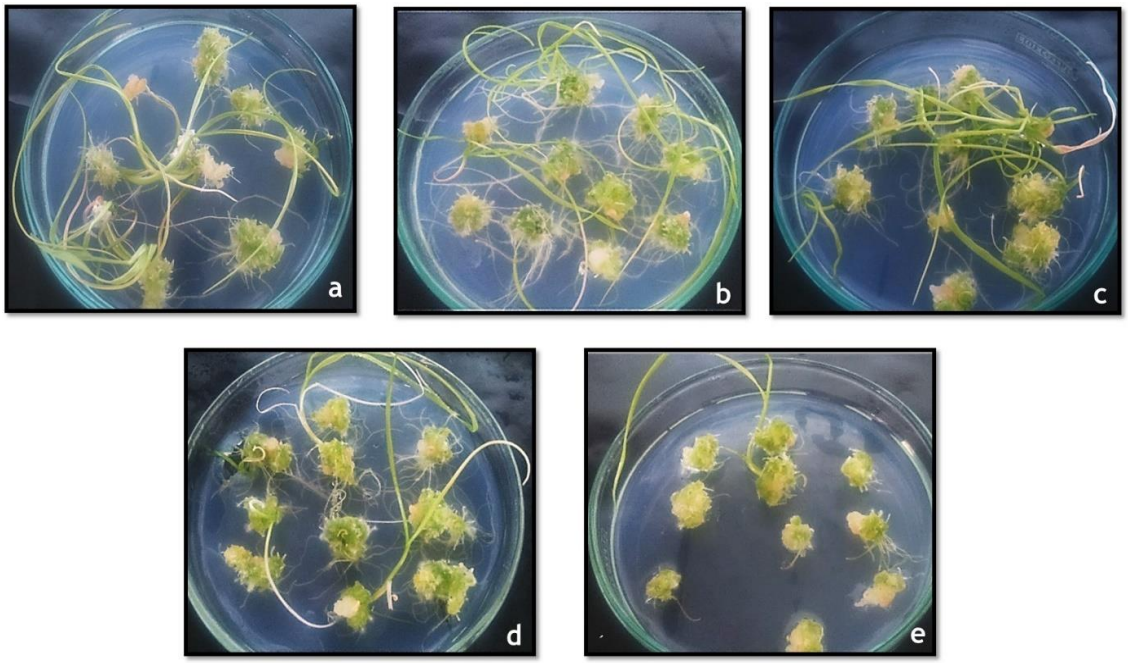

Figure 3. *Regeneration of Artuklu at different drought stress levels (a: control, b: -0.50 bar, c: -1.48 bar, d: -2.95 bar and e: -4.91 bar)*

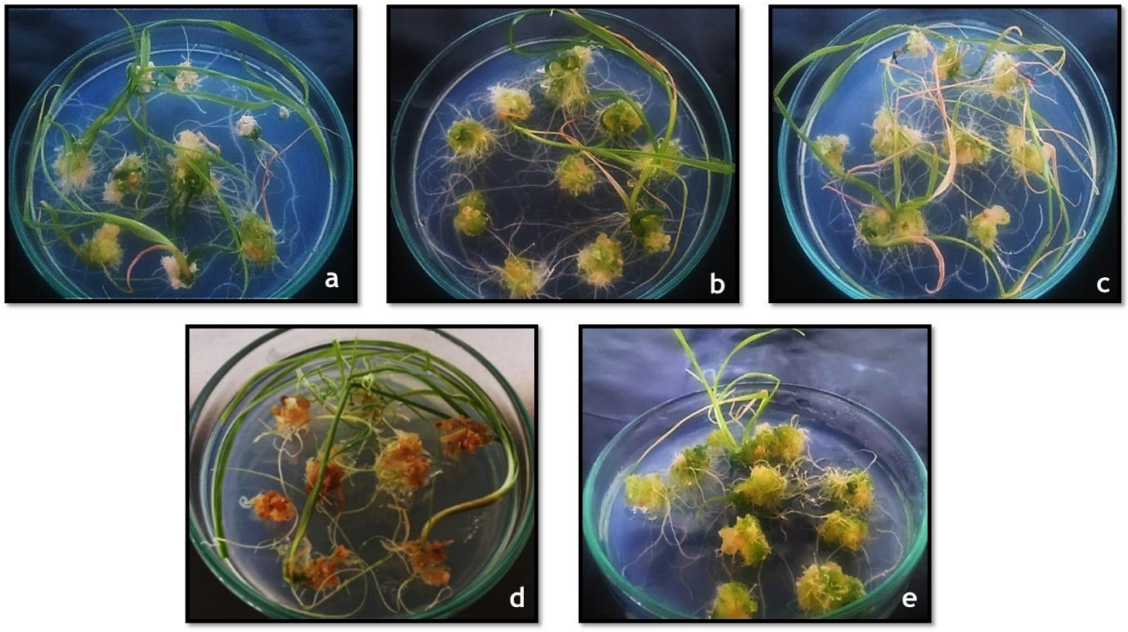

Figure 4*. Regeneration of Sarıçanak-98 at different drought stress levels (a: control, b: -0.50 bar, c: -1.48 bar, d: -2.95 bar and e: -4.91 bar)*

Discussion

The responses of durum wheat genotypes to tissue culture parameters differed significantly according to genetic structure. It has been clearly stated in previous studies that the genotype affects tissue culture parameters in wheat (Ozgen et al., 1998;

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Pellegrineschi et al., 2004; Zale et al., 2004; Grigoryeva and Shletser, 2006; Farshadfar et al., 2012a, b; Ozgen et al., 2017; Kacem et al., 2017: Miroshnichenko et al., 2019; Jasdeep et al., 2019).

Table 6. *Stress tolerance index (STI), stress sensitive index (SSI) and tolerance index values of durum wheat genotypes*

	Genotypes	Stress tolerance index				Stress sensitivity index				Tolerance index			
		-0.50	-1.48	-2.95	-4.91	-0.50	-1.48	-2.95	-4.91	-0.50	-1.48	-2.95	-4.91
Regeneration capacity	Altın-40/98	1.06	1.06	1.10	0.68	3.69	0.38	0.13	0.91	96.7	96.7	98.4	80.0
	Artuklu	1.14	1.14	1.10	0.99	0.00	0.00	0.13	0.30	100.0	100.0	98.4	93.4
	Çakmak-79	1.10	1.06	1.10	0.91	1.82	0.38	0.13	0.46	98.4	96.7	98.4	90.0
	Çeşit-1252	0.67	0.37	0.37	0.27	-10.46	1.90	1.26	1.19	76.7	58.4	58.4	51.7
	Eminbey	0.79	0.63	0.54	0.06	0.00	1.14	1.21	2.09	83.3	75.0	70.0	45.0
	Kızıltan-91	1.14	1.14	1.06	1.03	0.00	0.00	0.25	0.23	100.0	100.0	96.7	95.0
	Kunduru-1149	1.06	0.85	0.75	0.57	-3.95	0.81	0.95	0.98	96.7	86.7	81.7	73.3
	Meram-2002	1.03	0.92	0.66	0.59	1.94	0.79	1.44	1.02	95.0	90.0	78.4	75.0
	Mirzabey 2000	0.92	0.82	0.60	0.35	3.89	1.02	1.49	1.46	90.0	85.0	75.0	63.3
	Sarıçanak 98	1.14	1.14	1.14	1.14	0.00	0.00	0.00	0.00	100.0	100.0	100.0	100.0
	Selçuklu-97	0.85	0.55	0.48	0.37	11.39	2.76	2.09	1.49	86.7	73.4	70.0	65.0
	T. dicoccum	0.92	0.38	0.10	0.00	0.00	3.38	3.37	2.28	90.0	63.4	50.0	45.0
Plantlet formation capacity	Altın-40/98	0.75	0.25	0.37	0.12	0.71	1.24	0.92	1.03	23.4	16.7	18.4	15.0
	Artuklu	1.54	1.12	0.98	0.56	-0.63	0.18	0.33	0.65	33.4	28.4	26.7	21.7
	Çakmak-79	0.09	0.00	0.00	0.00	-1.38	1.66	1.48	1.17	8.4	3.4	3.4	3.4
	Çeşit-1252	0.14	0.09	0.05	0.05	0.00	0.55	0.99	0.78	10.0	8.4	6.7	6.7
	Eminbey	0.94	0.00	0.00	0.00	1.64	1.66	1.48	1.17	28.4	20.0	20.0	20.0
	Kızıltan-91	0.25	0.19	0.12	0.00	0.00	0.41	0.73	1.17	13.3	11.7	10.0	6.7
	Kunduru-1149	0.00	0.09	0.00	0.00	2.81	0.55	1.48	1.17	5.0	8.4	5.0	5.0
	Meram-2002	1.25	0.62	0.15	0.00	0.56	1.00	1.33	1.17	30.0	23.3	18.3	16.7
	Mirzabey 2000	0.22	0.33	0.33	0.00	2.00	0.95	0.84	1.17	15.0	16.7	16.7	11.7
	Sarıçanak-98	3.44	2.92	3.44	2.12	0.66	0.58	0.35	0.62	50.0	46.7	50.0	41.7
	Selçuklu-97	1.25	0.50	0.00	0.00	1.93	1.45	1.48	1.17	35.0	30.0	26.7	26.7
	T. dicoccum	0.16	0.08	0.08	0.00	1.68	1.33	1.18	1.17	11.7	10.0	10.0	8.4

In previous studies to determine the responses of genotypes to drought stress in wheat in vitro, generally immature embryos were used as an explant source (Galovic et al., 2005; Abdel-Haddy and El-Naggar, 2007; Bouiamrine and Diouri, 2012; Farshadfar et al., 2012a; Mahmood et al., 2012; Mahmoud et al., 2012). On the other hand, we used endosperm supported mature embryo culture method in our study (Ozgen et al., 1998); Thus, it was ensured that the calli benefit from the nutrients of the endosperm and develop. In most of the previous studies, drought stress was applied during callus formation or callus development stages (Hsissou and Bouharmont, 1994; Almansouri et al., 2001; Biswas et al., 2001; Bouiamrine and Diouri, 2012; Farshadfar et al., 2012a, b). The callus formed in our study were expected to reach sufficient maturity and the full totipotency feature was allowed to occur. In our study, to determine the true potentials of genotypes, no growth regulator used during drought stress and only endosperm used as a nutrient for callus induction.

Our findings show that the regeneration capacity of genotypes decreases with increasing PEG 6000 doses. PEG is used as a drought stress agent; our results are similar to the results of the studies conducted on paddy (Biswas et al., 2002; Wani et al., 2010), durum wheat (Bajji et al., 2000; Almansouri et al., 2001; Lutts et al., 2004; Abdel-Haddy and El-Naggar,

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2007; Bouiamrine and Diori, 2012; Razmjoo et al., 2015; Kacem et al., 2017), common bread wheat (Farshadfar et al., 2012a, b; Mahmood et al., 2012) and sorghum (Tsago et al., 2014).

In our study, Stress x Genotype interaction was found to be statistically significant in all parameters examined. In previous studies in which drought was induced by applying PEG in vitro conditions, durum wheat (Bouiamrine and Diouri, 2012; Razmjoo et al., 2015), common wheat (Farshadfar et al., 2012a, b; Mahmoud et al., 2012) and potato (Gopal and Iwama, 2007) were determined as the interaction of genotype x stress was statistically significant.

Abdel-Haddy and El-Naggar (2007); applied drought stresses by using different doses of PEG 6000 on callus they obtained from durum wheat. They stated that the regeneration capacity of the genotypes did not show a significant change at -0.50 bar stress level, but significant decreases occurred with -1.48 bar stress level. Most of the durum wheat genotypes we used in our study tolerated -1.48 bar of drought stress level. Besides, regeneration occurred at -4.91 bar stress level. Most of the durum wheat genotypes we used in our study tolerated -1.48 bar of drought stress level. Besides, regeneration occurred at -4.91 bar stress level. This difference is thought to be due to the genetic structures of genotypes. In addition, Abdel-Haddy and El-Naggar (2007); stated that among the varieties they used in their experiments, the most sensitive to drought stress was a local variety. In our study, we determined that emmer, which is a local wheat variety, is also sensitive to drought stress.

Bouiamrine and Diori (2012); applied different drought stresses with PEG to calli obtained from immature embryos of durum wheat genotypes. The researchers stated that the mean regeneration capacity was 88.73% in the control group and 26.91% at -4.91 bar stress level. In our study, the mean regeneration capacity of the genotypes was 93.6% in the control group and 52.5% at -4.91 bar stress level. While the regeneration capacity values in the control group are close to each other, there is a significant difference between mean of the regeneration capacity at -4.91 bar stress level. It is thought that this difference may be due to the source of the explant and the method used, as well as the genetic structure.

Farshadfar et al. (2012b); under in vitro conditions, by applying different drought stress to mature embryos of 20 bread wheat genotypes, they examined the genotypes callus induction and callus development. In their study, they stated that callus induction depends on the genotype, not the stress factor. In our study, drought stress was applied to mature calli and their regeneration ability was determined under stress conditions. It is thought that the application of the stress agent in the regeneration phase may be more determinant in measuring tolerance to drought stress.

Conclusion

The results confirmed a significant variation for plant regeneration ability in durum wheat genotypes under drought stress condition that can be used in durum wheat breeding programmes. We propose that this protocol to in vitro selection for drought tolerance would be a suitable and rapid way to characterize parental lines and to develop drought-tolerant lines in durum wheat.

According to the results, the genotypes with the most drought stress tolerance were determined as Sarıçanak-98 and Artuklu. On the other hand, Çakmak-79, Çeşit-2002, Eminbey, Kunduru-1149 and *Triticum dicoccum* (emmer) were determined as genotypes sensitive to drought stress.

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A 20-YEAR LONG TERM STUDY OF YIELD SUSTAINABILITY AND SOIL FERTILITY AFFECTED BY FERTILIZATION AND APSIM CLIMATIC CHANGE MODEL OF URUMQI, XINJIANG, CHINA

TAHIR, N.[1] – LI, J.[1*] – MA, Y.[2] – ULLAH, A.[1] – LIU, H.[3]

[1]*Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, 12 Southern Street of Zhongguancun, Beijing 100081, China*

[2]*Macau Environmental Research Institute, Macau University of Science and Technology, Macau 999078, China*

[3]*Institute of Soil Fertilizer and Agricultural Water Saving, Xinjiang Academy of Agricultural Sciences, Urumqi 830000, Xinjinag, China*
(phone: +86-1-5652-493-881)

**Corresponding author*
e-mail: lijumei@caas.cn

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Abstract. To achieve high crop production, long-term field experiments 20 years (1990-2010) were conducted at Urumqi-China. In this study, we investigate the effect of organic and inorganic fertilization treatments on maize, spring wheat, and winter wheat cropping system in Urumqi, Xinjiang China. We investigated the impact of soil fertility, crop yield sustainability index, nutrients balance, carbon sequestration (CSR), and climatic change model through the Agricultural Production Systems Simulator (APSIM). Five treatments were studied: CK (control); NPK (inorganic fertilizers nitrogen, phosphorus, and potassium); NPKM and manure; NPKS & straw and NPKM2 and manure (the rate of manure was double from NPKM. The study showed that the combined application of inorganic and manure application (NPKM2 and NPKM) significantly increased the crop grain yield and soil fertility and sustainable yield index were higher as compared to NPK and CK. The changes of maximum and minimum temperature (+2), decreasing precipitation (-10), and increased CO_2 level (350-650 ppm) from observed values, simulated in the climatic model will significantly decrease essential soil water (191.2-181.1 mm), runoff (0.43-0.40 mm), total NO_3^- (1444.0 to 1422.5) kg ha^{-1}, and leaching (0.75-0.73) and runoff (0.43 mm to 0.40 mm) while increased total NH_4^+(35.1 to 41.72) kg ha^{-1}.
Keywords: *long-term fertilization, nutrients balance, nutrient input, carbon sequestration rate*

Introduction

Nowadays the Chinese government is focusing on the increasing food crisis and the priority is to produce adequate food for their huge population. For sustainability in food supply to the whole world and especially in China, proper agriculture management practices are needed (Ullah et al., 2020). The application of synthetic fertilizers to achieve maximum crop yield is common practice in agriculture management (Gaind, 2007). Development in scientific data calculations and field management technologies has caused a significant increase in crop production (Doltra et al., 2019). Tong et al. (2003) reported that high-yielding varieties and large consumption of chemical fertilizers increased the annual production of crops in China by 3.37% during 1970-1990 (FAO, 2009). While 0.6% decrease in yield was noted during 1990-2006 (FAO, 2009), which might have been due to the impact of climate change (Zhang et al., 2013)

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and might be further affected by global climate changes (Lobell, 2003). Long-term application of synthetic fertilizers will cause soil deterioration that will decrease crop production, nutritional disorder, groundwater quality, and damage the environment through nonpoint source pollution (Jin et al., 2005; Zhao et al., 2020). Therefore, many scientists have suggested the use of organic fertilizers over chemical fertilizers (Bakht et al., 2009) that will increase soil carbon storage to tackle the problem of global climate change and food security (Zhao et al., 2020). However, use of inorganic fertilizers in excess form increase the water and atmospheric pollution (Derpsch, 2003), but mineral fertilizers through excessive usage in the soil system led to the environmental risk such as water quality (Chen et al., 2008).

To explore and assess the study of overall soil fertility the long-term fertility experiments have been important resources in modern-day science (Shahid et al., 2016). From the perspective of agricultural sustainability, long-term experimentation has provided a platform to investigate soil quality, productivity, and crop yield trends for a long period (Rasmussen et al., 1998). Irrigation and fertilization are considered important factors for nutrient deposition in the soil to obtain high agricultural production however, other factors, such as soil type, fertilization pattern, pests, climate, and cultivars also affect the crop productivity and soil sustainability in long-term cropping systems (He et al., 2020). The SYI stands for a sustainable yield index and represents the actual yields over a long period. It is a quantitative measuring unit to assess the sustainability of agricultural practice (Stull et al., 2004). Countries producing high yield over the years through advanced management and cultivation practices will have higher SYI and sustainability (Singh et al., 2017; Saha et al., 2018). Rasmussen et al. (1998) stated that the sustainability of an agricultural system can be measured through long-term experiments while Tong et al., 2003 reported that early warning systems for the future can be created from the data developed through long-term experiments.

The decline in soil fertility of Chinese soil has been reported by many long-term fertility experiments through extensive variation in crop yields. The continued nitrogen management is very important in this regard for sustainable crop production and soil protection (Tong et al., 2003; Xin et al., 2017; Hu et al., 2019). Chemical fertilizers should be replaced with organic fertilizers to improve phosphorous use efficiency and enhance soil fertility (Xin et al., 2017). Crop simulation models are considered as potentially valuable tools for the accompaniment and advancement of field observations. Crop simulation models deliver a clear demonstration of the essential biological and physical processes such as plant growth and development, nutrients, and water dynamics of single to multiple cropping seasons (Watson et al., 2002; Beaudoin et al., 2008; Kollas et al., 2015). These models provide the opportunity to study the short and long-term effects of new or ongoing agricultural management techniques on crop rotations under the range of different environmental and soil conditions. Worldwide, crop simulation models like APSIM (Keating et al., 2003a), STICS (Brisson, 2003) and DSSAT (Jones et al., 2003) were developed and used for crop simulation but few of these models were reported to have been used under maize-wheat crop rotation (Watson et al., 2002). Increasing the nitrogen fertilizer application in conventional crop rotation will decrease the weight of mineralization in nitrogen balances. Therefore, simple modeling and positive response of the crop to mineral N inputs are comparatively far significant than predicting nitrogen mineralization in upright model performance in these systems.

In this study, we evaluate the ability of the APSIM model to study the effects of different fertility management options in a mono-cropping region having maize, spring

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wheat, and winter wheat cropping system. The APSIM soil nitrogen and climatic model was used to identify nitrogen leaching, field water holding capacity (DUL), saturated water content (SAT), drainage, runoff, total NO_3^-, and NH_4^+, on the long-term soil fertilization treatments of soil over 20 years (1990-2010) in Urumqi Xinjiang, China. Data from the long-term field was used, such as soil, crop and climatic conditions provide a rich dataset for model testing and evaluation. The second objective of the study was to investigate the effects of a different combination of synthetic and organic (sheep-manure) fertilization on sustainable crop production, nutrient uptake, nutrient balance, and relative yield under a mono-cropping system in the Haplic calcisol (grey desert soil). The investigation of nutrients and yield was also carried out for better soil fertility and crop production.

Materials and methods

Location and site description

The long-term research station was established in Urumqi, Xinjiang, China. This region is a semi-arid and continental cold region, with a difference between winters and summer. The latitude and longitude of the area are about 43°49′31″N 87°37′00″E; elevation 600 m above sea level and it has warm and hot summers. The maximum temperature and precipitation were 15.2 °C and 360.1 mm while the minimum temperature and precipitation were 12.65 °C and 141 mm. The experiments were conducted under semi-arid land conditions on the well-fertilized Haplic calcisol (Grey desert soil based on the FAO soil classification) (Zhang et al., 2010). The dataset was used to parameterize the APSIM-Maize-wheat model, nitrogen, and climatic Model. The soil and crop data were also recorded for each crop. The mean annual temperature (MAT) and mean annual precipitation (MAP) for the experimental period were shown in *Figure 1*. The site detail, fertilizer input, and soil sampling procedure were described in *Table 1* and *Figure 2*.

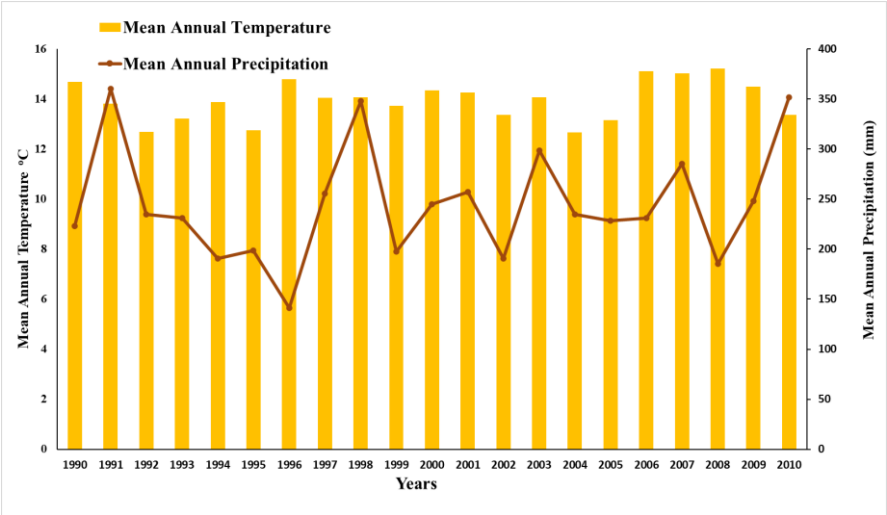

Figure 1. *The meteorological data mean annual temperature (MAT) and mean annual precipitation (MAP) during long term fertilization. The total precipitation was 4537.1 mm from the years (1990-2010). The annual average ambient temperature TAV was 7.86 (°C) and the annual amplitude in the mean monthly temperature was 41.03 (°C) calculated by the APSIM software. The weather data was daily data which was converted to mean annual average data*

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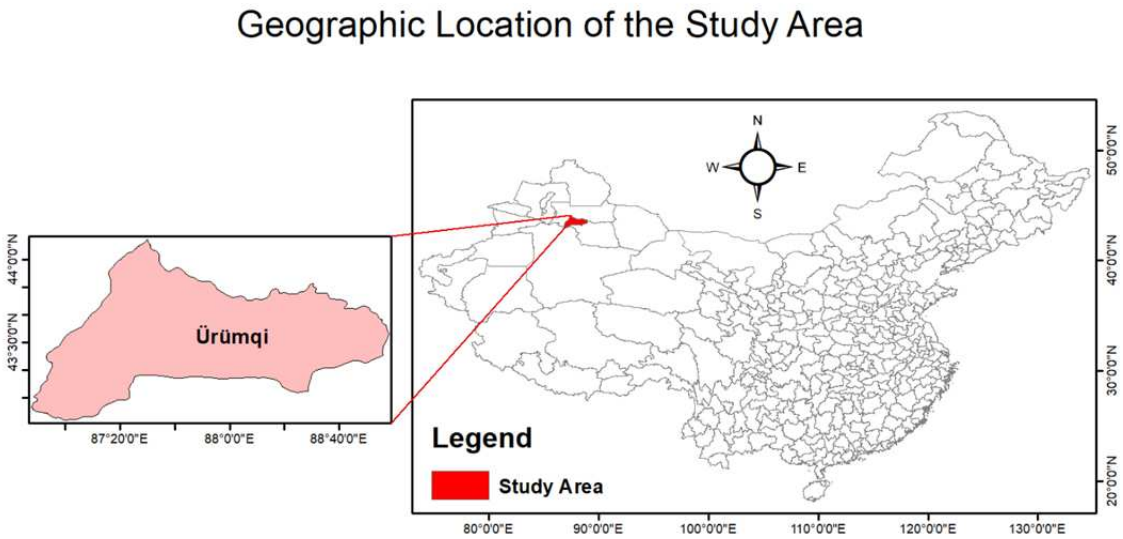

Figure 2. *Study site long term fertilization experiment in Urumqi, Xinjiang China the map was made by ARC-GIS using the coordinates of the location*

Table 1. *Initial soil physical and chemical properties at the long-term experimental site of Urumqi, Xinjiang, China*

Sites	Urumqi
China soil classification	Grey desert soil (Sandy loam)
FAO soil classification	Haplic Calcisol
Soil organic carbon[b] (g kg^{-1})	8.8
Total N[c] (g kg^{-1})	0.91
C/N ratio	10.4
Total P[d] (g kg^{-1})	0.67
Total K [e] (g kg^{-1})	23.0
Available N (mg kg^{-1})	55.2
Available-P (mg kg^{-1})	3.4
Available K (mg kg^{-1})	288
pH[a]	8.1
Clay content (<0.002 mm) (%)	20.9
Bulk density (g cm^{-3})	1.25
Altitude (m)	600
Cropping system	(Maize-Spring wheat-winter wheat)
Annual precipitation (mm)	280-320
Solar radiation (h)	2550-3500

Annual precipitation, temperature, effective accumulated temperature and sunshine hours are means over 20-years (1990-2010). MAT: mean annual temperature. EAT; effective accumulated temperature. (a) Measured in 1:5 soil: ratio, (b) Measured by the Walkley wet combustion method (Nelson and Sommers, 1996), (c) Total N measured by the Kjeldahl method (Nelson and Sommers, 1996), (d) Available P with 0.5 M NaHCO3 (Murphy and Riley, 1962), (e) Exchangeable K with N ammonium acetate (Richardson et al., 2009). Soil available N, P, and K were measured by Lu et al. (2000) (Olsen et al., 1954) and total nitrogen (TN), total phosphorus (TP) and total potassium (TK) in soils were determined using micro-Kjeldahl digestion, colorimetric analysis and a dissolution-flame photo- meter, separately (Page et al., 1982), respectively

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Experimental design

The study was conducted with different treatments consisting of inorganic and organic fertilization. The experiment consisted of five different treatments of fertilization and was conducted during 1990-2010. We selected five treatments in the fertilization dynamic study (*Table 2*): (1) CK (no fertilization); NPK (inorganic nitrogen, phosphorous and potassium fertilizer); (3) NPKM (inorganic NPK fertilizer and manure); (4) NPKS (inorganic NPK fertilizer and straw), and (5) NPKM2 (inorganic NPK fertilizer and manure with double the amount of NPKM). The study period was from 1990-2010. The fertilizer details, annual input rate of manure, and inorganic fertilizer are given in *Table 2*.

Crop and soil management

The cropping system was a mono-cropping system (maize-spring wheat-winter wheat). Extensively used local maize and wheat varieties were selected for cultivation, the variety detail and planting detail were given in *Table A1*. The crop samples were finely ground to pass a 0.15-mm sieve. To determine the total P (TP) content, the plant samples were digested with H_2SO_4–H_2O_2 following the method of (Jackson.,1969), and concentration in the digesting solution was measured using the molybdenum-blue colorimetric method (Page et al., 1982). The winter wheat average sowing date was September 28th; harvest date was July 10th. The average sowing date of spring wheat was April 3th; the harvest date was July 20th. The average sowing period of corn was May 1st; the harvest period started on September 20th. The irrigation time for winter wheat was 7 times, for spring wheat it is 5 times, and Maize 6 times. After the crops were harvested, they were irrigated in winter and turned in autumn at a depth of 30-40 cm. Before sowing, the soil is only raked and level' and furrowed. Inter tillage should be carried out during crop growth, winter wheat inter tillage once, and topdressing at the same time (April 18); spring wheat inter tillage once, and topdressing at the same time (May 13); corn inter tillage twice, in the seedling stage (May 10th), and (June 25th). In maize the plants ranged of 4,000 to 5,000 plants per ha with a row spacing of about 70 cm, and plant spacing of 25-30 cm. While wheat (winter and spring) row spacing was 40 cm. The nitrogen fertilizer was urea, phosphate fertilizer was superphosphate, potash fertilizer is potassium sulfate, organic fertilizer M was sheep manure, and S was straw. In the chemical fertilizer, 60% nitrogen fertilizer and all phosphorus and potassium fertilizers were used as base fertilizer, and 40% nitrogen fertilizer was used as topdressing. The crop was harvested; after harvest, the grain and straw were air-dried and weighed. The management practice were hand weeding and bird frightening methods were used to prevent grain loss from anthesis to harvest. Soil samples from 0-20 cm were taken once in the first week of October every year, approximately 15 days after crop harvesting in fall for all sites after every year. The samples were thoroughly mixed to make the composite sample, air-dried, and transferred to the laboratory for soil analysis. Soil bulk density was calculated on undisturbed soil samples that were collected with a cutting ring of 50.46-mm inner diameter, 100 cm^3 volume, and 50-mm depth of soil sampling (Pansu, 2006). After the crop was harvested, all air-dried and oven-dried grains and straw samples were kept for 30 min at 105 °C for the calculation of dry matter and nutrient contents. The plant nitrogen, phosphorous, and potassium content were measured through Kjeldahl digestion and vanadomolybdate yellow method, and flame photometer respectively. Nutrient uptake was determined by multiplying plant nutrient content with yield. To determine the chemical properties of the soil samples, all the method was described in the note section of *Table 1*.

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Table 2. *Annual input rate of organic (sheep manure) and inorganic N (nitrogen), P (phosphorous), K (potassium) fertilizer added to various treatments of a long-term experiment in a maize-wheat cropping system. Notes: CK (Control), no fertilizer; NPK, chemical nitrogen, phosphorus, and potassium; NPKM, chemical NPK, and manure; NPKS, chemical nitrogen, phosphorus, and potassium and straw; NPKM2, double in manure from NPKM*

Treatments	N (kg ha$^{-1)}$	P_2O_5 (kg ha^{-1})	K_2O (kg ha^{-1})	Organic fertilizer (kg ha^{-1})
CK	0	0	0	0
NPK	241	138	58	0
NPKM	85	51	12	30,000
NPKS	217	117	51	450
NPKM2	150	90	18	60,000

The nitrogen fertilizer was urea, the phosphate fertilizer was superphosphate, the potassium fertilizer was potassium sulfate and the organic fertilizer M was sheep manure. 60% of the nitrogen fertilizer and all the phosphorus and potassium fertilizers was used as the base fertilizer, and 40% of the nitrogen fertilizer was used as the top dressing. The sheep manure contains 7 g kg^{-1} of N, 3.2 g kg^{-1} of P, and 8 g kg^{-1} of K

Calculations

The SOC density content was calculated from SOC content by the equation (Lal, 2008):

$$\text{SOCdensity} = SOCcontent\ x\ d\ x\ BD\ x10 \quad \text{(Eq.1)}$$

where soil organic carbon density was in (Mg ha^{-1}) $SOC_{density}$; soil organic carbon content was in (g kg^{-1}) represented by $SOC_{content}$; d is the depth of the soil while BD is the depth of the soil layer (0.20 m) and soil bulk density (g cm^{-3}/kg m^{-3}). Soil organic carbon (SOC), as an index for soil fertility and a mean for carbon sequestration, has attracted much attention over the past decades. Soil fertility index was measured by soil organic carbon rate (SOC) and means of sequestration of carbon. The soil sequestration rate of organic carbon (CSR, t ha^{-1} year^{-1}) was calculated by the following equation (Zhang et al., 2012).

$$\text{CSR} = \frac{(\text{SOCT} - \text{SOCo})}{t} \quad \text{(Eq.2)}$$

where CSR is the Soil sequestration rate, SOCT and SOC_O is organic carbon (t ha^{-1}) stock at the time (t) and in the first year (1990), respectively and (t) was the period of experimentation (20 years). The relative yield (YR) was measured to study the comparison of individual treatment of yield data. YR was calculated through the following formula (Singh et al., 1990).

$$\text{YR} = \text{Ytreatment} - \text{Ycontrol} \quad \text{(Eq.3)}$$

where $Y_{treatment}$ is the yield of treatment of fertilizer applied (t ha^{-1}) in a particular year of experimentation and $Y_{control}$ is the yield (t ha^{-1}) in the same year of control treatment.

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Sustainable yield index (SYI) is a quantitative extent to measure the productivity of long-term land management (Stull et al., 2004). The SYI was measured by using the following equation (Singh et al., 1990):

$$\text{Sustainable yield index (SYI)} = \frac{\text{Ymean} - \sigma}{\text{Ymax}} \quad \text{(Eq.4)}$$

where Y_{mean} is a mean of yield treatment, σ is the treatment standard deviation, and Y_{max} is the maximum yield over the years for every treatment.

Apparent nutrient balance (AB) defined as the difference between entering nutrient inputs in the farming system and leaving nutrients from the farming system (OECD, 2013). AB (kg ha^{-1} $year^{-1}$) was measured using the equation as follows (Ouyang et al., 2017):

$$\text{Apparent nutrient balance} = \text{Sum of nutrient input} - \text{Sum of nutrient output} \quad \text{(Eq.5)}$$

where nutrient input (kg ha^{-1} $year^{-1}$) is annual nitrogen, phosphorus, and potassium input to the field through inorganic and organic fertilization. Nutrient output (kg ha^{-1}) is nitrogen, phosphorus, and potassium uptake (kg ha^{-1}) by the crop in above-ground biomass. If apparent nutrient balance indicates positive value then nutrients are in excess and surplus and if the negative value comes then nutrients are deficit, which indicates decreasing soil productiveness and fertility (OECD, 2013).

APSIM model

The Agricultural Production Systems Simulator (APSIM) was developed in Australia, the model enables the analysis of complex soil and crop issues for better agriculture production and simulation (McCown et al., 1996; Keating et al., 2003b). APSIM simulates the point scale system in soil and crop. It used daily weather data and utilized the soil physicochemical properties and crop physiological growth process was measured. In the present study, the following models were used such as crop (maize and wheat), soil N (SOILN2), soil water (SOILWAT2), crop residue (RESIDUE2), soil runoff (mm) and climatic change model. The soil runoff was measured by the APSIM climatic change model to check N losses from the soil system. All these models were linked in the APSIM framework. All the soil parameters and crop water requirements were adjusted with the help of the following website (https://www.apsim.info/wp-content/uploads/2019/10/Parameters-for-soil-water-Ver24.pdf). All the input parameters were described in *Table A2*. The date of the simulation was started from 1990-2010. The APSIM met data was calculated with the help of the following website. https://www.apsim.info/support/apsim-training-manuals/creating-an-apsim-met-file-using– excel/. The soil physical parameters requirement was given in *Tables A2* and *A3*. This table was calculated on the basis of bulk density and used as input measurement in the APSIM model.

Statistical analysis

The change among different fertilization treatments under long-term fertilization was divided into (1990-2000) and (2000-2010). The simple linear regression model was used for OC and yield graphs among different treatments. The pH, AN, AP, OC, TN, TP, and RY were used as dependent variables. The APSIM observed and simulated data were

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undertaken and verified from regression analysis methods. The correlation analysis and Principal component analysis (PCA) were performed through Software R (R version 3.6.1,) core team, Vienna, Australia, and this was used for graphical explanation of mean values of treatment effects throughout 1990-2010 on different parameters. The PCA was used for the classification and graphical representation of the components. It gives group accessions of the properties with the highest discrimination values among the variables.

Results

Crop yield and sustainability index

The result of the long-term fertilization on the maize-spring wheat-winter wheat cropping system showed significant influences over the fertilization years. The regression trend shown in *Figure 3* and grain yield (t ha^{-1}) in *Table 3* showed an increase in NPKM2, NPKS and NPKM, and a decrease in control while slight difference was observed in NPK among all the crops. The treatment (NPKM2) showed significant regression trend in maize crop ($R^2 = 0.5892$), spring wheat ($R^2 = 0.7872$), and winter wheat ($R^2 = 0.5447$). The annual grain yield in different fertilization years showed a significant decrease in control among all the treatments and it was the highest in NPKM, NPKS, and NPKM2. The treatment effects in fertilization from 1990-2000 were compared with (2000-2010), an increase in annual grain yield was observed in (2000-2010) in NPKM2, NPKM, and NPKS. In maize and spring and winter wheat, the control showed a decrease in annual grain yield while in NPK slight increase was observed. The sustainable yield index (SYI) increases in organic and inorganic (sheep manure) application in combined and pure doses while in NPK, and NPKM the sustainable yield index was high in winter wheat such as 0.64 ± 0.28 and 0.64 ± 0.29 followed by NPKM2 (0.61 ± 0.26). The SYI in maize crop showed the increased value in NPKS, NPKM2, NPKM, and NPKM2 treatment as compared to control. In spring wheat NPKS showed a higher sustainable yield index followed by NPK, NPKM and NPKM2. Moreover, the winter wheat was more sustainable as compared to spring wheat and maize crop.

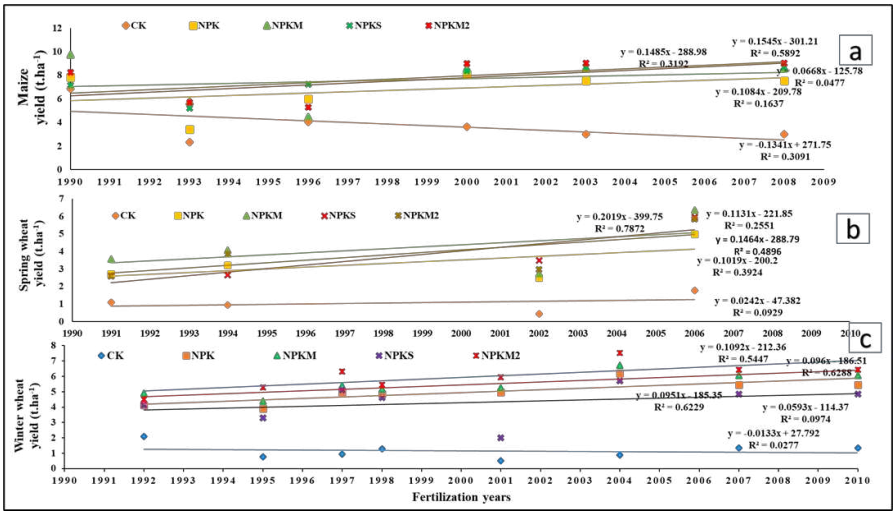

Figure 3. *Yield of crops vs fertilization years, the year of fertilization started from 1990-2010, hence each crop grows at different years as presented in the figure: maize (a), spring wheat (b), and winter wheat (c) under various fertilization years of a long-term experiment in the mono-cropping area of Urumqi, Xinjiang China*

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Table 3. *Sustainability yield index (SYI) winter wheat, (SYI) of maize and (SYI) of spring wheat under long term experiment having different fertilization dynamics in the mono-cropping system*

Treatments	SYI (winter wheat)	SYI (maize)	SYI (spring wheat)
CK	0.44 ± 0.09	0.76 ± 0.12	0.77 ± 0.15
NPK	0.64 ± 0.28	0.84 ± 0.11	0.84 ± 0.09
NPKM	0.64 ± 0.29	0.84 ± 0.16	0.83 ± 0.11
NPKS	0.56 ± 0.19	0.94 ± 0.04	0.89 ± 0.10
NPKM2	0.61 ± 0.26	0.89 ± 0.11	0.79 ± 0.04

Values are means of year ± standard deviations

Apparent nutrient balances and nutrient uptake

The Nutrient balance is defined as the difference between the nutrient enter into the soil system (input) and leaving the system (output), and provide information about environmental pressures. Nutrient input and apparent nutrient balance were presented in *Tables 4* and *A6*.

Table 4. *Annual nutrients uptake and apparent nutrient balance (N, P, and K) and crop yield in maize-spring wheat and winter wheat cropping system from the period of (1990-2010) and (2000-2010) under different fertilization of long-term experiment over 20 years of fertilization*

Years	Treatments	NU (kg ha^{-1})	PU (kg ha^{-1})	KU (kg ha^{-1})	N balance (kg ha^{-1} year^{-1})	P balance (kg ha^{-1} year^{-1})	K balance (kg ha^{-1} year^{-1})	Grain yield (t ha^{-1})
1990-2000 Maize	CK	76.05	7.11	0.00	-3.72	-7.11	0.00	4.41
	NPK	136.75	15.57	0.00	78.58	46.43	48.13	5.76
	NPKM	128.94	23.11	0.00	166.06	40.23	209.11	6.66
	NPKS	134.03	15.61	0.00	82.97	36.95	42.32	6.53
	NPKM2	134.61	24.26	0.00	435.39	97.04	413.23	6.41
Spring wheat	CK	21.59	2.73	4.65	-12.24	-0.94	4.65	1.02
	NPK	89.40	13.11	24.33	131.32	38.89	56.41	2.95
	NPKM	106.33	18.13	35.81	171.23	39.07	175.21	3.82
	NPKS	86.19	12.75	26.07	139.67	36.68	54.28	2.63
	NPKM2	102.12	18.20	27.86	347.95	77.70	303.35	3.23
Winter wheat	CK	34.62	3.93	2.13	-34.62	-3.93	-2.13	1.28
	NPK	139.19	19.11	17.56	101.81	42.89	30.57	4.46
	NPKM	125.42	22.78	17.75	169.58	40.57	191.35	4.96
	NPKS	88.76	18.40	17.28	128.24	34.17	25.04	4.29
	NPKM2	126.48	26.34	20.99	463.30	99.48	413.23	5.40
2000-2010 Maize	CK	61.10	8.00	134.57	-61.10	-8.00	-134.57	3.22
	NPK	168.89	30.68	273.93	72.11	31.32	-225.80	7.73
	NPKM	176.26	29.17	312.12	118.74	34.18	-103.01	8.58
	NPKS	178.35	32.22	317.81	38.65	20.34	-275.49	8.57
	NPKM2	200.96	43.53	337.26	369.04	77.77	75.97	9.01
Spring wheat	CK	35.36	4.93	29.71	-35.36	-4.93	-29.71	0.69
	NPK	155.12	17.00	110.62	85.88	45.00	-62.49	2.85
	NPKM	150.82	21.38	158.23	144.18	41.97	50.88	3.39
	NPKS	136.72	18.96	120.42	80.28	33.60	-78.11	3.08
	NPKM2	195.84	23.97	165.25	374.16	97.33	247.99	3.42
Winter wheat	CK	18.58	7.75	7.61	-18.58	-7.75	-7.61	1.02
	NPK	125.07	43.71	74.38	115.93	18.29	-26.25	5.52
	NPKM	139.33	54.15	87.00	155.67	9.19	122.11	6.02
	NPKS	89.68	34.93	49.86	127.32	17.64	-7.54	4.35
	NPKM2	153.90	54.80	77.00	416.10	66.50	336.24	6.58

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Among all the fertilization years (1990-2010), in all treatments the highest nutrient uptake (nitrogen, phosphorous, and potassium) were observed in NPKM2 and the lowest was in CK. Among all the crops the nitrogen uptake (NU) showed a decrease in the year of fertilization from 1990-2010 in NPKM, NPKS, NPKM2 while increased from 2000 to 2010. Phosphorus and potassium uptake also showed the same trend as NU uptake, the highest nutrient uptake was in NPKM2 among all the fertilization years. A minor change was observed among the control treatment in all crops. The apparent nutrient balance was significantly different from control as compared to treatments. The control showed a negative value and a deficit in nutrient balance among the crop from 1990-2010. The nitrogen, phosphorus, and potassium showed a positive value and surplus nutrient balance was showed in NPKM2 and significantly different from other treatments. Nitrogen and phosphorous balance among all treatments were positive during all the cropping periods. The potassium balance showed a negative value for all the treatments except NPKM2 and NPKM during all cropping periods except from 2000-2010 the NPKM showed negative value in the maize and winter wheat cropping system. The potassium balance in maize ranges from 77.77 kg ha^{-1} $year^{-1}$ in NPKM to -8.00 kg ha^{-1} $year^{-1}$, in Spring wheat 247.99 kg ha^{-1} $year^{-1}$ to -29.79 kg ha^{-1} $year^{-1}$ while in winter wheat 247.99 kg ha^{-1} $year^{-1}$ to -7.61 kg ha^{-1} $year^{-1}$. The nutrient balance over 20 years of fertilization in nitrogen and phosphorous showed a decreasing trend among all the treatments in 2000-2010 as compared to 1990-2000.

Dynamics of soil nutrients and soil organic carbon sequestration rate (CSR)

The soil pH, nutrients, and soil organic carbon sequestration were significantly influenced by long-term fertilization. During the two long-term fertilization periods 1990-2000 and 2000-2010, the soil pH fluctuated from 8.12 in NPK and 8.40 in NPKS. Soil pH increased in the second period of soil fertilization as compared to the first year 1990-2000 (*Table 5*). Soil TN, TP, and TK in the maize cropping system showed an increasing trend in NPKM2 followed by NPKM, NPK, and NPKS in the fertilization year of 1990-2000 while the same trend was followed by the second year of fertilization with minor variation. All the nutrients showed an increasing trend while AK showed decreasing values during the years of fertilization. In the cropping system of spring wheat, the TN, TP, and AN, AP and AK showed a higher value in NPKM2 followed by NPKM, NPK, and NPKS respectively in the fertilization period 1990-2000. The comparison of two periods of fertilization showed that all the nutrients decreased in the second year of fertilization except NPK and NPKS in TN, NPKM in TP, NPK, and NPKM in AN and NPKM in AP respectively. In the winter wheat cropping system the TN, TP, and AN, AP, and AK showed a higher value in NPKM2, NPKM and NPKS and NPK, respectively. The NPKM indicated an increasing trend when compared with the initial year of fertilization while all other nutrients decreased in the second year of fertilization (2000-2010). The combined application of synthetic fertilizer and organic fertilizer (sheep manure) significantly increases the soil nutrient level among all treatments such as available and total nitrogen, available and total phosphorous, and total potassium. Manure increases soil fertility and also soil structure and it significantly increases the nutrient input to the soil system. Soil organic carbon in the maize cropping system showed a positive relationship in the year of fertilization and increased with time, higher SOC was observed in NPKM2 followed by NPKM. The highest OC g kg^{-1} was found in NPKM2 followed by NPKM, NPK, and NPKS and control. The RY t ha^{-1} also followed the same trend. The spring wheat showed the highest value of OC g kg^{-1}

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in NPKM followed by NPKM, NPKS, and NPK, the lowest was found in control treatment among all the cropping systems in fertilization years. The RY also had the same trend of increasing value. In winter wheat OC g kg^{-1} showed a higher value in the soil NPKS with the minute increase from NPKM2 in both years of fertilization and a similar trend was followed in both years with little increase. The soil carbon change was shown in *Table A6* and *Figure A1*. The RY t ha^{-1} was higher in NPKM2 followed by NPKM and NPKS. Soil carbon sequestration rate (CSR t ha^{-1} yr^{-1}) showed negative value among all the CK, NPKM, and NPKS while it showed positive value for both NPKM and NPK. Overall, the change in all fertilization treatments was no sign of CSR.

Table 5. *Soil nutrient contents, annual organic carbon, and soil organic sequestration rate CSR in maize-winter/spring wheat cropping system form the period of 1990-2010 under different fertilization treatments in long term experimental site*

Year/crop	Treatments	TN (g kg^{-1})	TP (g kg^{-1})	AN (mg kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})	OC (g kg^{-1})	pH	RY (t ha^{-1})	CSR (t ha^{-1} y^{-1})
Initial year		1.01	0.35	84.6	9	461	9.78	8.01	-	-
1990-2000 Maize	CK	0.98	0.69	56.20	2.73	144.00	8.48	8.30	1.28	-0.116
	NPK	0.98	0.79	53.07	10.53	135.00	9.32	8.12	4.46	0.000
	NPKM	1.12	0.77	67.77	12.27	335.33	10.16	8.28	4.96	0.000
	NPKS	0.95	0.61	54.80	7.73	160.83	8.63	8.40	4.29	0.000
	NPKM2	1.15	0.82	73.30	32.03	351.67	11.94	8.21	5.40	-0.001
2000-2010	CK	0.68	0.60	45.77	4.06	165.67	7.98	8.40	1.02	-0.051
	NPK	0.92	0.91	62.83	13.00	219.73	9.05	8.50	5.52	0.001
	NPKM	1.47	0.80	99.37	50.70	390.07	11.37	8.29	6.02	0.000
	NPKS	0.70	0.63	54.63	8.57	124.87	8.57	8.28	4.35	-0.002
	NPKM2	1.21	0.77	81.90	30.70	391.83	14.10	8.40	6.58	-0.002
1990-2000 Spring wheat	CK	0.80	0.50	52.50	2.13	129.80	8.41	8.35	1.02	-0.1232
	NPK	0.89	0.83	54.67	13.23	194.07	8.78	8.11	2.95	0.0004
	NPKM	1.17	0.75	71.20	18.77	351.20	8.86	8.32	3.82	0.0002
	NPKS	0.86	0.70	54.37	8.67	160.83	9.63	8.36	2.63	-0.0001
	NPKM2	1.40	0.98	84.70	37.30	413.90	10.98	8.23	3.23	-0.0013
2000-2010	CK	0.73	0.55	38.75	2.87	153.50	7.86	8.41	0.69	-0.076
	NPK	0.96	0.79	56.25	14.95	185.00	8.76	8.29	2.85	0.000
	NPKM	0.96	0.73	86.25	29.55	325.50	8.14	8.28	3.39	0.000
	NPKS	1.17	0.66	51.55	16.30	157.50	9.84	8.40	3.08	-0.003
	NPKM2	1.20	0.68	71.45	23.60	314.50	10.60	8.42	3.42	0.000
1990-2000 Winter wheat	CK	0.85	0.70	48.65	3.65	172.08	7.96	8.40	4.41	-0.097
	NPK	0.92	0.95	74.10	10.63	148.75	8.69	8.26	5.76	0.000
	NPKM	1.19	0.76	75.88	12.73	385.75	10.93	8.28	6.66	0.000
	NPKS	0.94	0.74	69.23	7.95	205.63	8.53	8.40	6.53	-0.001
	NPKM2	1.44	0.88	94.53	33.30	374.75	12.88	8.24	6.41	-0.002
2000-2010	CK	0.72	0.67	50.17	3.39	125.33	7.87	8.42	3.22	-0.064
	NPK	0.81	0.87	57.47	11.40	290.00	8.52	8.29	7.73	0.001
	NPKM	1.41	0.89	92.63	52.00	583.33	11.24	8.28	8.58	0.000
	NPKS	0.72	0.71	79.53	10.63	180.67	8.22	8.40	8.57	-0.002
	NPKM2	1.14	0.67	70.03	20.71	375.00	12.71	8.38	9.01	-0.003

Means followed by an average of ten years' data in which every year data with different crop means considered separately for maize, spring wheat, and winter wheat, values of means are (n = 3). Abbreviations: TN, total N; TP, total P; AN, available N; AP, Available P; RY, Relative Yield; CSR, soil carbon sequestration rate

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Principal component analysis (PCA)

The Principal Component Analysis (PCA) clustering processes are usually performed to measure the variation among the treatment effect and show the relationship between the variables. It interprets the data more expressively than other tools. There are two components in the principal component analysis as PC1 and PC2. The PCA analysis was performed to check the variation effect among the treatments on different variables such as (NU, PU, KU, N balance, P balance, K balance, and grain yield) over the year of fertilization from 1990-2000 and 2000-2010. The principal component analysis from the year of fertilization 1900-2000 among different variables (*Fig. 4a*) showed that all the values were more diverse and showed variation over different treatments. The first PC1 and second PC2 together described 87.3 of the total variation among all the observed values. The principal component analysis (PC1) (*Figure 4*) variation primarily attributes to NU, PU, KU, N bal, P bal, K bal, and GY for which eigenvectors were 4.65, 1.45, 0.66, 0.122, 0.051, 0.038, and 0.004, respectively.

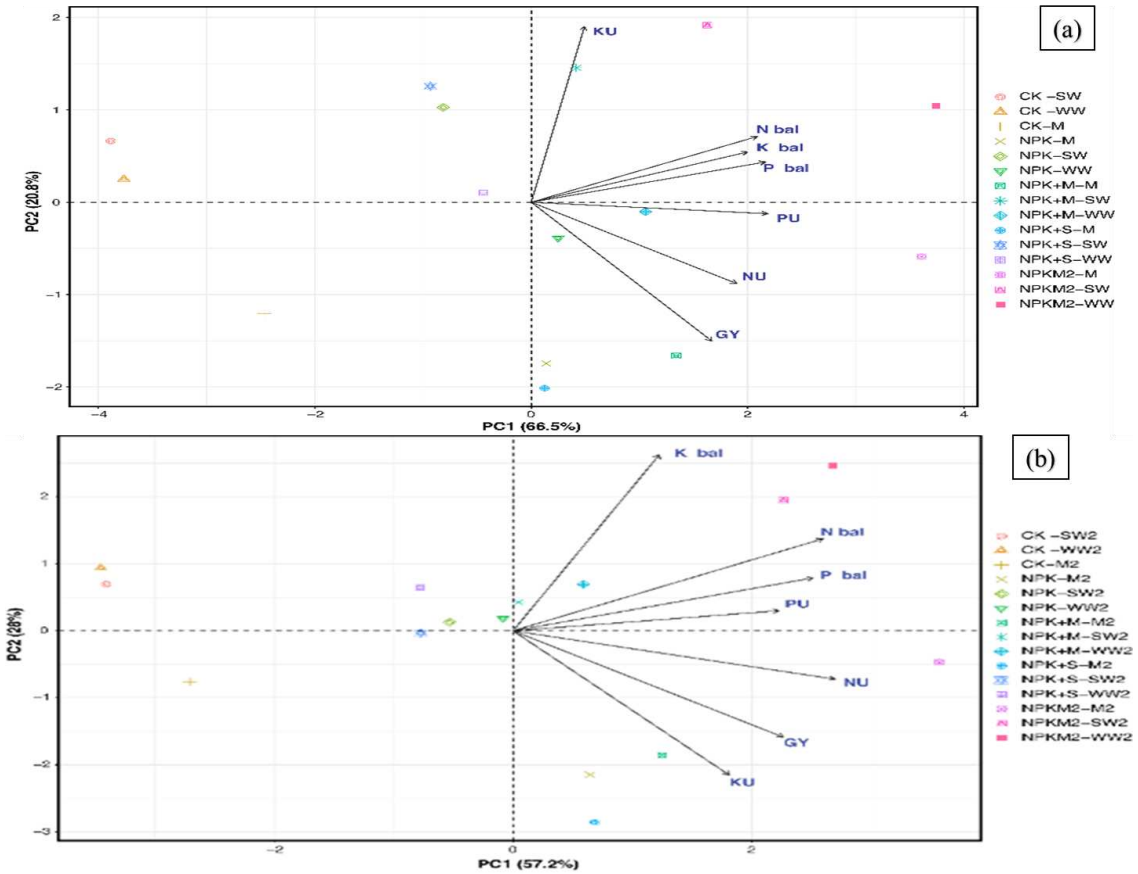

Figure 4. *Principal component analysis (PCA) (a) (1990-2000) of nutrient Uptake (kg ha^{-1} Year^{-1}) (NU, PU, and KU) and nutrient balance ((kg ha^{-1} Year^{-1})) representing through N bal (nitrogen balance); P bal (Phosphorous balance) and K bal (potassium balance) and GY (grain yield (t ha^{-1})) while PC: principal component. (b) Principal component analysis (PCA) on the years from 2000-2010. Every point on the scatter plot showed the effect of a single treatment on nutrients. Control (CK) and NPK of maize, spring wheat and winter wheat showed less variation on nutrient uptake, balance, and grain yield while higher variation was showed by treatment NPKM2 followed by NPKM and NPKS respectively*

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The second figure (*Fig. 4b*) of PCA showed the variation among the nutrient uptake, balance, and grain yield for the year of fertilization 2000-2010. The PC1 and PC2 contribute 85.2 of variation in treatment effect from the 2000-2010 fertilization year. The variation in PC1 is attributed to NU, PU, KU, N bal, P bal, K bal, and GY with eigenvector of 4.00, 1.96, 0.81, 0.15, 0.04, 0.008, and 0.003, respectively. The treatment effect on the different years of fertilization was shown in *Figure 4,* and variation among the different years was observed in both graphs of PCA. The control, NPK, NPKM, NPKS, and NPKM2 showed a significant effect on the nutrient uptake and balance under the different treatments over 20 years. The different nutrient poses major attribute and influence on the yield of the crop. Moreover, the correlation was positive among all the nutrient uptake, balance, and grain yield. The treatments having a combined application of organic and inorganic fertilizer showed higher yield and it improved the soil fertility and crop yield.

Correlation of nutrient uptake, nutrient balance, and grain yield.

Pearson's correlation analysis is the best tool for complex data visualizing. Pearson's correlation analysis was performed to study nutrient uptake, nutrient balance, and grain yield throughout the 20 years. The different nutrient uptake balances showed a positive relationship among the nutrient uptake by crop and grain yield (*Fig. 5*).

Figure 5. *The correlation analysis between the nutrient uptake, nutrient balance, pH, and grain yield. The above diagonal characterizes the coefficient correlation of the p-value $p < 0.05$, 0.01, and 0.001; denote significance at *, **, and *** correspondingly. The lower plot of diagonal showed NU, Nitrogen uptake; PU, phosphorus uptake; KU, Potassium uptake; N balance; P balance; K balance (kg ha^{-1}), pH and grain yield (t ha^{-1})*

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Grain yield showed a positive relationship with nutrient uptake of N, P, and K, the correlation was positive, it represented that it had a significant effect on nutrient uptake and showed positive correlation with nutrient balance PU followed by NU respectively. The grain yield showed a highly significant correlation with NU (r = 0.82***) followed by KU (r = 0.79**) and PU (r = 0.74**) while pH had a negative effect on it. Nutrient balance showed highly significant effect on each other and showed a positive relationship with each other. The N balance showed highly positive correlation with P balance (r = 0.95***) followed by K balance (r = 0.91***). N nutrient uptake showed a positive correlation with k uptake (r = 0.78***) and P uptake (r = 0.75***). The pH had a negative effect on all the nutrient uptake and balance and showed a negative relationship with each other.

APSIM results on climatic change and nitrogen model

Agricultural Production System Simulator (APSIM) well simulated the climatic change and nitrogen model in wheat-maize cropping system in Urumqi China. The APSIM showed significant results on the climatic change model of the current weather condition. The precipitation over the years of fertilization showed variations throughout the years. The runoff showed a positive relationship with the precipitation. The higher the precipitation, the higher the runoff, and the higher the nutrient losses from the soil system. The surface runoff and leaching normally occur with rainfall events. This leads to the environmental risk of phosphorus transportation from agroecosystems to rivers and lakes. Hence it was proved from the overall nutrient balance (*Table 4*) that the highest phosphorous balance was observed in NPKM2 and NPKM. So APSIM also showed surface runoff with precipitation, moreover, the rates of P inputs should be reduced to prevent the nutrient losses in to soil, air and water environment. This will lead to the achievement of sustainable crop production and decreasing environmental pollution risk (*Fig. 8*). The soil hydrodynamic properties among each layer of the soil were measured through APSIM through saturated water content (SAT) and field water holding capacity (DUL), SAT was calculated from soil bulk density. It gives complete changes in soil dynamics of different nutrients. In this current study, we used eight layers up to 160 cm showed in *Table A2*. The soil hydrodynamic was shown in *Figure 6a, b*. There are two major crops such as maize and wheat. The crop and soil parameters were the crop's lower limit (LL) and the crop water absorption coefficient (KL) at 8 different layers and depths. The LL and KL were also calculated separately for maize and wheat crops. There were no values based on which we measure but it is adjusted and estimated based on APSIM software. The effect of water balance runoff, drainage, and esw (essential soil water) was affected by soil water capacity and weather. The climatic change model showed a significant change in nitrogen balance throughout 20 years (*Figs. 7* and *8; Table A4*). The runoff (mm), esw (mm), leaching (0-1), total NO_3^- (kg ha^{-1}), and total NH_4^+(kg ha^{-1}) and precipitation showed significant results with both CO_2 levels (350-650) ppm. The observed and predicted graph with linear regression showed significant changes when the climatic model was applied. The level of CO_2 from 350 ppm to 650 ppm was changed and precipitation was changed to -10 and maximum and minimum temperature was changed to + 2 °C. The average precipitation over 20 years of simulation received was 0.63 mm in observed while in predicted 0.57 mm. The runoff was changed from 0.43 mm to 0.40 mm. The change observed in total NO_3^- (1444.0 to 1422.5) kg ha^{-1} and total NH_4^+ (35.1 to 41.72) kg ha^{-1} respectively. Leaching defined as the loss of water-soluble plant nutrients due to

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precipitation and irrigation from the soil. Leaching fraction is the LF = effluent / influent. The nitrogen leaching was observed between (0.75-0.73) and no change in the drainage was observed. Nitrogen balance (kg ha^{-1}) was 14.38-19.22 and essential soil water (esw (mm)) was 191.2-181.1. The changes in the climatic model showed that the change in future environmental parameters had a significant effect on soil, crop, and water parameters (*Table A4*). Proper measurements are needed to be taken to overcome the significant changes in the environment and increase crop production and improve soil fertility.

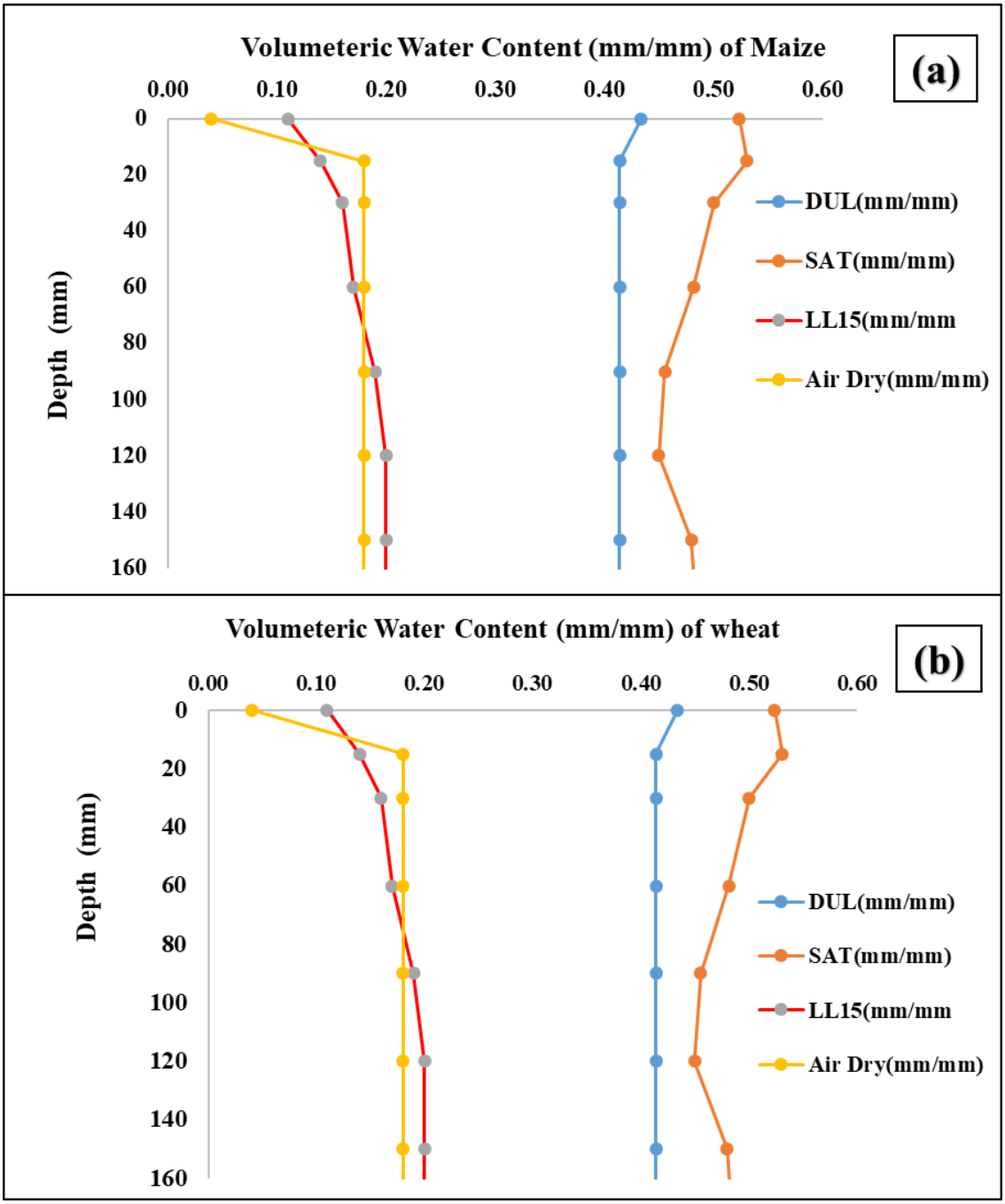

Figure 6. *Volumetric water content under different layers (1-160) divided into 8 different layers. (a) Maize crop and (b) wheat crop*

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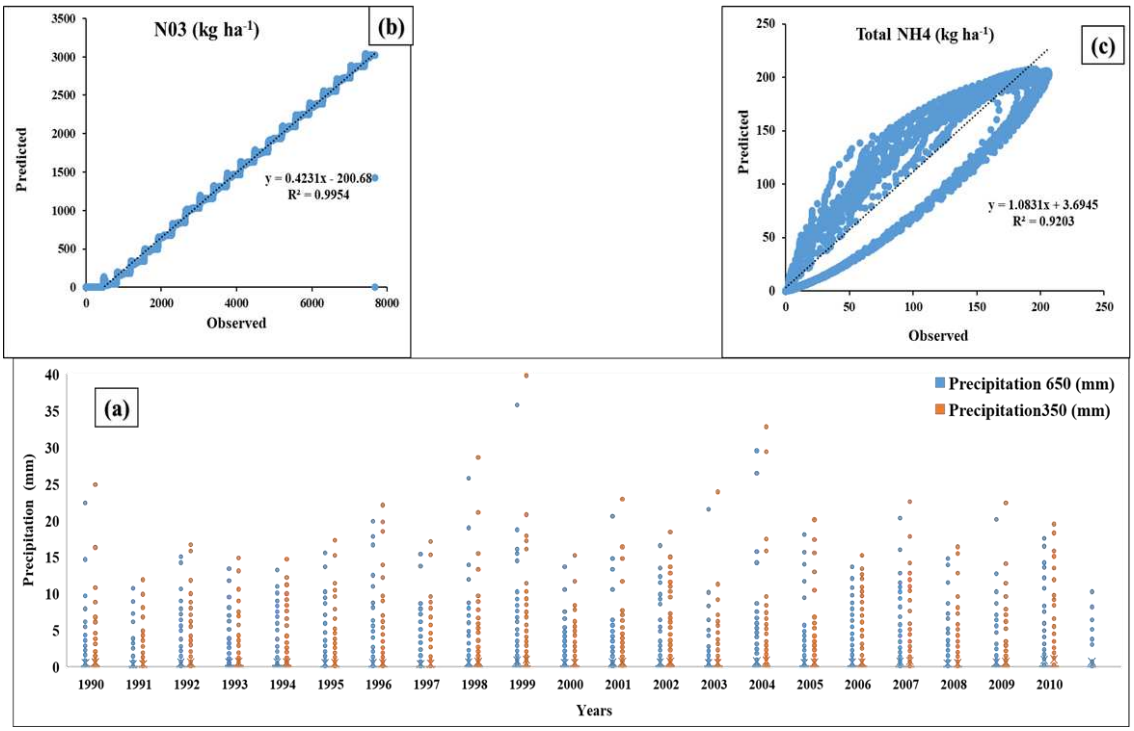

Figure 7. *The graph was created through APSIM climatic change and nitrogen model with 305 ppm and 650 ppm scenario of CO_2 and change in temperature (Maximum and minimum) (+2) and precipitation (-10). (a) Years vs precipitation (mm) during climatic model when precipitation was change to (-10) on 20 years' simulation. (b) NO_3^-(kg ha^{-1}) during observed and simulated values. (c) NH_4^+leaching observed and simulated and trend lines showed relationship*

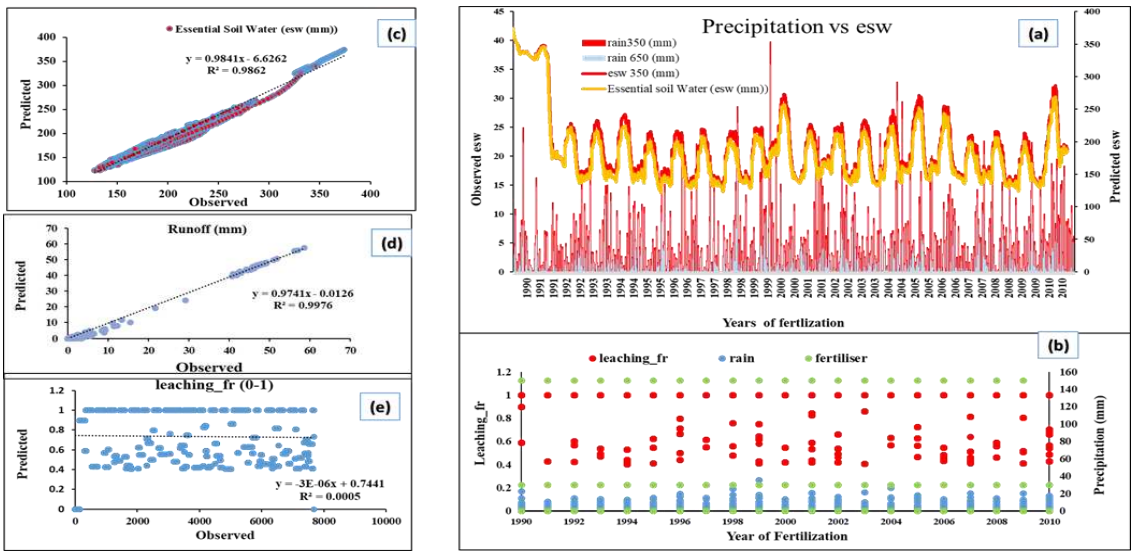

Figure 8. *(a) Precipitation vs essential soil water (esw) over fertilization years 1990-2010 (b) years of fertilization vs leaching and rain fall. (c) Essential soil water (esw) (mm) observed vs predicted (d) Total runoff (mm) during observed and predicted simulation, and (e) leaching (0-1) observed vs predicted over the 20 years of fertilization*

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Discussion

In our findings it was clearly showed that over 20 years of fertilization, the combined application of inorganic and organic (sheep manure) significantly enhanced crop growth, yield, and soil fertility as compared to a single application of organic fertilizer. Crop yields of CK (control) among all crops (maize, spring wheat, and winter wheat) showed a decrease over the years of fertilization. The grain yield in all treatments increased in NPKM2, NPKM, NPKS, and NPK over the years (1990-2010) (*Table 4; Fig. 3*) (Das et al., 2014). Manure is reported to be used to effectively improve the agricultural fertility of the soil and soil health (Gai et al., 2018). The long-term fertilization treatments of grain yield during organic manure treatments may be attributed to the enhanced availability of a reservoir of organic minerals by applying manure to the soil. Long-term fertilizer application and organic manure significantly enhanced deposition of soil organic carbon as well as nitrogen and also microbial soil biomass, obtaining maximum SMBC consisting of 3.7% of SOC, SMBN accounted for 4.5% of total nitrogen, and SMBP contributed for 2.1% of total phosphorous. These soil organic carbon and nitrogen deposits were effective in slowing the decomposition of inborn soil carbon (Qiu et al., 2018). Our result was supported by the previous finding, the use of such synthetic and organic (manures) fertilizers improved significantly yield and quality of wheat, maize, and rice by an overall gain of only 29% compared to other organic fertilizers and by 8% compared to the synthetic fertilizer application (Han et al., 2004). The previous results showed that the positive residual impacts on agricultural productivity were observed in various long-term fertilization dynamics (Chavas et al., 2009; La et al., 2016). The key reasons for reduced agricultural productivity in CK and NPK may be soil acidification, as constant use of synthetic fertilizers tends to decrease soil productivity by reducing soil pH and has a higher influence on CO_2 fixation and soil microbial growth (Sarkar et al., 2018). In previous studies a high reduction of crop yield was observed under chemical fertilization in paddy soils over the time (Yang et al., 2018). The result of (Zhu et al., 2018) suggested that to reduce soil acidification high chemical nitrogen fertilization can be partially supplemented by composite or natural fertilizer in tea crop (Zhang et al., 2010, 2018). The organic and inorganic fertilizer application increased nitrogen, phosphorous, and potassium as compared to CK and NPK shown in *Table 4,* which were also similar to the findings of (Wei et al. 2020) and Yang et al., 2020). With the combined application of organic and synthetic fertilizer, we found that the nutrient balance was positive and the highest in NPKM2 followed by NPKM, NPKS, and NPK. The CK showed a negative balance among all the crops showed in *Table 4*. The result was similar to the finding of (Hu et al., 2019) who stated that the amount of crop production, grain yield, and nitrogen was high when organic and inorganic fertilizers were used as treatment. The nitrogen use effectiveness was also high in manure. The yield and nutrients were balanced under these treatments. The manure treatment showed higher results as compared to other treatments among all the crops over 20 years of fertilization showed in *Table 4* and *5,* similar findings were showed in organic and inorganic fertilizer treatments (NPKM and NPKM2) the activities of the enzymes were enhanced and it is the reason of soil nutrients increase and the soil pH was not change (Zhang et al., 2019). The soil nutrient balance of nitrogen and phosphorus was high in the years 1990-2000 as compared to 2000-2010. The nutrient balance was high in NPKM2 followed by NPKM, NPKS, and NPKM over the years of fertilization. The correlation in *Figure 5* also showed a positive correlation among NU, PU, and N balance and P balance. The positive N and P balance was due to

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lower N uptake by crops. Previous studies indicated that nitrogen effectiveness of nutrient usages (NUE) and improper fertilization significantly reduced nitrogen uptake (NU) and increased nutrient balance and nutrient depletion in the soil (Shi et al., 2012; Egan et al., 2019). The analysis indicates that long-term organic fertilizer has the most positive effects on grain yield and soil fertility (Liu et al., 2010). Various research studies showed that the soil properties (physical and chemical) were significantly affected by organic matter addition (Liu et al., 2010). The study of maize-spring wheat-winter wheat in the Urumqi region showed that the addition of organic manure with synthetic fertilizer enhanced crop yield and nutrient availability in increasing trend and significant change was observed in NPKM2 and NPKM as shown in *Figure 3* and *Table 4*. Similar increasing findings were also experienced by (Wei et al., 2015, 2020). Soil organic carbon increased in the combined treatments NPKM2 followed by NPKM (*Table A6*). The minor change was experienced in syntactic fertilizer application alone NPKS and NPK while significantly decrease in CK from 9.8 g kg^{-1} to 7.8 g kg^{-1} (*Table 5*). A similar result was experienced by (Jiang et al., 2018), they concluded that in the long-term treatments of an experiment it is necessary to continuously use of manure with chemical fertilizers for better soil fertility, crop production, and soil organic carbon sustainability and sequestration. Nutrient content of soil both available and total increased with the application of inorganic plus manure as compared to a single application of fertilizer with time (*Table 5*), a similar result was found by (Wei et al. 2015 and Yin et al. 2018). The soil physicochemical and biological properties were significantly affected by soil organic carbon (Hayne et al., 1997). Soil organic carbon was increased over time in NPKM2 followed by NPKM, NPKS, NPK, and significantly decreased in CK. The result in our study indicates that the significant increase in organic carbon was due to manure application which enhances soil organic carbon and soil structure (Wagner et al., 2007). The grain yield under the maize-wheat cropping system over different fertilization times also increases in the combined application of organic and inorganic fertilization (Yang et al., 2016). The plant growth and biomass enhanced with manure amendment and regular soil inputs also enhance soil physical, chemical, and biological processes in the soil (Flie et al., 2007; Gul et al., 2015). The manure application increases the soil microbial community in soil and improved soil crop growth and soil health condition similar finding was observed by (Zhiyong et al. 2020 and Ozlu et al., 2019). The principal component analysis also showed that the fertilization input directly affected nutrients availability. The nitrogen, phosphorous, and potassium uptake increase the grain yield. The NU, PU, and KU showed a positive correlation in long term fertilization over 20 years (1990-2010) similar finding was observed by (Atli et al., 2011; Cambrolle et al. 2013, and Bessa et al. 2016) that relative yield was directly affected by organic and inorganic fertilization. These cluster methods (PCA and PLS-DA) generally showed differences among the samples of various groups by detecting the main variable which contributes and finding a correlation among the variables (Ramadan et al., 2006). Climate change plays a major role in crop production (Azam et al., 2020). The high relative yield was showed by NPKM2 and NPKM treatments as compared to the CK (*Table 5*). The increased fertilizer level is considered the main driving force of nitrogen and phosphorus losses from agricultural areas. In long-term fertilization dynamics, crop growth and soil health improved with combined application of inorganic and organic fertilization. The surface runoff and precipitation over the year of fertilization showed a positive relationship and when there was precipitation event runoff also occurred similar result was experienced by (Zhang et al.,

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2011). The crop parameter KL refers to the crop extraction of water and its ability, it was 0.08 day^{-1} from 0.8 and decreased with depth, similar to (Hammer et al., 2009). Soil water dynamics in the root zone required the best soil properties to simulate the use of soil water balance and requirement for the particular crop. Worldwide warming influences and changes the water cycle by altering the rainfall levels and variability in temperature, which affect the soil water moisture and flow reported by Ritchie (1998) and Holsten et al. (2009). An environmentally friendly and combined practice of modeling agriculture system can enhance agriculture production and it will not mitigate climatic changes. (Bai et al., 2013; Yi et al., 2018; Hong et al., 2019) suggested the threshold level of nitrogen and phosphorous fertilizer input to the soil system. Our result finding of NPKM was the best fit with his level while the level of NPKM2 exceeds which will result in the environmental hazards. Moreover (Pote et al., 1996) found that an extractable P concentration in a grassland soil of 50 mg kg^{-1} (equivalent to c. 20 mg P kg^{-1}) gave a DRP concentration in overland flow of 0.5 mg $liter^{-1}$. For cultivated soils the quantity of P lost in overland flow can be much greater due to an additional particulate component (Sharpley et al., 2001). The nitrogen level in NPKM2 also showed increasing level, similar finding was showed by (Yuan et al., 2018). These results also showed that the level of nitrogen and phosphorous need to be minimized and the best management strategies need to be adopted to get high yield and reduce the risk of environmental pollution. The outcomes of the study indicate that chemical fertilizer and manure application is the best management strategy for regulating crop yield and improving soil health and crop yields.

Conclusions

The aim was to study the climatic impact over the 20-years and combined application of manure and chemical fertilizers. Significant differences in soil fertility and crop yields among different fertilization treatments were found. Sheep manure was applied with a rate of 30,000 kg ha^{-1} and inorganic fertilizers over 20 years (1990-2010) significantly increased the crop yield, sustainability yield index, soil nutrients content, soil organic carbon, and organic carbon sequestration as compared to inorganic fertilization. Soil available nitrogen, total nitrogen, and organic matter (OC) play a vital role in crop grain production. The major driving force for crop yield in the combined use of long-term manure and inorganic fertilization was the available soil N, OC and overall N. Soil pH, which was directly influenced by N input, showed a direct impact on AP and an indirect impact on relative crop yield. Carbon production affected relative crop yield indirectly by directly influencing soil OC. The pH of the soil did not change significantly over the year of fertilization and non-significant effect on relative yield. Organic carbon and phosphorous significantly increased under the application of the treatment of inorganic and organic manure (sheep). Apparent phosphorous sand nitrogen balance exceeded the environmental risk threshold level under the NPKM2 treatment. The phosphorus and nitrogen balance exceeds the environmental risk threshold level in the treatments NPKM2 and NPKM, respectively. Our study concluded the level of phosphorus and nitrogen fertilizer needs to be decreased in organic and inorganic fertilizer. Therefore, the rate of phosphorous and nitrogen application needs to be minimized to reduced surplus nitrogen and phosphorous in the soil. The potassium showed a positive balance in inorganic and organic fertilization during 1990-2010 while negative balance was observed during 2000-2010. The

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precipitation and runoff were calculated through APSIM software and runoff exceeded the precipitation, which increases the nutrient losses and leads to environmental risk. The study showed the highest yield of grain in (maize-spring wheat-winter wheat) under inorganic and organic NPKM2 and NPKM as compared to inorganic treatments. The soil microbial activities enhanced with manure addition and also increased carbon level in the soil which leads to increased yield. Climate change due to the rising emissions of greenhouse gases, particularly carbon dioxide (CO_2), it is essential to evaluate its prospective influence on crop production and development. Applying the APSIM model to simulate the wheat-maize cropping system and climate model to study the impact of climatic change on the study sites. The climatic model in APSIM results showed that with a decrease of annual precipitation (-10) and by increasing the temperature maximum and minimum (+2, -2) and CO_2 level (600 ppm) these changes in the future climate will significantly affect the soil and environmental situations.

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APPENDIX

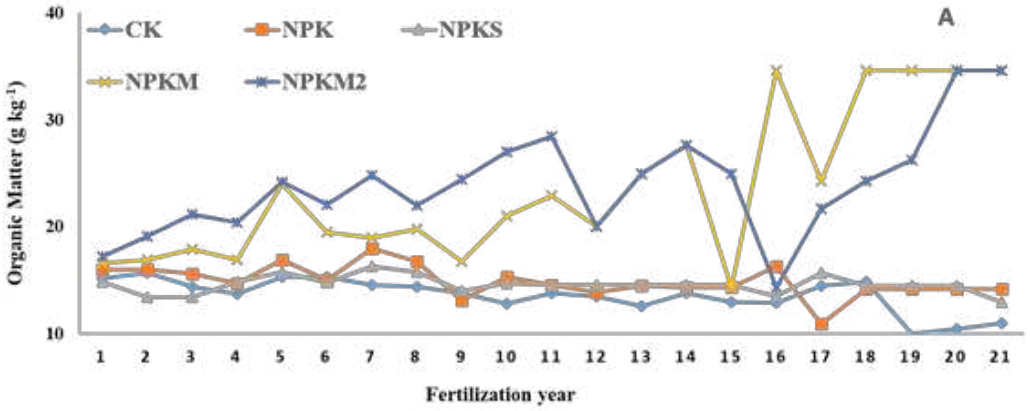

Figure A1. *Fertilization years' vs organic matter g. kg^{-1} changes over 20 years*

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Table A1. *Crop type with crop variety, sowing dates and seedling time and harvesting date of all the crop at Urumqi, Xinxiang China*

Year	Crop type	Crop variety	Sowing period	Seedling stage	Heading period/trolling period	Harvest period
1990	Maize	SC-704	1990-4-21, 22	1990-5-1		1990-9-23, 24
1991	Spring wheat	Xinchun 2	1991-4-4	1991-4-12	1991-6-5	1991-7-15
1992	Winter wheat	Xindong 15	1991-9-23	1991-10-3		1992-7-14
1993	Maize	SC-704	1993-4-22, 24	1993-5-2	1993-7-8	1993-9-28, 29
1994	Spring wheat	Xinchun 2	1994-4-3	1994-4-12		1994-7-18
1995	Winter wheat	5148	1994-9-21	1994-10-2		1995-7-15
1996	Maize	SC-704	1996-4-26	1996-5-4		1996-10-1, 2
1997	Spring wheat	Xindong 19	1996-10-1	1996-10-12		1997-7-2
1998	Winter wheat	Xindong 19	1997-9-22	1997-10-1		1998-7-21
2000	Maize	Xinyu 7	2000-5-1	2000-5-10	2000-7-8	2000-9-25, 26
2001	Maize	SC-704	2001-4-2122	2001-5-1		2001-9-23, 24
2002	Spring wheat	Xinchun 2	2002-4-4	2002-4-12	2002-6-5	2002-7-15
2003	Winter wheat		2003-9-23	2003-10-3		2003-7-14
2004	Maize	Xindong 15	2004-4-22, 24	2004-5-2	2004-7-8	2004-9-28,29
2005	Spring wheat	SC-704	2005-4-3	2005-4-12		2005-7-18
2006	Winter wheat		2006-9-21	20064-10-2		2006-7-15
2007	Maize	Xinchun 2	2007-4-26	2007-5-4		2006-10-1, 2
2008	Spring wheat		2008-10-1	2008-10-12		2007-7-2
2009	Winter wheat	5148	2009-9-22	2009-10-1		2009-7-21
2010	Maize	SC-704	2010-5-1	2010-5-10	2000-7-8	2010-9-25, 26

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Table A2. *Bulk density and SAT, DUL all calculation table depth wise*

BULK DENSITY & DRAINED UPPER LIMIT										
Sample No	**Depth range (cm)**	**DUL Gravimetric (g/g) G** ***((Wet-Dry)/Dry) = (E-F)/F***	**DUL Gravimetric (%) H** ***Grav(g/g)*100 = G x 100***	**Bulk density (g/cc) I** ***DrwWt/Core Vol = F/D***	**DUL Volumetric (mm/mm) J** **Grav(g/g)*BD** ***= G x I***	**DUL Volumetric (%) K** ***Grav% x BD = H x I***	**PO Volumetric (mm/mm) L** ***(1-BD/2.65) = (1-I/2.65)***	**SAT Volumetric (mm/mm) M** ***(PO-0.03) = L-0.03***	**SAT Volumetric (%) N** ***SAT(mm/mm) *100 = M x 100***	**SAT-DUL (mm/mm) O** ***SAT-DUL = M-J***
1	0-15	0.368	36.8	1.35	0.497	49.74	0.49	0.461	46.06	-0.04
2	15-30	0.365	36.5	1.36	0.497	49.70	0.49	0.457	45.68	-0.04
3	30-60	0.341	34.1	1.44	0.491	49.14	0.46	0.427	42.66	-0.06
4	60-90	0.344	34.4	1.43	0.492	49.24	0.46	0.430	43.04	-0.06
5	90-100	0.365	36.5	1.36	0.497	49.70	0.49	0.457	45.68	-0.04
6	100-12	0.381	38.1	1.31	0.498	49.85	0.51	0.476	47.57	-0.02
7	120-140	0.405	40.5	1.23	0.498	49.78	0.54	0.506	50.58	0.01
8	140-160	0.399	39.9	1.25	0.498	49.83	0.53	0.499	49.88	0.00

Table A3. *Crop and soil parameters used in APSIM*

Depth	**BD (g/cc)**	**Air Dry (mm/mm)**	**LL15 (mm/mm)**	**DUL (mm/mm)**	**SAT (mm/mm)**	**KS (mm/day)**	**Maize LL (mm/mm)**	**Maize PAWC 285.0**	**Maize KL (/day**	**Maize XF (0-1)**	**Wheat LL (mm/mm)**	**Wheat PAWC 324.0**	**Wheat KL (/day)**	**Wheat XF (0-1)**
0-15	1.25	0.18	0.2	0.497	0.461	0.11	0.11	36	0.08	1	0.11	36	0.06	1
15-30	1.25	0.18	0.2	0.497	0.457	0.14	0.14	37.5	0.08	1	0.13	39	0.06	1
30-60	1.25	0.18	0.2	0.491	0.427	0.16	0.16	34.5	0.08	1	0.13	39	0.04	1
60-90	1.26	0.18	0.19	0.492	0.430	0.17	0.17	33	0.08	1	0.15	36	0.04	0.9
90-100	1.27	0.18	0.17	0.497	0.457	0.19	0.19	30	0.06	1	0.15	36	0.04	0.9
100-120	1.27	0.18	0.16	0.498	0.476	0.2	0.2	57	0.04	1	0.16	69	0.04	0.7
120-140	1.27	0.18	0.14	0.498	0.506	0.2	0.2	57	0.03	1	0.16	69	0.02	0.7
140-160	1.27	0.18	0.14	0.498	0.499	0.2	0.2	57	0.03	1	0.16	69	0.02	0.7

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Table A4. *Climatic change model observed and predicted values over 20 years of simulation under the climatic change*

1990-2010	Observed	Predicted
Rain 650 (mm)	0.64	0.57
Runoff (mm)	0.43	0.40
N03 (kg ha^{-1})	1444.00	1422.54
Leaching_ fr (0-1)	0.75	0.73
Drain (mm)	0.00	0.00
Total NH_4 (kg/ha)	35.12	41.73
Organic matter	-0.45	-0.45
Nitrogen balance (kg/ha)	14.38	19.23
Essential soil water (esw (mm))	191.21	181.54

Table A5. *Climatic change model output variables*

Output frequency:
end_day
Output variables:
dd/mm/yyyy as Date, year,day,esw
no_3() as no_3_Tot, cropsta as crop_stage
dae,wagt as total_biomass, wso as storage_organs
wrr as rice_yield
tnsoil as N_avail
rain,dul(1),sw(1)
no_3(),NH_4,drain,irrigation
biomass as wheat_biom
yield as wheat_yield
runoff, fertiliser
nh4(), rlai as LAI(rice)
wheat.lai as LAI(wheat)
leaftotaln, nflv
sw_stress_expan,sw_stress_photo,sw_stress_pheno

Table A6. *Annual soil organic carbon stock and organic matter vs fertilization graph*

Treatments	Carbon rate	A*bd* depth/10
CK	0.18	0.45
NPK	0.12	0.31
NPKM	0.90	2.2
NPKS	0.02	0.06
NPKM2	0.44	1.08

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Table A7. *Descriptive statistics of the data*

Crop	Treatments	Mean	Minimum	Maximum	Std. deviation	Std. error
Maize	NU	122.08	76.05	136.75	25.89	11.58
1990-2000	PU	17.13	7.11	24.26	6.92	3.10
	KU	0.00	0.00	0.00	0.00	0.00
	NBal	151.86	-3.72	435.39	169.49	75.80
	PBal	42.71	-7.11	97.04	37.04	16.56
	KBal	142.40	0.00	413.00	170.97	76.46
	GY	5.95	4.41	6.66	0.93	0.42
Corn	NU	157.11	61.10	200.96	54.99	54.99
2000-2010	PU	28.72	8.00	43.53	12.89	12.89
	KU	275.14	134.57	337.26	81.87	81.87
	NBal	107.49	-61.10	369.04	160.43	160.43
	PBal	31.12	-8.00	77.77	30.95	30.95
	KBal	-132.58	-275.49	75.97	135.54	135.54
	GY	7.42	3.22	9.01	2.39	2.39
Spring wheat	NU	81.13	21.59	106.33	15.35	34.33
1990-2000	PU	12.98	2.73	18.20	2.82	6.30
	KU	23.74	4.65	35.81	5.16	11.54
	NBal	155.59	-12.24	347.95	57.56	128.70
	PBal	38.28	-0.94	77.70	12.44	27.82
	KBal	118.60	5.00	303.00	53.94	120.60
	GY	2.73	1.02	3.82	0.47	1.05
2000-2010	NU	134.77	35.36	195.84	26.73	59.77
	PU	17.25	4.93	23.97	3.29	7.37
	KU	116.85	29.71	165.25	24.19	54.08
	NBal	129.83	-35.36	374.16	67.67	151.31
	PBal	42.59	-4.93	97.33	16.34	36.54
	KBal	25.71	-78.11	247.99	59.86	133.86
	GY	2.69	0.69	3.42	0.51	1.14
Winter wheat	NU	157.11	61.10	200.96	24.59	54.99
1990-2000	PU	28.72	8.00	43.53	5.77	12.89
	KU	275.14	134.57	337.26	36.61	81.87
	NBal	107.49	-61.10	369.04	71.75	160.43
	PBal	31.12	-8.00	77.77	13.84	30.95
	KBal	-132.58	-275.49	75.97	60.62	135.54
	GY	7.42	3.22	9.01	1.07	2.39
2000-2010	NU	105.31	18.58	153.90	24.16	54.03
	PU	39.07	7.75	54.80	8.64	19.33
	KU	59.17	7.61	87.00	14.26	31.89
	NBal	159.29	-18.58	416.10	70.89	158.51
	PBal	20.77	-7.75	66.50	12.36	27.63
	KBal	83.39	-26.25	336.24	68.56	153.30
	GY	4.70	1.02	6.58	0.99	2.21

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THE EFFECTS OF VARIOUS ROW SPACING AND SOWING PERIODS ON THE PLANT PROPERTIES OF QUINOA (*Chenopodium quinoa* Willd.)

ZULKADIR, G.

Mersin University, Applied Technology and Management School of Silifke, Organic Farming Management Department, Silifke, Mersin/Turkey
(e-mail: gulayzulkadir@gmail.com; phone: +90-507-995-2958)

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Abstract. Quinoa is a highly nutritional plant that could adapt to different growth conditions. Thus, it is cultivated and consumed globally. However, to grow quinoa outside its indigenous geography, factors such as the sowing period and row spacing should be determined in advance to maximize yield. Thus, the present study aimed to determine the differences that could be observed throughout the total growth period of quinoa with different sowing periods and row spacing applications under Mediterranean climatic conditions. The study findings demonstrated that the plant branch count varied between 1.3 and 20.2, the plant height varied between 30.9 and 104.0 cm, the main panicle length varied between 15.0 and 41.2 cm, the plant weight varied between 0.01 and 52.2 g, the plant yield in parcel varied between 71.2 and 3199.1 g and the harvest index varied between 5.1 and 52.5%. According to the results quinoa should be sowed in the first or second half of April in the region based on the climate, and the ideal row spacing should be 40 cm. The analysis revealed that this row spacing leads to maximum yield, while sowing period could vary due to the impact of climatic factors.
Keywords: *ecologycal impact, harvest index, panicle length, plant height, plant yield*

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a plant that has been known for a long time cultivated in the Andean region for centuries to consume the seeds and leaves (Jacobsen, 2017). However, the cultivation and consumption of quinoa became popular globally during the last three decades (Wu et al., 2017). Today, it is cultivated in more than 90 countries, 80% of the cultivation is in Bolivia and Peru, while the remaining 20% is distributed among several countries (Bazile et al., 2016).

The popularity of quinoa is due to its high nutritional content, adaptability and ability to grow in harsh climatic conditions, making it an ideal crop for drought-prone and saline agricultural lands (Bazile et al., 2016). Due to its outstanding nutritional properties, the global quinoa market is currently increasing rapidly (Jacobsen, 2017). In addition to its high protein content, the plant is rich in nutrients including balanced amino acids and high mineral concentrations. Studies demonstrated that only a limited number of grain seeds do not contain gluten and the quality of the mineral, vitamin, antioxidant and protein content is comparable to casein. It was also evidenced to have high levels of essential amino acids such as lysine (Wu et al., 2016).

Another factor in the prevalence of quinoa cultivation is its adaptation to various ecological conditions. In other words, quinoa could survive 4000 m above the sea level and in temperatures between -8 and 38 °C. However, despite its wide ecological range, significant yield differences are observed based on the variety, and soil, water and climate conditions (Scanlin and Lewis, 2017). For instance, high temperatures during flowering and grain filling periods significantly reduces yield (Hinojosa et al., 2018). In fact, during the flowering period, night temperatures between 20 and 22 °C reduces grain yield by

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23% to 31% (Lesjak and Calderini, 2017). On the other hand, the impact of heat stress, duration, intensity and the rate of temperature increase is a complex function (Wahid et al., 2007), and heat stress leads to different reactions among plant species based on the duration of temperature increase and the plant development period in which heat stress occurs. In general, the plant flowering stage is more susceptible to heat stress when compared to the vegetative stages (Prasad et al., 2017).

Perhaps the most important agricultural application that affect crop yield is the sowing density. In each cultivation system, there is a plant density that would maximize the consumption of available resources (such as water, nutrients and daylight) and allows the achievement of the maximum yield (Sangoi et al., 2000).

Based on the above-mentioned information, determination of the most adequate sowing period and sowing density would balance the plant requirements and environmental conditions to maximize the yield and quality in quinoa cultivation. Due to these requirements, the present study aimed to determine the possible variations based on different sowing periods and row spacing within the overall quinoa plant growth period and identify certain properties of the quinoa plant in Mediterranean climate conditions.

Materials and Methods

The present study was conducted for two years in Kahramanmaras province ecological conditions during 2016 and 2017 in Turkey. In the experiments, "Q52" quinoa variety, compatible with the Mediterranean climate conditions, was employed. The study area soil analysis results are presented in *Table 1* and climate data are presented in *Table 2*.

Table 1. *Some soil characteristics of experimental areas in the 2017-2018*

Soil properties		Saturation (%)	pH	EC dS m $^{-1}$	Lime (%)	Organic Matter (%)	P_2O_5 (kg da $^{-1}$)	K_2O (kg da $^{-1}$)
2017	Values	58.00	7.76	0.32	24.48	2.28	3.20	98.64
	Comments	Clay-Loamy	Light Alkaline	Light Saline	More Lime	Middle	Low	High
2018	Values	79.00	7.40	0.11	23.00	2.09	5.62	61.2
	Comments	Clay	Neutral	Saltless	Limy	Middle	Poor	High

As seen in *Table 1*, the test site soil content was low in phosphorus content, adequate in potassium, and moderate in organic matter content for both years of experiment. However, based on the year and location, soil saturation changed from clayey-loamy to clayey, and it was determined that the soil was slightly alkaline-neutral, slightly salty-salt-free, highly calcareous-calcareous.

It was observed that the total historical precipitation was 135.5 mm and the mean precipitation in the season that the experiment was conducted was 22.6 mm as seen in *Table 2*. During the experiments, total and average precipitation figures were 176.2 and 29.4 mm for the first year, 140.0 and 23.3 mm for the second year. It was determined that the average precipitation in both years was higher than the historical average precipitation. The seasonal average temperatures in the region were 21.7 °C and 11.5 °C, respectively. In the study, the mean temperature was 23.2 °C in the 2017 cultivation period, and the mean temperature was 23.5 °C in the 2018 cultivation period, and both figures were higher than the historical average.

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Table 2. *Some meteorological parameters of experimental areas at Kahramanmaras in 2017 and 2018 and irrigation dates*

Months	Max. Temperature (°C)			Min. Temperature (°C)			Average temperature (°C)			Total rainfall (mm)			Average relative humidity (%)		
	2017	2018	1963-2018	2017	2018	1963-2018	2017	2018	1963-2018	2017	2018	1963-2018	2017	2018	1963-2018
March	17.9	19.7	15.9	7.2	9.6	5.8	12.2	14.2	10.4	74.2	47.4	97.5	55.1	60.8	60.4
April	21.8	25.5	21.3	10.1	12	9.9	15.7	18.4	15.2	68.1	71.6	72.7	49.7	45.3	57.5
May	26.2	28.8	26.7	14.2	15.7	14.2	19.6	21.7	20.0	105.0	28.1	40.0	54.9	52.6	54.6
June	33.3	32.5	32.0	19.9	19.9	18.9	26.2	25.4	24.9	3.1	39.4	7.8	43.3	49.1	48.8
July	39.1	35.6	35.7	23.9	23.2	22.2	30.9	28.6	28.3	0.0	0.3	2.7	34.9	46.2	49.9
August	37.9	36.8	36.1	23.7	23.3	22.2	29.8	29.1	28.4	0.0	0.0	2.3	46.2	43.8	51.2
September	36.4	34.7	32.6	21.1	21.0	18.5	27.7	27.2	24.9	0.0	0.6	10.0	38.3	38.4	48.8
Total (Season)	212.6	213.6	200.3	120.1	124.7	111.7	162.1	164.6	152.1	176.2	140.0	135.5	322.4	336.2	371.2
Average (Season)	30.4	30.5	28.6	17.2	17.8	16.0	23.2	23.5	21.7	29.4	23.3	22.6	46.1	48.0	53.0

Irrigations									
2017	March 24	April 20	April 28	May 11	June 09	June 21	July 01	July 10	July 25
2018	March 26	April 17	April 28	May 13	June 08	June 21	June 28	July 21	August 09

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The mean relative humidity in Kahramanmaras was 53.0% in the season, 58.1% throughout the year, while it was 46.1% and 48.0%, respectively in the 2017 and 2018 cultivation seasons (Anonymous, 2019).

The experiment was set up with random plots with different sowing times (ST) (March 23, April 6, 20, May 11 in 2017; March 26, April 2, 13, and 26 in 2018), and different row spacing (RS) (20, 40 and 60 cm) in sub-plots and 3 repetitions. The plants were sowed with 20 cm, 40 cm and 60 cm (4 rows per lot) row spacing on the lines marked with a hand marker and at 1-2 cm depth. The size of the plots was 4 m^2, 8 m^2 and 12 m^2. The TKW value of the seed material used was 2.15 g, and the amount of seed sown in the experimental plots was 1.2 kg da^{-1}. Based on the soil nutrient content (*Table 1*), pre-sowing fertilization was conducted with 0.6 kg ha^{-1} N, 0.6 kg ha^{-1} P and 0.6 kg ha^{-1} K. After sowing, when plants were about 20 cm high, net 0.7 kg ha^{-1} N was applied in the second fertilization. Based on the climate conditions, the plots were irrigated based on the water requirement of the quinoa plant (*Table 2*). Weed control was conducted manually based on the weed prevalence in the field.

The observations in the research were determined as the number of brances in the plant (NBP), the plant height (PH), the main panicle length (MPL), plant weight (PW), the plant yield per parcel (PY) and harvest index (HI) (Kır and Temel, 2017). The data obtained from the research were analyzed through variance analysis by means of SAS (version 6.03) program, and Duncan multiple comparison test was implemented to determine the significance levels of the differences among the implementations.

Results and Discussion

The results of the analysis of variance conducted on the data obtained with various sowing time and row spacing applications during the two years of experiment and the comparison of the data averages are presented in *Table 3* (*Tables 3.1, 3.2, 3.3*).

Table 3.1. *Means and F values of different years, sowing dates and row spacings on the phenological characteristics of quinoa in 2017 and 2018*

	NBP	**PH**	**MPL**	**PW**	**PY**	**HI**
Year						
2017	4.173 B	77.628 B	24.410 B	14.331 B	1378.05 B	31.703 A
2018	15.653 A	84.162 A	28.150 A	25.448 A	2271.21 A	24.875 B
F values	**4146.52****	**27.76****	**96.53****	**1186.07****	**893.55****	**295.26****
Sowing Time						
23/26 March (I)	8.139 C	89.636 A	33.228 A	32.109 A	2067.26 A	28.876 B
06/02 April (II)	8.504 C	81.122 B	22.444 C	21.779 C	1826.71 B	31.326 A
20/13 April (III)	10.241 B	87.822 A	23.336 C	24.366 B	1884.32 B	29.202 B
04 May/26 April (IV)	12.767 A	65.000 C	26.111 B	1.302 D	1520.23 C	23.752 C
F values	**140.36****	**81.71****	**164.86****	**1658.62****	**57.91****	**65.40****
Row Spacing						
20 cm	9.489 B	76.846 B	25.202 C	16.844 C	1636.39 C	30.891 A
40 cm	10.183 A	82.944 A	27.408 A	22.152 A	2048.02 A	30.821 A
60 cm	10.067 A	82.896 A	26.229 B	20.673 B	1789.49 B	23.155 B
F values	**4.60***	**11.66****	**10.47****	**67.18****	**80.72****	**302.20****

*: $p<0.05$, **: $p<0.01$, IS: insignificant, NBP: the number of brances in the plant, PH: the plant height, MPL: the main panicle length, PW: single plant weight, PY: the plant yield per parcel, HI: harvest index

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Table 3.2. *Means and F values of year x sowing time, year x row spacing and sowing time x row spacing interactions on the phenological characteristics of quinoa in 2017 and 2018*

Year X Sowing Time							
2017	I	2.533	95.689	39.211	21.387	1803.387	27.851
	II	3.587	87.311	19.978	16.257	1579.199	34.241
	III	4.594	93.222	20.139	17.090	1870.377	27.163
	IV	5.978	34.289	18.311	2.591	259.256	37.558
2018	I	13.744	83.583	27.244	42.832	2331.136	29.900
	II	13.422	74.933	24.911	27.302	2074.228	28.411
	III	15.889	82.422	26.533	31.642	1898.271	31.241
	IV	19.556	95.711	33.911	0.013	2781.207	9.947
F values		**18.91****	**217.66****	**227.60****	**244.77****	**344.78****	**332.78****
Year X Row Spacing							
2017	20 cm	3.944	72.042	24.404	12.198	1281.360	35.305
	40 cm	4.858	82.567	25.633	15.010	1772.133	36.277
	60 cm	3.717	78.275	23.192	15.785	1080.670	23.528
2018	20 cm	15.033	81.650	26.000	21.489	1991.417	26.477
	40 cm	15.508	83.321	29.183	29.293	2323.908	25.365
	60 cm	16.417	87.517	29.267	25.560	2498.307	22.783
F values		**9.68****	**5.95****	**10.83****	**16.97****	**99.11****	**110.21****
Sowing Time X Row Spacing							
I	20 cm	7.500	83.367	32.383	24.823	1454.722	27.170
	40 cm	8.167	94.008	35.167	36.618	2615.743	33.065
	60 cm	8.750	91.533	32.133	34.887	2131.318	26.392
II	20 cm	8.113	75.333	20.467	20.647	1792.382	36.810
	40 cm	8.783	80.467	21.333	24.073	2093.707	30.745
	60 cm	8.617	87.567	25.533	20.618	1594.052	26.423
III	20 cm	9.608	84.033	20.925	20.560	1579.998	30.602
	40 cm	10.550	92.367	26.600	26.162	1961.240	27.925
	60 cm	10.567	87.067	22.483	26.377	2111.733	29.080
IV	20 cm	12.733	64.650	27.033	1.345	1718.452	28.982
	40 cm	13.233	64.933	26.533	1.753	1521.392	31.548
	60 cm	12.333	65.417	24.767	0.808	1320.850	10.727
F values		**1.28 IS**	**2.96***	**10.96****	**19.10****	**53.59****	**117.43****

*: p<0.05, **: p<0.01, IS: insignificant, NBP: the number of brances in the plant, PH: the plant height, MPL: the main panicle length, PW: single plant weight, PY: the plant yield per parcel, HI: harvest index

The number of branches per plant

Based on the study findings, there were statistically significant differences between Y, ST, Y x ST, Y x RS, Y x ST x RS factors ($p < 0.01$) and RS ($p < 0.05$) based on NBP property, while ST x RS interaction was insignificant. NBP was higher in 2018 (15.7) when compared to 2017 (4.2). The analysis of the applications based on ST demonstrated that the highest figure was obtained in the 4th sowing (12.8), and the lowest figures were obtained in the 1st and 2nd sowing. The analysis of RS application demonstrated that the lowest value was obtained with 20 cm row spacing and the other two RS applications yielded statistically the same figures.

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Table 3.3. *Means and F values of year x sowing time x row spacing on the phenological characteristics of quinoa in 2017 and 2018*

Year X Sowing Time X Row Spacing							
2017	20 cm	1.267	87.533	41.233	16.540	1151.840	25.373
I	40 cm	3.767	103.133	39.800	21.080	2704.600	35.003
	60 cm	2.567	96.400	36.600	26.540	1553.720	23.177
	20 cm	4.360	80.200	19.000	14.287	2029.697	45.370
II	40 cm	3.033	86.133	17.667	16.107	1801.383	32.067
	60 cm	3.367	95.600	23.267	18.377	906.527	25.287
	20 cm	4.083	85.467	17.050	15.287	1706.073	26.667
III	40 cm	5.233	104.000	25.467	19.357	2113.863	25.503
	60 cm	4.467	90.200	17.900	16.627	1791.193	29.320
	20 cm	6.067	34.967	20.333	2.680	237.830	43.810
IV	40 cm	7.400	37.000	19.600	3.497	468.697	52.533
	60 cm	4.467	30.900	15.000	1.597	71.240	16.330
2018	20 cm	13.733	79.200	23.533	33.107	1757.603	28.967
I	40 cm	12.567	84.883	30.533	52.157	2526.887	31.127
	60 cm	14.933	86.667	27.667	43.233	2708.917	29.607
	20 cm	11.867	70.467	21.933	27.007	1555.067	28.250
II	40 cm	14.533	74.800	25.000	32.040	2386.040	29.423
	60 cm	13.867	79.533	27.800	22.860	2281.577	27.560
	20 cm	15.133	82.600	24.800	25.833	1453.923	34.537
III	40 cm	15.867	80.733	27.733	32.967	1808.617	30.347
	60 cm	16.667	83.933	27.067	36.127	2432.273	28.840
	20 cm	19.400	94.333	33.733	0.010	3199.073	14.153
IV	40 cm	19.067	92.867	33.467	0.010	2574.087	10.563
	60 cm	20.200	99.933	34.533	0.020	2570.460	5.123
F values		**6.14****	**2.08 IS**	**6.16****	**17.86****	**35.98****	**86.71****

*: p<0.05, **: p<0.01, IS: insignificant, NBP: the number of brances in the plant, PH: the plant height, MPL: the main panicle length, PW: single plant weight, PY: the plant yield per parcel, HI: harvest index

In the Y x ST interaction, the lowest value was obtained in the first sowing (2.5) in 2017 and the highest value was obtained in the fourth sowing (19.5) in 2018. In the Y x RS interaction, the lowest value was determined with 60 cm RS (3.7) in 2017 and the highest was determined with 60 cm RS (16.4) in 2018. The lowest value in Y x ST x RS interaction was 1.3 (1st sowing in 2017) and the highest value was 20.2 (4th sowing in 2018).

Previous studies reported different values on the branching characteristics of the quinoa plant. Thus, Naik et al. (2020) reported 17.70 branches per plant, Onkur and Keskin (2019) reported 19.9-26.4 per plant, Afiah et al. (2018) reported 2.1-4.4 per plant, Al-Naggar et al. (2017) reported 7.0-20.0 per plant, Kır and Temel (2017) reported 15.1-26.0 per plant, Dumanoglu et al. (2016) reported 4.0-8.0 per plant, and Long (2016) reported 4.28-15.75 branches per plant. The wide range in reported values were due to employment of different varieties, ecological, climatic, soil structure differences, row spacing and sowing method. Although the present study data were compatible with these reports, the differences observed in the present study are explained below.

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The plant height (cm)

In the study, it was determined that there were statistical differences between Y, ST, RS, Y x ST, Y x RS, factors ($p < 0.01$) and ST x RS ($p < 0.05$) based on PH, while Y x ST x RS interaction was found to be insignificant. PH was taller in 2018 (84.2 cm) when compared to 2017 (77.6 cm). The analysis of the applications based on ST revealed that the highest values were obtained in first and third sowings (89.6 and 87.8 cm), while the lowest value was obtained in the second sowing (81.1 cm). The analysis of the RS application revealed that the lowest value was obtained with 20 cm row spacing and the other two RS produced statistically same values.

In Y x ST interaction, the lowest value was obtained in 4th sowing in 2017 (34.3 cm) and the highest was obtained in 4th sowing in 2018 (95.7 cm). Furthermore, 95.7 cm PH obtained in first sowing in 2017 was the second highest value. In the Y x RS interaction, the lowest PH was obtained with 20 cm RS in 2017 (72.0 cm), and the highest was obtained with 60 cm RS (87.5 cm) in 2018. The ST x RS interaction data revealed that the lowest value was 94.0 cm (1st; 40 cm) and the highest value was 64.7 cm (4th; 20 cm).

The PH data reported in other studies were 122.3 cm (Naik et al., 2020), 66.5-116.4 cm (Tan and Temel, 2018), 35.3-71.6 cm (Eltahan et al., 2019), 73.9-90.3 cm (Altuner et al., 2019), 49.3-101.5 cm (Geren and Gure, 2017), 55.4-101.0 cm (Tan and Temel, 2017), 48.5-94.1 cm (Geren, 2015) and 82-118 cm (Hirich et al., 2014), it was stated that the differences were due to varietal and ecological differences and various stress factors.

The main panicle length (cm)

The analysis of the differences in MPL property based on the applications, it was determined that the Y, ST, RS, Y x ST, YxRS, ST x RS, Y x ST x RS interactions ($p < 0.01$) were significant. MPL was higher in 2018 (28.2 cm) when compared to 2017 (24.4 cm). The analysis of the applications based on ST demonstrated that the highest value was obtained in the first sowing (33.2 cm), and the lowest value was obtained in the second planting (22.4 cm). The analysis of the RS application findings revealed that the lowest value was obtained with 20 cm row spacing and the highest value was obtained with 40 cm row spacing.

The lowest Y x ST interaction was obtained in fourth sowing in 2017 (18.3 cm) and the highest was obtained in fourth sowing in 2018 (33.9 cm). The lowest Y x RS interaction was obtained with 60 cm RS in 2017 (23.2 cm), and the highest were obtained with 60 cm RS (29.3 cm) and 40 cm RS (29.2 cm) in 2018. The lowest ST x RS interaction was 20.5 cm (1st; 20 cm) and the highest was 35.2 cm (1st; 40 cm). Finally, the lowest Y x ST x RS interaction was 15.0 cm (2017; 4th; 60 cm) and the highest was 41.2 cm (2017; 1st; 20 cm).

Previous studies reported variable panicle length figures such as 31.1-42.8 cm (Altuner et al., 2019), 20.0-36.0 cm (Reguera et al., 2018), 17.8-25.3 cm (Rames et al., 2017), 24.3-29.1 cm (Long, 2016), 38.3-53.3 cm (Geren et al., 2015), 28.6-53.3 cm (Geren et al., 2014) and 15-57 cm (Hirich et al., 2014). The present study data were consistent with other studies.

Plant weight (g plant^{-1})

The analysis of the differences between the applications based on PW of the harvested samples revealed that Y, ST, RS, Y x ST, Y x RS, ST x RS, Y x ST x RS interactions ($p < 0.01$) were statistically significant. PW was higher in 2018 (25.4 g plant^{-1}) when

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compared to 2017 (14.3 g plant^{-1}). The analysis of the applications based on ST demonstrated that the highest value was obtained in the first sowing (32.1 g plant^{-1}), and the lowest value was obtained in the fourth sowing (1.3 g plant^{-1}). The analysis of the applications based on RS revealed that the lowest value was 16.8 cm with 20 cm RS, while the highest value was 22.2 g with 40 cm RS.

The lowest Y x ST interaction was obtained in 4th sowing in 2017 (2.6 g plant^{-1}) and the highest was obtained in 1st sowing in 2018 (42.8 g plant^{-1}). The highest Y x RS interaction in 2017 was determined with 20 cm RS (12.2 g plant^{-1}), and in 2018, the highest value was determined with 40 cm RS (29.3 g plant^{-1}). The lowest ST x RS interaction was 1.3 g plant^{-1} in the 4th sowing with 20 cm row spacing, while the highest value was 36.6 g with 40 cm row spacing in the first sowing. The lowest Y x ST x RS interaction was 0.010 g plant^{-1} (2018; 4th; 20 and 40 cm) and the highest value was 52.2 g plant^{-1} (2018; 1st; 40 cm).

Afiah et al. (2018) analyzed 6 quinoa genotypes under the first and second crop conditions and found that the PW value ranged between 17.5 and 53.9 g plant^{-1}. On the other hand, Alandia et al. (2016) reported that different water regimes and different nitrogen applications led to changes in quinoa plant PW between 8.2 and 37.2 g plant^{-1}. While it was observed that the present study data were consistent with previous studies, it was suggested that the differences were due to the plant variety, and climate and cultural processes (Pulvento et al., 2010).

The plant yield per parcel (g ha^{-1})

In the experiment, the analysis of the differences between the harvested plant samples based on PY demonstrated that the Y, ST, RS, Y x ST, Y x RS, ST x RS, Y x ST x RS interactions ($p < 0.01$) were statistically significant. The analysis of the PY variable based on the years revealed that PY was higher in 2018 (227.12 g ha^{-1}) when compared to 2017 (137.81 g ha^{-1}). The analysis of the applications based on ST, the highest value was observed in the first sowing (206.73 g ha^{-1}), and the lowest was obtained in the fourth sowing (152.02 g ha^{-1}). The analysis based on the RS application revealed that the lowest value was 163.64 g ha^{-1} (20 cm) and the highest value was obtained with 40 cm row spacing (204.80 g ha^{-1}).

The lowest Y x ST interaction was observed in the 4th sowing in 2017 (25.93 g ha^{-1}) and the highest was observed in the fourth sowing in 2018 (278.12 g ha^{-1}). In Y x RS interaction, the lowest value was determined with 60 cm RS (108.07 g ha^{-1}) in 2017, and the highest value was observed with 60 cm RS (249.83 g ha^{-1}) in 2018. The ST x RS interaction data revealed that the lowest value was 132.09 g ha^{-1} (4th; 60 cm) and the highest value was 261.57 g ha^{-1} (1st; 40 cm). The analysis based on Y x ST x RS interaction revealed that the lowest value was 7.12 g ha^{-1} (2017; 4th; 60 cm) and the highest value was 319.91 g ha^{-1} (2018; 4th; 20 cm).

Altuner et al. (2019) investigated the effects of 2 quinoa varieties and 3 sowing times and determined the plant yield between 15.4 and 29.2 m^{-2} and Maliro et al. (2017) analyzed 11 quinoa genotypes under different water regimes and determined that the plant yield varied between 0.2 and 9.9 kg da^{-1}. Based on these data, the present study findings were consistent with other studies.

Harvest index (%)

The analysis of the differences between the HI data obtained with various applications in the study demonstrated that Y, ST, RS, Y x ST, Y x RS, ST x RS, Y x ST x RS

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interactions ($p < 0.01$) were statistically significant. The analysis based on the years revealed that HI was higher in 2017 (31.7%) when compared to 2018 (24.9%). The analysis based on ST revealed that the highest value was obtained in the second sowing (31.3%), and the lowest was obtained in the fourth sowing (23.8%). The analysis based on the RS application showed that the lowest value was 23.2% (60 cm RS) and the highest value was obtained with 20 cm row spacing (30.9%). On the other hand, HI value obtained with 40 cm row spacing was the second highest (30.8%) and it was within the same group with 20 cm row spacing.

In Y x ST interaction, the lowest value was obtained in fourth sowing in 2018 (9.9%), and the highest value was observed in fourth sowing in 2018 (37.6%). In Y x RS interaction, the lowest value was determined with 60 cm row spacing in 2018 (22.8%), and the highest was determined with 40 cm row spacing in 2017 (36.3%). It was determined that the lowest value was 10.7% (4^{th}; 60 cm) and the highest value was 36.8% (2^{nd}; 20 cm) based on the ST x RS interaction data. Based on the Y x ST x RS interaction, the lowest value was 5.1% (2018; 4^{th}; 60 cm) and the highest value was 52.5% (2017; 4^{th}; 40 cm).

Eltahan et al. (2019) reported that HI varied between 16.4 and 46.6% in quinoa plants exposed to different salt stress in different row spacing applications, Onkur and Keskin (2019) reported that HI varied between 40.2 and 50.1% at different row spacing applications, Tan and Temel (2018) reported that HI varied between 5.6 and 38.0% in 10 genotypes at two different locations. Reguera et al. (2018) analyzed 3 quinoa genotypes in 4 different locations and reported HI values between 40 and 50% and analyzed 6 quinoa varieties in the first and second growth seasons and reported that HI varied between 31.4 and 40.3%. Geren and Gure (2017) reported that the HI value varied between 25.2 and 50.3% based on the administration of different nitrogen and phosphorus doses. Geren (2015) reported that the HI varied between 13.3-46.6 % at different nitrogen levels applications.

Conclusion

In the present study conducted in Kahramanmaras province, where the Mediterranean climate prevails, winters are temperate and rainy, and summers are hot and dry, it was determined that the differences between the applications conducted with different sowing times and row spacing were significant. These results demonstrated that the cultivation of 'Q52' quinoa cultivar was adequate for Kahramanmaras region.

On the other hand, it was determined that quinoa sowing should be conducted in the first or second half of April based on the climate conditions, and the ideal row spacing is 40 cm. While this spacing provides maximum benefits based on all investigated properties, it was suggested that the sowing time may vary since it could be effected by climatic factors more.

To improve the cultivation of quinoa, which is a novel crop in Turkey, it is very important to initially plan a good sowing calendar. The determination of the dates where the plant is exposed to the required temperatures, precipitation and relative humidity would increase the yield, which in turn would increase the interest in the cultivation of the plant.

Finally, agronomic guidelines should be developed for quinoa cultivation in Turkey and should be provided with adequate scientific knowledge for local communities, which would in turn will cultivate the crop nationwide. It was concluded that the varieties

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resistant to biotic and abiotic stress conditions that could be experienced regionally and nationwide should be determined, and further studies should be conducted on the development of new varieties.

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GEOGRAPHICAL DISTRIBUTION OF TOXIC ELEMENTS IN NORTHEAST MARMARA SEA SEDIMENTS AND ANALYSIS OF TOXIC ELEMENT POLLUTION BY VARIOUS POLLUTION INDEX METHODS (ISTANBUL/TURKEY)

KAM, E.[1] – YÜMÜN, Z. Ü.[2*]

[1]*Environmental Engineering Department, Çorlu Engineering Faculty, Namık Kemal University, 59860 Çorlu, Tekirdağ, Turkey*
(e-mail: erolkam@yildiz.edu.tr; ORCID ID: 0000-0001-5850-5464)

[2]*Physics Department, Faculty of Arts and Sciences, Yildiz Technical University, Davutpaşa Campus, 34220 Esenler/Istanbul, Turkey*

**Corresponding author*
e-mail: zyumun@nku.edu.tr; ORCID ID: 0000-0003-0658-0416

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Abstract. In this study, the geographic distribution of toxic element concentrations was determined in Northeast Marmara Sea (Istanbul/Turkey) sediments. In addition, the pollution degree of the environment was analyzed using various pollution index methods. Geochemical analysis of 28 elements were performed in sediment samples. The concentrations of several elements (especially Fe, Mn, Ti, Zn, and Cr) were found to be very high in some locations. At sites, where heavy metal concentrations were high, foraminifera genera and species numbers and number of individuals were very low. The low number of foraminifera in the samples taken from some regions could be due to uncontrolled ship traffic and domestic and industrial discharges.
Keywords: *geochemical analysis, marine sediments, elements, environmental index, pollution factors, eastern Marmara, Istanbul*

Introduction

Historically, settlements were generally built near water bodies (seas, lakes, rivers) in order to meet the water needs of cleaning, nutrition, and agriculture, and to eliminate their waste. Pollution was not a serious issue due to low human population. However, in the last 30 years, as the population and the corresponding amount of waste has increased, the carrying capacity of the receiving aquatic environments has decreased (Algon et al., 2004; Yümün and Önce, 2017). Although many scientists have stated that the seas have been extremely polluted in the last 30 years, pollution has been ongoing since earlier times. The seas, which have been one of the major accumulation areas, are the largest water bodies affected by anthropogenic pollution. Apart from paper, plastic, and metal wastes, heavy metals, organic wastes, and inorganic wastes have also accumulated in the seabed sediment. Sediments provide necessary habitat for many freshwater, estuary, and marine organisms. Contaminated sediments endanger aquatic life and human health through direct toxicity as well as bioaccumulation in the food chain (Bakan et al., 1999; Knezovıch and Harrison, 1987; Bampton, 1999). Contaminated sediments can cause lethal and sub-lethal effects in benthic zones and on other sediment-related organisms (Long et al., 1995). Therefore, it is important to measure the sediment quality when determining the quality of a water body. To determine the quality of sediment, it is necessary to perform toxic element analysis and

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to determine benthic health. These toxic elements are deposited in the sediment by precipitating towards the seabed without undergoing biodegradation. This accumulation causes morphological changes in the crust structures through limited movement or by passing to the sea floor. These organisms play a vital role as bioindicators in the determination of pollution in the seas. In recent years, many studies have used bioindicators and toxic element analysis to determine sediment quality (Yümün and Önce, 2017; Yümün, 2017, 2016; Kam and Önce, 2016; Meriç et al., 2012, 2009; Balkıs et al., 2007; Baştürk et al., 1988; Yümün and Kam, 2019; Yümün et al., 2019; Yıldırım et al., 2010). In this study, heavy metal concentrations were determined in marine sediments of the Istanbul coast of Marmara Sea using core samples taken from 20 locations.

Some genera and species of Foraminifera cannot survive in dirty environments and a decrease is observed in their numerical abundance. In addition, elements such as Fe, S, Mn and Mg found in contaminated environments cause color changes in foraminiferal shells. Although the elements with toxic effects disrupt the living environment of the foraminifera, they may also cause the formation of anomalies in the foraminifera. For this reason, Foraminifers have been used as an indicator in terms of environmental pollution in many scientific studies (Yümün and Önce, 2017; Yümün, 2017; Meriç et al., 2012, 2009).

Therefore, the effects of pollution on the ecological system (study area) have been evaluated using foraminifera as bioindicators. Foraminiferal assemblages of the Marmara Sea sediments were identified in Istanbul. Foraminifer genera and species were examined to determine whether they originated from the Sea of Marmara.

In this study, toxic element concentrations and marine sediment pollution determination methods were applied to marine sediments of Northeast Marmara Sea. In addition, benthonic foraminifera assemblages of sediment samples taken in the Istanbul part of Marmara Sea were determined (*Fig. 1*).

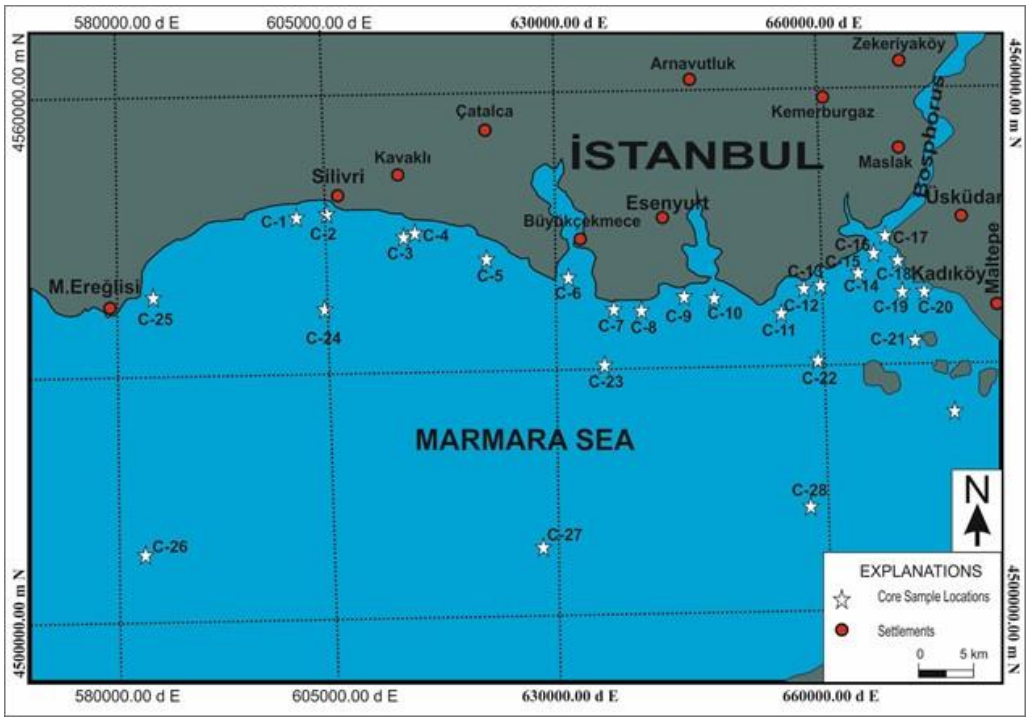

Figure 1. *Location map of the study area*

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Sources of the pollutants

Causes of marine environment pollution; Domestic wastes generated with population growth, pesticides and fertilizers used in agricultural activities, industrial wastes, maritime transport and the geological structure of the neighboring terrestrial area.

While the population of Istanbul 1971 was 3,126,400 (State Institute of Statistics (DIE)), the population in 2012 was 14,160,467 and the current population (2021) is 15,634,257. Here, it is seen that the population of Istanbul has increased approximately 5 times in 50 years. With this population growth, solid and liquid wastes and industrial wastes have also increased in parallel. In parallel with the population growth and the development of the industry, a serious increase is observed in maritime transport. The increase in the population has led to a decrease in agricultural land. If we evaluate the land use types between 1971 and 2012 in general, 32.7% of the agricultural lands; State-owned forest areas are observed to have decreased by 9.0% (*Fig. 2*). Between the two periods, residential areas are 409.8%; quarry - sand - pasture - stony areas - nursery - warehouse - facility - swamp - ENH - highway land group 269.8%; water surfaces increased by 64.3%. The most significant change in land use types occurred in agriculture and residential areas (Şahin, 2014).

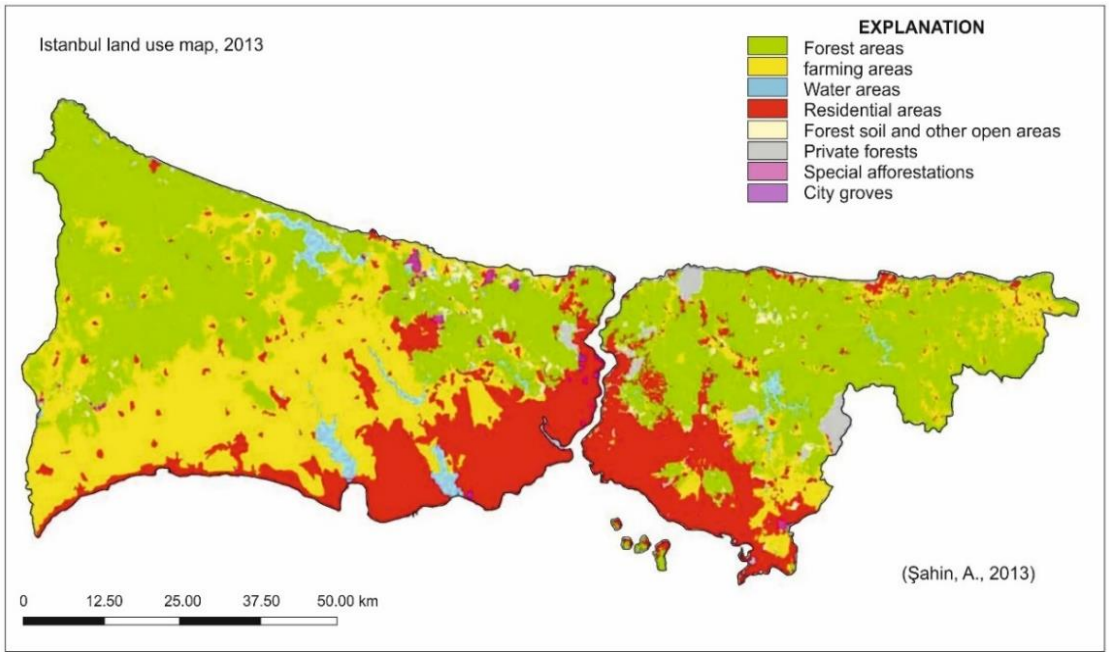

Figure 2. *Istanbul land use map (Şahin, 2014)*

Environmental pollution caused by sea transportation can be divided into two groups as “sea pollution” and “air pollution”. Ships navigating international waters can cause unnatural displacement of different species. This displacement can negatively affect the ecosystem and thus affect human life negatively.

According to the data of IMO (International Maritime Organization), which is the top organization of the maritime sector at the international level, 8% of the wastes that cause pollution in the sea are from natural resources, 0.5% from offshore production, 11% from maritime transport and 30% from the atmosphere, 40% from flood and land-based discharges, 10% from illegal discharge into the world seas (Küçük and Topçu, 2012).

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The most important causes of ship-related pollution in the seas: The discharge of bilge, dirty ballast or washing water to the sea, throwing garbage and similar domestic wastes into the sea, giving the oily and detergent water to the sea as a result of washing the decks, surface cleaning and painting of the outer surface discharge of the wastes arising from the transported loads into the sea, discharge of pollutant wastes on the deck with rainwater or ballast overflow water, leakage to the sea during fuel transfer, oil mixing with the cooling water of the ship engine and flowing into the sea with the cooling water, the leakage of the shaft sealing oil into the sea, the explosion of the hydraulic system on the decks the result is the flowing of the oil flowing into the sea and leaving the polluted water caused by life on the ship to the sea without treatment (Özdemir, 2012). *Figure 3* shows the Turkey international Ro-Ro and roads on the map. Here, it is seen that the ship traffic is concentrated in Tekirdağ and Istanbul parts of the Marmara Sea. This density indicates that the Marmara Sea may cause the Tekirdağ and Istanbul parts to be contaminated (Kutluk, 2018).

Figure 3. *Turkish International Ro-Ro Lines (UP, 2011)*

Lead is the most important heavy metal contaminant caused by transportation vehicles. Other metals that are polluted by transportation vehicles are Cd, Cu, Cr, Ni and Zn. These heavy metals are caused by the wear on the vehicle (Karaca, 1997).

Materials and methods

Core samples (seabed sediment) from 20 locations (Istanbul/Turkey) were used in the study. Core samples were obtained by a specially designed core-free method. Samples were taken from both clean areas and areas with high pollution potential, including domestic, industrial and port waste disposal sites. In this way, it is thought that the samples will represent the entire study area. Sample coordinates are given in *Table 1* (Silivri-1, Silivri-2, Selimpaşa-1, Selimpaşa-2, Kumburgaz, Büyükçekmece, Gürpınar, Ambarlı, Avcılar, Küçükçekmece, Yeşilköy, Zeytinburnu, Yenikapı-1, Yenikapı-2, Kumkapi-1, Kumkapi-2, Bosphorus, Haydarpasa, Uskudar, and Kadikoy). Laboratory studies were carried out in two parts: Sieve analysis and Geochemical analysis.

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Table 1. *Core samples and their coordinates taken from the shores of the West Marmara Sea in Istanbul*

Core sample No	Core sample location	Sample date	Depth (M)	Geographic position	
				North	East
C-1	Silivri-1	10.06.2019	25	4546298	0603555
C-2	Silivri-2	10.06.2019	22	4546290	0603450
C-3	Selimpaşa-1	10.06.2019	24	4543800	0614748
C-4	Selimpaşa-2	10.06.2019	20	4543825	0614600
C-5	Kumburgaz	10.06.2019	38	4540935	0621468
C-6	Büyükçekmece	10.06.2019	20	4538709	0631551
C-7	Gürpınar	10.06.2019	23	4534925	0636700
C-8	Ambarlı	10.06.2019	22	4535178	0639489
C-9	Avcılar	10.06.2019	21	4536219	0644673
C-10	Küçükçekmece	10.06.2019	28	4536629	0648076
C-11	Yeşilköy	10.06.2019	17	4534694	0656542
C-12	Zeytinburnu	10.06.2019	18	4536482	0659244
C-13	Yenikapı-1	10.06.2019	21	4539708	0664909
C-14	Yenikapı-2	11.06.2019	30	4539598	0665458
C-15	Kumkapı-1	11.06.2019	32	4539722	0666198
C-16	Kumkapı-2	11.06.2019	18	4540490	0666165
C-17	Boğaziçi	11.06.2019	51	4541007	0667563
C-18	Haydarpaşa	11.06.2019	18	4540766	0668134
C-19	Üsküdar	11.06.2019	16	4538517	0668634
C-20	Kadıköy	11.06.2019	16	4535730	0669586
C-21	Kınalı Island	11.06.2019	35	4531005.94	671256.44
C-22	C-I.22	11.06.2019	65	4528966.90	660167.75
C-23	C-I.23	11.06.2019	75	4528463.42	635770.20
C-24	C-I.24	11.06.2019	85	4534723.57	603312.94
C-25	M. Ereğlisi	11.06.2019	32	4537387.00	582550.00

Sieve analysis

Core samples obtained from the study area were divided into 10 cm sections. From these sections, 15 g samples were placed in beakers. In order to obtain foraminifera, the sediment samples were kept in 10% H_2O_2 for 24 h. Following this procedure, the sediment samples were washed with water in a 63-micron sieve. The washed samples were dried in a 50 °C oven and examined under a binocular microscope to differentiate the foraminiferal shells.

Geochemical analysis

Geochemical analysis for the elements (Fe, Zn, Al, Mn, As, B, Co, Cr, Cu, Ni, Sb, Na, Mg, K, Ca, P, Pb, Hg, Cd, Ag, Bi, Cd, Mo, Pb, Pt, Sn, Se, and Hg) was carried out using the SPECTROBLUE model Induced Matched Plasma-Optic Emission Spectrometer (ICP-OES) device. Samples to be analyzed with ICP-OES devices, firstly being dissolved by the suitable method (King water method, triple acid method, melting method, TS ISO 14869-1, TS ISO 14869-2 etc.). Then samples which dissolved have been analyzed with TSE, 2004/a-b method.

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Approximately 20 g samples were taken from the elementary levels for wet sieve analysis. After drying, the sediment samples were beaten with the help of mortar and the grains were separated. From these, 0.5 g samples were extracted and 12 ml of HNO_3 and 4 ml of HCl were added to the samples, which were then placed in incineration tubes and burned for 1 h at 98 °C and 1.5 h at 200 °C. After the cooling tubes opened in the fume hood, they were filled with 50 ml of ultra-pure water and filtered using filter papers. Prepared samples were put into the measurement unit of ICP-OES and readings were recorded (Yümün and Önce, 2017; Morillo et al., 2002; Galuszka et al., 2014). Sedimentary samples were taken by the Gravity Core method and the sample sizes are 50-100 cm. In order to evaluate the samples homogeneously, analyzes were made by taking them at 10 cm intervals. The average value of the geochemical analysis results of all samples taken from each core sample was taken and used in evaluations.

Sediment pollution analysis methods

Sediment Contamination Assessment Methods were applied to the geochemical analysis results of sediment samples taken in the study area. These methods are; Enrichment Factor (EF) (Buat et al., 1979; Mason and Moore, 1982). Contamination factor (*Cfi*) (Hakanson, 1980), Pollution Load Index (PLI) (Tomlinson et al., 1980) and Pollution Index (PI) (Yümün, 2017). The results obtained with the methods were interpreted by correlation.

Results and discussion

Geochemical analysis

Geochemical analysis of sediment samples taken from seabed was performed by ICP-OES method. Geochemical analysis results show that heavy metal concentrations are very high in most of the locations (especially Küçükçekmece, Ambarlı, Büyükçekmece) (*Tables 2* and *3*). Geochemical analysis results show very high differences between the elements in ppm. Those with concentration values in the range of 0–1000 ppm were considered as First Group Elements (*Table 2*) and those with values greater than 1000 ppm as Second Group Elements (*Table 3*).

From the elements defined as the first group of elements, there are locations where Mn, P, Cr, Ti, Zn and Cu concentrations are higher than average values. Of the elements evaluated as the second group of elements, Ca and K are high in some locations, while other elements are close to average values. Sediment Pollution Analysis was applied to determine the total pollution status and the polluting elements for each location.

Sediment pollution analysis

Enrichment factor (EF)

Enrichment Factor is a method of determining the rate of heavy metal pollution from anthropogenic origin in soil or wet environment sediments. It is very common to calculate the enrichment factor in order to determine the anthropogenic effects in sediments (Galuszka et al., 2014). The purpose of this method is the ratio of contamination to natural concentrations by a normalization factor (Daskalakis and O'Connor, 1995; Feng et al., 2004).

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Table 2. *First group element concentrations and pollution index of Marmara Sea in Istanbul*

Sample No	Zn	Mn	As	B	Co	Cr	Cu	Ni	Ti	P
Silivri-1	63.86	352.00	4.04	34.25	13.63	74.11	24.39	104.05	203.90	390.10
Silivri-2	63.49	358.2	2.61	38.73	13.94	82.05	24.30	105.19	277.86	396.13
Selimpaşa-1	54.26	310.30	3.04	63.98	10.97	69.63	21.47	76.71	288.80	331.80
Selimpaşa-2	62.28	358.63	4.14	60.68	15.09	81.58	30.76	110.4	298.89	363.73
Kumburgaz	63.21	374.32	2.25	57.00	12.72	74.25	27.32	82.17	259.06	261.75
Büyükçekmece	69.42	460.88	10.03	53.81	14.69	80.22	38.40	96.67	248.63	273.75
Gürpınar	51.10	246.50	2.39	27.96	8.51	43.58	26.79	42.50	167.96	261.12
Ambarlı	72.40	252.03	3.01	32.97	7.64	49.87	51.81	45.57	138.77	269.25
Avcılar	76.03	214.60	2.82	24.17	7.61	42.13	39.06	38.42	121.28	302.22
Küçükçekmece	87.00	259.00	2.28	28.65	7.71	44.43	48.53	36.87	116.69	580.70
Yeşilköy	62.23	170.26	3.46	31.23	4.89	86.24	40.17	25.64	142.87	284.43
Zeytinburnu	219.8	288.6	4.75	55.66	6.84	197.64	135.4	42.03	138.45	545.76
Yenikapı-1	83.15	218.2	7.47	48.64	6.56	133.15	62.01	30.82	126.02	485.35
Yenikapı-2	65.43	180.25	4.78	30.62	5.31	87.00	40.26	23.75	114.94	341.4
Kumkapı -1	106.72	235.6	7.87	31.18	6.41	103.50	88.54	29.51	117.31	931.95
Kumkapı -2	160.47	225.35	8.62	45.62	6.44	174.12	126.44	33.45	152.17	850.9
Boğaziçi	66.37	201.60	3.40	20.52	5.79	36.72	51.50	23.59	90.63	385.10
Haydarpaşa	52.99	223.36	2.57	16.93	6.51	35.17	26.31	24.93	94.96	321.5
Üsküdar	88.47	335.73	3.80	28.98	10.17	58.92	58.42	45.41	152.75	675.13
Kadıköy	79.77	292.73	3.50	27.75	8.33	49.69	55.00	35.15	156.41	341.96
Kınalı Island	28.00	125.00	1.55	11.01	5.20	45.00	28.50	35.00	125.01	201.10
C-I.22	25.05	135.02	2.02	21.06	7.03	65.21	23.32	45.01	123.03	213.02
C-I.23	34.02	134.01	2.50	22.11	8.70	56.05	22.25	32.05	89.00	211.01
C-I.24	45.03	154.05	3.20	23.15	6.50	55.00	26.10	34.05	98.05	223.05
M. Ereğlisi	42.30	163.20	23.57	67.24	38.69	46.10	9.35	106.4	1.29	285.20
Main value of Istanbul region in Marmara Sea	74.2	254.40	4.01	34.85	8.63	**76.04**	**46.52**	49.95	160.14	393.42
The main values of Marmara Sea	88.54	388.9	18.05	61.7	30.36	62.17	30.3	78.63	170.4	741.5

In calculating this index, background values of various normalization elements determined by different methods are used. By taking iron (Fe) as the reference element, the effect of large differences in grain size, carbonate dilution and mineral content is eliminated. Bekground elements (Cr, Mn, Ni, Cu, Zn, As, Ti, Al, Ca and Fe) are used to reduce metal variations caused by grain size and mineral structure, to detect metal anomalies and to ensure geochemical normalization of metals. Elements (Al, Fe, Zirkon, Li), which are not geochemically active and can be found easily in fine-grained materials, are used as normalization factor (Rodríguez-Barroso et al., 2009]. Among these elements, Al is the dominant element in the earth's crust and Fe is the dominant element in the structure of clay minerals (Morillo et al., 2002; Adamo, 2005; Valdes et al., 2005). In many studies, iron is used as a normalization element since it is thought that the distribution of iron is not related to other heavy metals in the enrichment factor calculations (Niencheski, 1994). Since this study analyzed the element contents of sea sediments (sandy, silty clay and silty clay), Fe was used as the normalization element in the enrichment factor calculations. In the absence of anthropogenic inputs, the average shale metal concentrations are taken into consideration in the evaluation of the metal concentrations of the marine sediments (Algan et al., 2004; Pekey, 2004; Taylor and McLennan, 1995; Aksu et al., 1997; Sarı and Çağatay, 2001; Sarı, 2004). Because shales in marine environments best represent the top level of the earth's crust. For this reason, the shale concentrations (Mason and Moore, 1982; Turekian and Wedepohl, 1961; Krauskopf, 1985) given in *Table 4* were used in this study. In this study,

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enrichment factor (EF) of metals (Zn, As, Co, Cu, Ni, Pb and Mn) was calculated using heavy metal analysis results. The Enrichment Factor is calculated (*Table 5*) by the *Equation 1* below, defined by Buat et al. (1979):

$$EF = (Cn / Cref) / (Bn / Bref) \quad \text{(Eq.1)}$$

In the formula, EF: Enrichment factor, Cn: Metal value measured in the study, Cref: Value of the reference element (Fe) measured in the study, Bn: Background (shale) value of the measured element, Bref: Background (shale) value of the reference element. The calculated EF value result close to 1 (EF < 1) indicates the shell origin, while 1 < EF < 3 shows little enrichment. The fact that it is between 3 < EF < 5 is arguably accepted that it is of shell origin (very enrichment) and if EF > 5 is definitely not of shell origin (Galuszka et al., 2014; Halstead et al., 2000).

Table 3. *Second group element concentrations and pollution index of Marmara Sea in Istanbul*

Sample No	S	Al	Fe	Na	Mg	K	Ca
Silivri-1	3621.1	9610.6	24425.20	10851.10	13685.30	4267.00	92360.80
Silivri-2	3641.86	13606.3	25687.83	10496.3	14196.13	5055.36	77036.43
Selimpaşa-1	4852.1	14509.5	23896.90	16612.50	15718.70	7550.30	136555.00
Selimpaşa-2	4307.63	15762.6	28407.26	13868.2	15829.93	7890.56	143896.33
Kumburgaz	3536.72	10112.40	26039.22	15505.70	9195.28	9544.93	4218.64
Büyükçekmece	5372.88	11055.57	29073.15	15002.20	9162.75	9702.57	3060.70
Gürpınar	3102.73	7703.62	17021.82	10103.10	6207.23	5562.85	69675.42
Ambarlı	2835.43	6982.60	15735.95	13266.90	7055.73	5850.90	85185.90
Avcılar	2674.47	6438.17	13430.07	9760.65	5732.68	4587.20	63675.02
Küçükçekmece	3044.38	7044.40	14044.15	13100.13	6057.83	4773.10	32710.13
Yeşilköy	3509.16	8537.46	12065.13	9757.1	7367.7	3910.36	133015.33
Zeytinburnu	6483	12746.06	19752.96	14073.13	16002.73	6593.83	19230.70
Yenikapı-1 (Iç)	6293.3	11518.35	14065.5	11634.35	10549.95	5271.2	183733
Yenikapı-2 (Dış)	4254.35	8836.15	17746.9	7813.8	7283.2	3532.35	116516.5
Kumkapı -1	4325.9	8673.7	16428.5	9154.9	7358.75	3512.3	113774.85
Kumkapı -2	7726	9827.3	18727.55	11370.45	9941.3	4918.4	125067.35
Boğaziçi	1674.3	7075.9	13969.60	5865.20	6599.70	2207.20	112445.00
Haydarpaşa	1581.43	7354.76	14567.36	6232.46	2098.9	90617.03	6232.46
Üsküdar	2694.3	12293.8	22336.7	7614.56	7511.63	3739.16	43245
Kadıköy	2527.86	11572.26	19843.67	7995.03	6863.46	3752.60	.52046.2
Kınalı Island	856.4	856.00	11025.12	1520.10	2050.05	1250.15	2850.10
C-I.22	768.00	900.05	11567.10	1566.15	2312.10	1125.10	2675.15
C-I.23	769.05	875.10	12546.21	1377.02	3451.02	2311.20	2543.20
C-I.24	789.10	1056.10	13456.20	1432.05	3532.05	245421	2345.00
M. Ereğlisi	776.03	17768.2	20936.90	6046.80	4800.40	3290.80	80924.20
Main value of Istanbul region in Marmara Sea	3385.05	8539.52	18160.81	9415.53	**8156.83**	**8332.47**	67670.57
The main values of Marmara Sea	3775.62	15291.00	25210.92	9741.96	7536.66	7214.30	71985.69

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Table 4. *Heavy metal concentrations of some geological reference rocks (Turekian and Wedepohl, 1961; Krauskopf, 1985)*

Elements	Unit	Earth crust	Shale	Sandstone	Limestone	Ultrabasics	Basalt	Deep sea clays
Fe	%	5.00	4.70	0.98	0.38	9.40	8.60	6.50
Zr	ppm	165.00	180.00	19.00	-	45.00	140.00	150.00
Cr	ppm	100.00	90.00	35.00	11.00	1600.00	170.00	90.00
Mn	ppm	950.00	850.00	50.00	1100.00	1620.00	1500.00	6700.00
Ni	ppm	75.00	70.00	2.00	20.00	2000.00	130.00	225.00
Cu	ppm	55.00	45.00	5.00	4.00	10.00	87.00	250.00
Zn	ppm	70.00	95.00	16.00	20.00	50.00	105.00	165.00
Cd	ppm	0.10	0.30	-	-	-	0.20	0.40
Pb	ppm	13.00	20.00	7.00	9.00	1.00	6.00	80.00
As	ppm	1.80	13.00	1.00	1.00	1.00	2.00	13.00
V	ppm	135.00	130.00	20.00	20.00	40.00	250.00	120.00
Sb	ppm	0.20	1.50	-	0.20	0.10	0.20	1.00

While the enrichment of Cu and Zn is seen in many locations, the Ni enrichment in Silivri and Gürpınar is remarkable. In the analysis, Ni enrichment was determined to be very rich in Silivri (3.2) and Gürpınar (4.1) samples. Copper (Cu) enrichment is very high in Selimpaşa-2 (3.2), Kumburgaz (3.1), Büyükçekmece (3.1), Yenikapı-1 (4.9), Boğaziçi (4.1), Ambarlı (3.7), Küçükçekmece (3.5). In Kumkapı-1 (6.0), Kumkapı-2 (7.5), Yeşilköy (7.9), Gürpınar (5.3) and Zeytinburnu (7.6), there is excessive enrichment. Zinc (Zn) enrichment is very rich in Yenikapı-1 (3.1), Kumkapı-1 (3.4), Kumkapı-2 (4.5) and Küçükçekmece (3.3); Excessive enrichment was observed in Zeytinburnu (5.9). Too much enrichment definitely shows that the sources of pollution are not natural. In all other locations, the enrichment factor of all elements was found to be less than 3, and these values show that there is little or no enrichment.

Contamination factor (Cfi)

Contamination factor is a method that is used frequently in studies investigating the origin of heavy metal concentrations in sediments and defines the current situation. Contamination factor calculations for the study area are given in *Table 6* and pollution factor classification is given in *Table 7*. The Contamination Factor was calculated by the correlation (2) defined by Hakanson (1980).

$$Cfi = C\ i\ /\ Cn\ i \qquad \text{(Eq.2)}$$

In the formula, Ci is the metal value measured in sediment and Cni is the pre-industry reference value of the metal. Nickel contamination (CfNi); Silivri-1 (1.5), Silivri-2 (1.5), Selimpaşa-1 (1.1), Selimpaşa-2 (1.6), Kumburgaz (1.2), Büyükçekmece (1.3), Gürpınar (1.4). Copper contamination (CfCu); Selimpaşa-2 (1.8), Kumburgaz (1.7), Büyükçekmece (1.8), Gürpınar (1.8), Ambarlı (1.15) and Kumkapı (1.9) are also seen. Zinc (CfZn) contamination value is seen at medium level in Kumkapı-1 (1.12). Moderate and little contamination is observed for all elements in all other locations.

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Table 5. *Enrichment factor values calculated in the study area*

Silivri -1		**Silivri-2**	**Selimpaşa-1**	**Selimpaşa-2**
Toxic elements	**Enrichment value**	**Enrichment value**	**enrichment value**	**Enrichment value**
EF_{Mn}	0.85	0.80	0.80	0.75
EF_{Co}	1.50	1.40	1.20	1.40
EF_{Ni}	**3.10**	**2.90**	**2.30**	**2.80**
EF_{Cu}	1.10	1.10	1.00	3.20
EF_{Zn}	1.40	1.30	1.20	1.20
EF_{As}	0.60	0.40	0.50	0.56
	Kumburgaz	**Büyükçekmece**	**Yenikapı-1**	**Yenikapı-2**
Toxic elements	**Enrichment value**	**Enrichment value**	**Enrichment value**	**Enrichment value**
EF_{Mn}	0.80	1.20	0.90	0.60
EF_{Co}	1.20	1.30	1.20	0.80
EF_{Ni}	2.30	2.40	1.60	0.90
EF_{Cu}	**3.10**	**3.10**	**4.90**	**2.50**
EF_{Zn}	1.30	1.30	3.10	1.90
EF_{As}	0.30	1.30	2.10	1.10
	Kumkapı-1	**Kumkapı-2**	**Boğaziçi**	**Üsküdar**
Toxic elements	**Enrichment value**	**Enrichment value**	**Enrichment value**	**Enrichment value**
EF_{Mn}	0.80	0.70	0.80	0.60
EF_{Co}	1.10	0.90	1.10	1.20
EF_{Ni}	1.30	1.30	1.20	1.50
EF_{Cu}	**6.00**	**7.50**	**4.10**	**2.90**
EF_{Zn}	**3.40**	**4.50**	**2.50**	**2.10**
EF_{As}	1.80	1.80	0.90	0.70
	Gürpınar	**Ambarlı**	**Avcılar**	**Küçükçekmece**
Toxic elements	**Enrichment value**	**Enrichment value**	**Enrichment value**	**Enrichment value**
EF_{Mn}	0.80	0.90	0.90	1.10
EF_{Co}	2.30	1.30	1.50	1.40
EF_{Ni}	4.10	2.10	2.10	1.90
EF_{Cu}	**5.30**	3.70	3.50	3.50
EF_{Zn}	1.50	2.40	2.90	4.38
EF_{As}	0.50	0.70	0.80	0.60
	Yeşilköy	**Zeytinburnu**	**Haydarpaşa**	**Kadıköy**
Toxic elements	**Enrichment value**	**Enrichment value**	**Enrichment value**	**Enrichment value**
EF_{Mn}	0.80	0.90	0.80	0.90
EF_{Co}	1.10	0.90	1.20	1.10
EF_{Ni}	1.50	1.50	1.20	1.20
EF_{Cu}	7.90	7.60	2.00	3.10
EF_{Zn}	2.70	5.90	1.90	2.10
EF_{As}	1.10	0.90	0.70	0.60

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Table 6. *Contamination factor values calculated for the study area*

Sample locations	C_f Mn	C_f Fe	C_f Ni	C_f Cu	C_f Zn	C_f Co	C_f As
Silivri-1	0.40	0.51	**1.50**	0.50	0.70	0.70	0.30
Silivri-2	0.40	0.55	**1.50**	0.54	0.70	0.73	0.20
Selimpaşa-1	0.40	0.50	**1.10**	0.50	0.60	0.60	0.20
Selimpaşa-2	0.42	0.60	**1.60**	**1.80**	0.70	0.80	0.30
Kumburgaz	0.40	0.55	**1.20**	**1.70**	0.70	0.70	0.20
Büyükçekmece	0.50	0.60	**1.30**	**1.80**	0.70	0.80	0.80
Gürpınar	0.30	0.36	**1.40**	**1.80**	0.53	0.77	0.18
Ambarlı	0.29	0.33	0.65	**1.15**	0.76	0.40	0.23
Avcılar	0.25	0.28	0.55	0.93	0.80	0.40	0.21
Küçükçekmece	0.30	0.29	0.53	0.98	0.95	0.40	0.17
Yeşilköy	0.20	0.25	0.36	1.91	0.65	0.25	0.26
Zeytinburnu	0.30	0.40	0.60	**3.00**	**2.30**	0.36	0.36
Yenikapı-1	0.21	0.37	0.34	0.89	0.69	0.27	0.36
Yenikapı-2	0.20	0.35	0.32	0.73	0.93	0.21	0.32
Kumkapı-1	0.27	0.35	0.42	**1.90**	**1.12**	0.33	0.60
Kumkapı-2	0.26	0.39	0.48	**2.80**	**1.60**	0.33	0.70
Boğaziçi	0.23	0.29	0.33	**1.20**	0.69	0.30	0.26
Haydarpaşa	0.24	0.30	0.35	0.58	0.55	0.34	0.19
Üsküdar	0.26	0.47	0.65	**1.30**	0.93	0.53	0.29
Kadıköy	0.30	0.40	0.50	**1.20**	0.83	0.43	0.27

Table 7. *Pollution factor (Cf) classification Hakanson (1982)*

C_f	Sediment quality
$C_f < 1$	Little contamination
$1 < C_f < 3$	Midle contamination
$3 < C_f < 6$	Significant contamination
$C_f > 6$	Very high contamination

Pollution load index (PLI)

Pollution Load Index is a method that evaluates the pollution level of heavy metals. PLI is defined by the n root of the product of the ratio of the concentration of each element measured to the background values (Tomlinson, 1980).

$$PLI = (C^1_f \times C^2_f \times C^3_f \times \cdots \times C^n_f)^{\wedge}(1/n) \quad \text{(Eq.3)}$$

$$C^i_f = C^i_s / C^i_n \quad \text{(Eq.4)}$$

Here, Cis: represents the contamination value of (i) metal, Cin: represents the background value of (i) metal. If the PLI value > 1 indicates the presence of contamination, and PLI < 1 indicates no contamination (Tomlinson, 1980). This method was applied to the element concentrations obtained from the study area (*Table 8*).

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Shale bekground values defined by Turekian et al. (1961) and Krauskopf et al. (1985) were used in the Pollution Load Index calculations of the study area. In the data obtained, the smallest PLI value was calculated as 0.29 (Kınalı island) and the largest PLI value was calculated as 0.90 (Zeytinburmu). The reason for the absence of pollution is considered as Bekdround values compiled from world averages that cannot represent Marmara Sea Sediments. For this reason, calculations were made again using the "Marmara Sea Element Concentration Averages" obtained in the studies performed by Yümün (2017) (*Tables 8* and 9). Correlation was performed in *Table 9* by giving Pollution Load Index (PLI) together with Pollution Index (PI) values.

In the calculations made by taking the average values of the element concentrations of the Marmara Sea drilling samples as the back ground, again the smallest value was found in Kınalı island (0.38), while the highest value was calculated in Zeytinburnu sample (1.16). Here, Zeytinburnu (1.16), Kumkapı (1.09) and Büyükçekmece (1.03) Pollution Load Index (PLI) values show that there is contamination because it is > 1. It is seen that other locations are not dirty.

Table 8. *Pollution load ındex (PLI) values calculated in the study area*

Sample locations	Zn	Mn	As	Cr	Cu	Ni	Fe	Pollution load index (PLI)
Silivri-1	63.86	352.00	4.04	74.11	24.39	104.05	24425.20	0.61
Silivri-2	63.49	358.20	2.61	82.05	24.30	105.19	25687.83	0.58
Selimpaşa-1	54.26	310.30	3.04	69.63	21.47	76.71	23896.90	0.52
Selimpaşa-2	62.28	358.63	4.14	81.58	30.76	110.40	28407.26	0.66
Kumburgaz	63.21	374.32	2.25	74.25	27.32	82.17	26039.22	0.56
Büyükçekmece	69.42	460.88	10.03	80.22	38.40	96.67	29073.15	0.79
Gürpınar	51.10	246.50	2.39	43.58	26.79	42.50	17021.82	0.41
Ambarlı	72.40	252.03	3.01	49.87	51.81	45.57	15735.95	0.49
Avcılar	76.03	214.60	2.82	42.13	39.06	38.42	13430.07	0.43
Küçükçekmece	87.00	259.00	2.28	44.43	48.53	36.87	14044.15	0.46
Yeşilköy	62.23	170.26	3.46	86.24	40.17	25.64	12065.13	0.43
Zeytinburnu	219.80	288.60	4.75	197.64	135.40	42.03	19752.96	0.90
Yenikapı-1	83.15	218.20	7.47	133.15	62.01	30.82	14065.50	0.62
Yenikapı-2	65.43	180.25	4.78	87.00	40.26	23.75	17746.90	0.48
Kumkapı -1	106.72	235.60	7.87	103.50	88.54	29.51	16428.50	0.67
Kumkapı -2	160.47	225.35	8.62	174.12	126.44	33.45	18727.55	0.84
Boğaziçi	66.37	201.60	3.40	36.72	51.50	23.59	13969.60	0.41
Haydarpaşa	52.99	223.36	2.57	35.17	26.31	24.93	14567.36	0.36
Üsküdar	88.47	335.73	3.80	58.92	58.42	45.41	22336.70	0.60
Kadıköy	79.77	292.73	3.50	49.69	55.00	35.15	19843.67	0.53
Kınalı Ada	28.00	125.00	1.55	45.00	28.50	35.00	11025.12	0.29
C-I.22	25.05	135.02	2.02	65.21	23.32	45.01	11567.10	0.33
C-I.23	34.02	134.01	2.50	56.05	22.25	32.05	12546.21	0.33
C-I.24	45.03	154.05	3.20	55.00	26.10	34.05	13456.20	0.38
M. Ereğlisi	42.30	163.20	23.57	46.10	9.35	106.40	20936.90	0.53
Background of shale	95.00	850.00	13.00	90.00	45.00	70.00	47000.00	1.00

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Table 9. *Pollution load index (PLI) values calculated in the study area*

Sample locations	Zn	Mn	As	Cr	Cu	Ni	Fe	Pollution load index (PLI)	Pollution index (PI)
Silivri-1	63.86	352.00	4.04	74.11	24.39	104.05	24425.20	0.78	0.90
Silivri-2	63.49	358.20	2.61	82.05	24.30	105.19	25687.83	0.75	0.95
Selimpaşa-1	54.26	310.30	3.04	69.63	21.47	76.71	23896.90	0.67	**1.05**
Selimpaşa-2	62.28	358.63	4.14	81.58	30.76	110.40	28407.26	0.85	**1.13**
Kumburgaz	63.21	374.32	2.25	74.25	27.32	82.17	26039.22	0.72	0.88
Büyükçekmece	69.42	460.88	10.03	80.22	38.40	96.67	29073.15	**1.03**	**1.00**
Gürpınar	51.10	246.50	2.39	43.58	26.79	42.50	17021.82	0.52	0.66
Ambarlı	72.40	252.03	3.01	49.87	51.81	45.57	15735.95	0.64	0.75
Avcılar	76.03	214.60	2.82	42.13	39.06	38.42	13430.07	0.56	0.63
Küçükçekmece	87.00	259.00	2.28	44.43	48.53	36.87	14044.15	0.59	0.70
Yeşilköy	62.23	170.26	3.46	86.24	40.17	25.64	12065.13	0.56	0.74
Zeytinburnu	219.80	288.60	4.75	197.64	135.40	42.03	19752.96	**1.16**	**1.32**
Yenikapı-1	83.15	218.20	7.47	133.15	62.01	30.82	14065.50	0.80	**1.04**
Yenikapı-2	65.43	180.25	4.78	87.00	40.26	23.75	17746.90	0.62	0.74
Kumkapı -1	106.72	235.60	7.87	103.50	88.54	29.51	16428.50	0.87	0.95
Kumkapı -2	160.47	225.35	8.62	174.12	126.44	33.45	18727.55	**1.09**	**1.27**
Boğaziçi	66.37	201.60	3.40	36.72	51.50	23.59	13969.60	0.53	0.61
Haydarpaşa	52.99	223.36	2.57	35.17	26.31	24.93	14567.36	0.46	**1.15**
Üsküdar	88.47	335.73	3.80	58.92	58.42	45.41	22336.70	0.78	0.79
Kadıköy	79.77	292.73	3.50	49.69	55.00	35.15	19843.67	0.68	0.72
Kınalı Ada	28.00	125.00	1.55	45.00	28.50	35.00	11025.12	0.38	0.33
C-I.22	25.05	135.02	2.02	65.21	23.32	45.01	11567.10	0.42	0.36
C-I.23	34.02	134.01	2.50	56.05	22.25	32.05	12546.21	0.42	0.36
C-I.24	45.03	154.05	3.20	55.00	26.10	34.05	13456.20	0.48	0.38
M. Ereğlisi	42.30	163.20	23.57	46.10	9.35	106.40	20936.90	0.68	0.73
Main value of Marmara Sea	88.54	388.9	18.05	62.17	30.3	78.63	25211	1.00	**1.00**

Pollution index (PI)

To make the results of geochemical analysis more visual and interpretable, the Pollution Index (PI) method, as defined by Yümün (2017), was applied. PI values are the proportionality coefficients containing the arithmetic mean of the values of each element obtained by geochemical analysis together with the values obtained from the previous studies in the Sea of Marmara. PI was calculated using the following equation (*Eq. 5*):

$$PI = [(MV_1 / MV_{avg}) + (MV_2 / MV_{avg}) + \cdots + (MV_n / MV_{avg})] / n \qquad \text{(Eq.5)}$$

The parameters used in the equation are: PI: Pollution Index, MV_1: Heavy metal measurement value (ppm), MV_{avg}: Heavy metal measurement value average (ppm), n: The number of the heavy metals measured. PI maps were produced by Kriging method (Krige, 1951) using PI values. In this map, PI values are defined as 0–0.50 (max. clean zone), 0.5- 0.85 (clean zone), 0.85–1.00 (clean-dirty transition zone), 1.00-1.15

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(polluted zone), and PI > 1.15 (high polluted zone). In areas where heavy metal concentrations are high, PI values are higher than critical values (PI = 1) and are defined as dirty areas.

The number of genera, species and individuals of foraminifera is quite low in the places where pollution is high. It is thought that the low number of foraminifera samples in samples taken from Küçükçekmece, Büyükçekmece, Ambarlı and Avcılar regions are due to ship traffic and discharge of domestic and industrial wastewater into the sea (*Table 10; Figs. 4* and *5*).

Table 10. *Values of pollution index (PI)*

Sample No	Pollution index (PI)	Sample No	Pollution index (PI)
Silivri-1	0.898	Yenikapı-2	0.741
Silivri-2	0.947	Kumkapı -1	0.954
Selimpaşa-1	**1.049**	Kumkapı -2	**1.270**
Selimpaşa-2	**1.131**	Boğaziçi	0.613
Kumburgaz	0.881	Haydarpaşa	**1.152**
Büyükçekmece	0.996	Üsküdar	0.791
Gürpınar	0.655	Kadıköy	0.718
Ambarlı	0.754	Kınalı Ada	0.326
Avcılar	0.631	C-I.22	0.358
Küçükçekmece	0.696	C-I.23	0.357
Yeşilköy	0.741	C-I.24	0.384
Zeytinburnu	**1.319**	M. Ereğlisi	0.729
Yenikapı-1	**1.044**		
Main value of Istanbul region in Marmara Sea	0.809	The main values of Marmara Sea	**1.000**

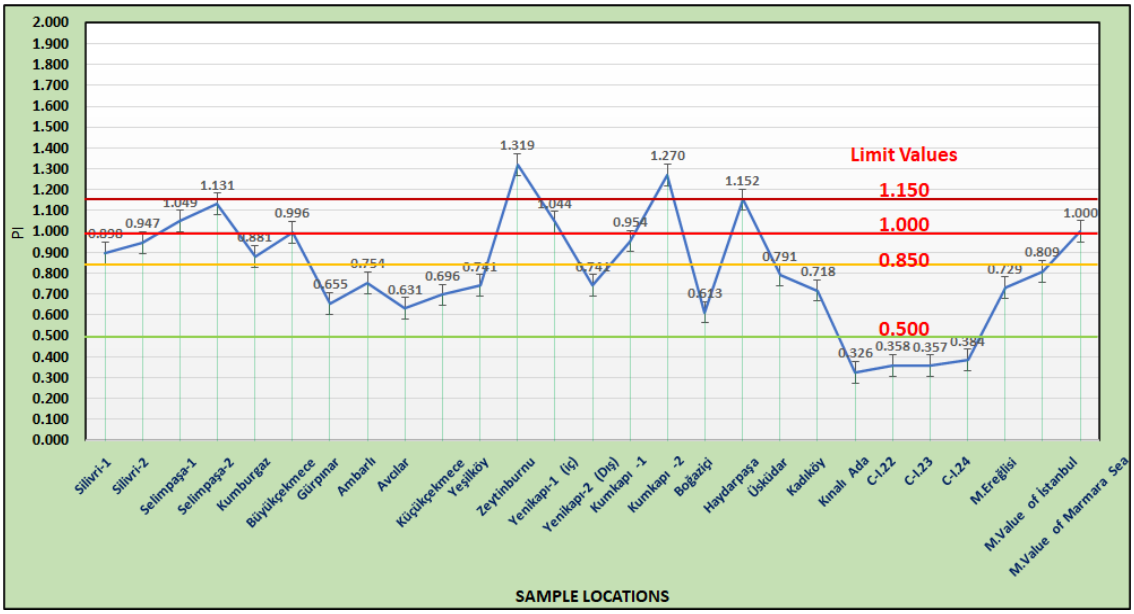

Figure 4. *Pollution index of Marmara Sea in Istanbul*

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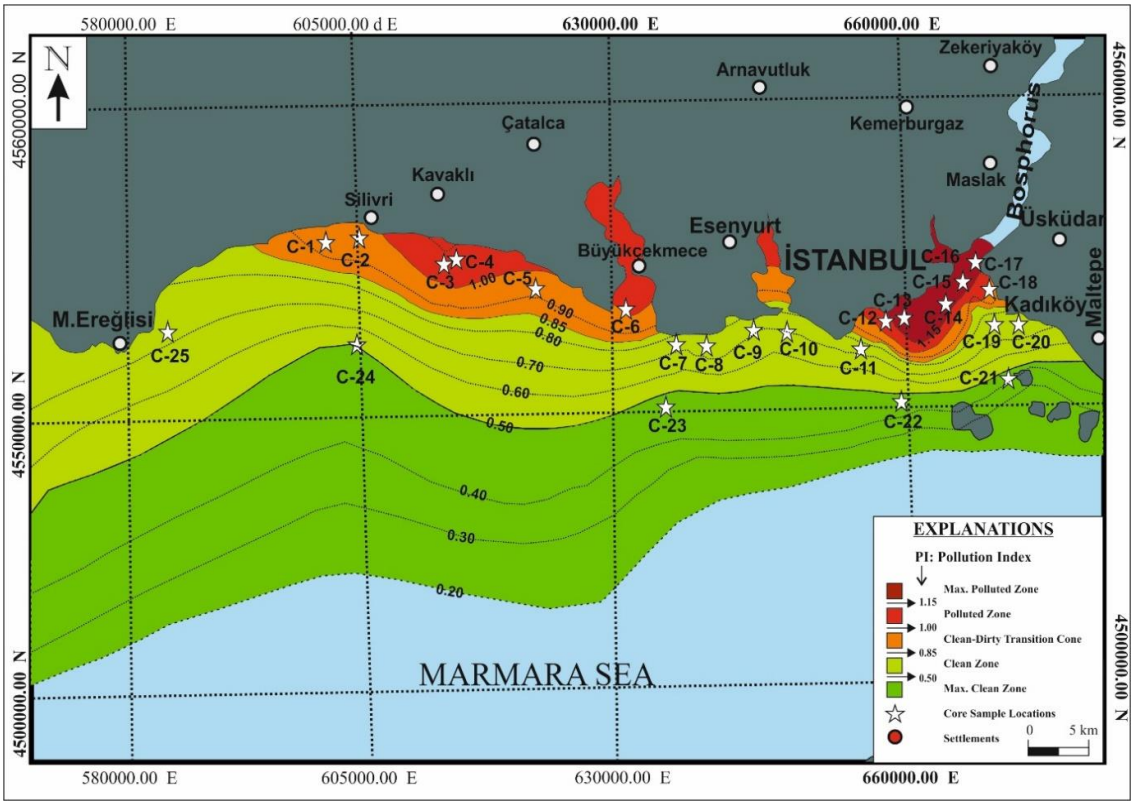

Figure 5. *Pollution index (PI) map of the study area (North Marmara)*

Foraminifer communities

Sediment core samples taken from the study area were divided into 10 cm sections and granulometric analysis was conducted for each sample. In the granulometric analysis, the granules remaining on the 63 µm sieve were examined using a stereo-zoom microscope. The foraminifera were extracted, and micro-photographs were taken. In this study, a rich foraminifera group consisting of 15 genera and 30 species was identified (*Table 11; Figs. 6-9*). Color changes were observed in *Ammonia compacta*. The changes in the foraminifer shells were investigated by examining the concentrations of toxic elements at the levels where morphological changes occurred in the foraminifer shells. Significant color changes were observed in the foraminiferal shells. The shells demonstrated different shades, ranging from yellowish brown to black. To determine the causes of these discolorations, surface element analysis was conducted on the shells using a scanning electron microscope (SEM) (*Fig. 10*). The shell structures of the foraminifera are limestone (CaCO 3), agglutinate (Rock Pieces) and silica (SiO2). During the growth of foraminifer, CaCO3 and SiO2, which are dissolved in water, are chemically added to the shell structure. During the growth of the shells, it undergoes color change by adding elements that are in the form of molten or ions in the water. In places where the S values were high, the color had turned into dark grey-black colors. At high Fe and Mn values, the color turned yellow-yellowish brown.

The numbers given in *Table 11* give the numbers of foraminifera species found in 10 g of sediment. The high number of foraminifera species indicates that the environment conditions are ideal for micro-living life, and the low number of foraminifera species indicates that the environment is not ideal for the habitat.

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Table 11. *Foraminifer communities identified in the study area*

	Silivri-1	**Silivri-2**	**Selimpaşa-1**	**Selimpaşa-2**	**Kumburgaz**	**Büyükçekmece**	**Gürpınar**	**Ambarlı**	**Avcılar**	**Küçükçekmece**	**Yeşilköy**	**Zeytinburnu**	**Yenikapı-1**	**Yenikapı-2**	**Kumkapı-1**	**Kumkapı-2**	**Boğaziçi**	**Haydarpaşa**	**Üsküdar**	**Kadıköy**
Adelosina cliarensis																3		10	10	4
Adelosina duthersi	2	1	4	2			4		7											
Adelosina mediteranensis	1								1									2		
Adelosina partschi																				
Ammonia compacta	47	40	39	30	19	**6**	23	8	68	22	14	**33**	25	11	17	18	1	60	38	28
Ammonia parkinsoniana																		7	8	
Ammonia tepida	3	2	2	5			1		8			**5**			1		1	9	7	
Cribroelphidium poeyanum			2										2						1	
Cycloforina contorta			3	6									6	2			1			
Cycloforina villafrance							3		7				8							
Elphidium crispum	36	40	50	45	4	**4**	46	5	50	15	19	**40**	40	7	5	20		15	32	13
Elphidium complanatum							5	4	2							3				
Eponides concomeratus	1		10	6		**1**							11	3			1		8	5
Lachlanella bicornis	1										1		4					2		
Lobatula lobatula	3	2	26	15	1		4	2	14	7		**19**	16	2	1	4	1	18	15	13
Miliolinella subrotunda			3				3					**2**	5			3		3	1	11
Miliolinella circularis																				
Pseudotriloculina oblonga																				
Pyrgo elongata																				
Pygro Inornata		2	2		2															
Plonorbulına mediterranensis									1	1										
Quinqueloculina bidentata																				
Quinqueloculina jugosa	1		3	4				1	8		1						1		4	
Quinqueloculina lamarckiana																				
Quinqueloculina laevigata												**3**								9
Quinqueloculina seminula			9	12		**1**	3	1			1	**5**	23	3	4	7	4	31	26	26
Quinqueloculina stelligera																				
Rosalina bradyi			2										3							
Spiriloculina excavata			2	4	1		1		6				6			1				
Textularia bocki																				

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Figure 6. *1a. Quinqueloculina jugosa, Cushman (Ambarlı-A)2a,b. Adelosina duthersi (Avcılar-a), 3a,b. Cycloforina contorta, (d'Orbigny), Boğaziçi-2, 4a,b. Cycloforina villafrance, (le Calvez, J & Y.), Gürpınar-2, 5a,b. Pseudotriloculina oblonga, (Montagu), Haydarpaşa-1, 6a,b. Miliolinella subrotunda, (Montagu), Haydarpaşa-2, 7a,b. Miliolinella subrotunda, (Montagu), Kumkapı-2/1, 8a,b. Pygro inornata, (d'Orbigny), Selimpaşa-1, 9a,b. Pygro inornata, (d'Orbigny), Selimpaşa-1, 10a,b. Pygro inornata, (d'Orbigny), Selimpaşa-2, 11a,b. Pygro inornata, (d'Orbigny), Selimpaşa-2, 12a,b. Cycloforina contorta, (d'Orbigny), Selimpaşa-1, 13a,b. Adelosina mediteranensis, (le Calvez, J & Y.), Silivri-1, 14a,b. Lachlanella bicornis, (Walker & Jacob), Silivri-2, 15a,b. Miliolinella sp. Yenikapı-1/1, 16. Textularia bocki, Höglund, Haydarpaşa-2. (scale lengths are mm)*

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Figure 7. *1. Elphidium crispum, (Linne), Ambarlı-1, 2. Elphidium crispum, (Linne), Avcılar-4, 3. Elphidium crispum, (Linne), Büyükçekmece-1, 4. Elphidium crispum, (Linne), Kumkapı-,2/2, 5a,b. Eponides concomeratus, (Williamson). Boğaziçi-1, 6a,b. Ammonia tepida, (Cushman), Selimpaşa-1, 7a,b. Ammonia tepida, (Cushman), Yenikapı-1/1, 8a, b. Lobatula lobatula (Walker & Jacob), Avcılar-1, 9a,b. Lobatula lobatula (Walker & Jacob),Boğaziçi-1, 10a,b. Lobatula lobatula (Walker & Jacob),Gürpınar-2, 11a,b. Lobatula lobatula (Walker & Jacob),Haydarpaşa-1, 12a,b. Lobatula lobatula (Walker & Jacob),Haydarpaşa-2, 13a,b. Lobatula lobatula (Walker & Jacob),Kumkapı-2/1, 14a,b. Ammonia compacta, Hofker, Silivri-1, 15a,b. Ammonia compacta, Hofker Avcılar-4, 16a,b. Ammonia tepida, (Cushman), Gürpınar-1, 17a,b. Ammonia tepida, (Cushman), Bostancı-2. (scale lengths are mm)*

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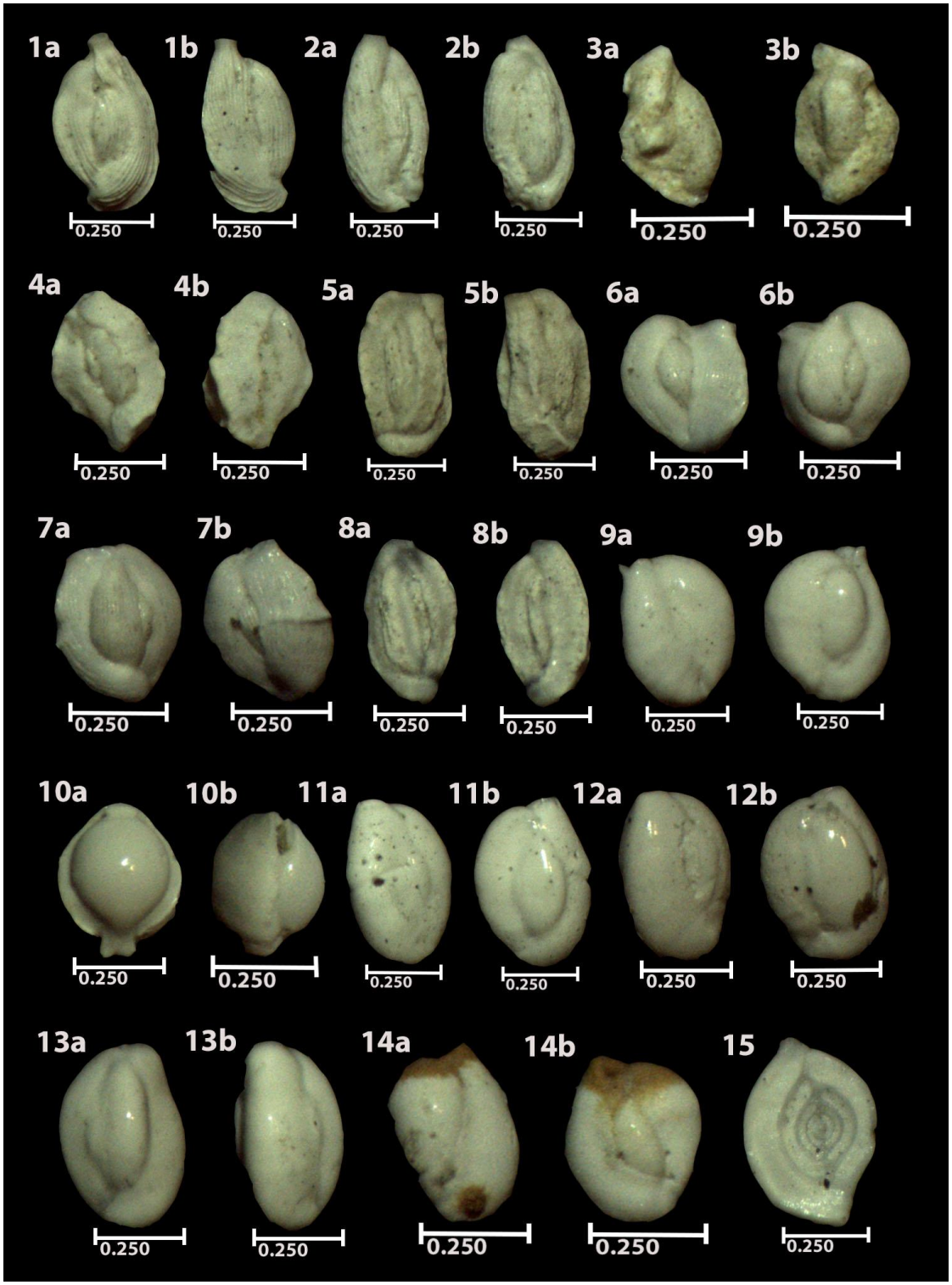

Figure 8. *1a,b: Adelosina partschi (Avcılar -b), 2 a,b: Sycloforina villafrance (Avcılar-b), 3 a,b: Quinqueloculina stalkeri (Kadıköy-a), 4a,b: Quinqueloculina bidentata (Maltepe-a), 5 a,b: Quinqueloculina bidentata (Selimpaşa-c), 6 a,b. Quinqueloculina lamarckiana (Yenikapı-1-b), 7 a,b: Quinqueloculina lamarckiana (Yeşilköy-b), 8 a,b: Quinqueloculina stelligera (Yenikapı-1-b), 9 a,b: Miliolinella circularis (Zeytinburnu-a), 10 a,b: Pyrgo elongata (Selimpaşa-c), 11 a,b: Quinqueloculina laevigata (Yenikapı-1-b), 12 a,b: Quinqueloculina laevigata (Yenikapı-1-c), 13 a,b: Quinqueloculina laevigata (Yenikapı-2-a), 14 a,b: Spiriloculina excavata (Yeşilköy-b), 15 : Spiriloculina excavata (Yenikapı-1-b). (scale lengths are mm)*

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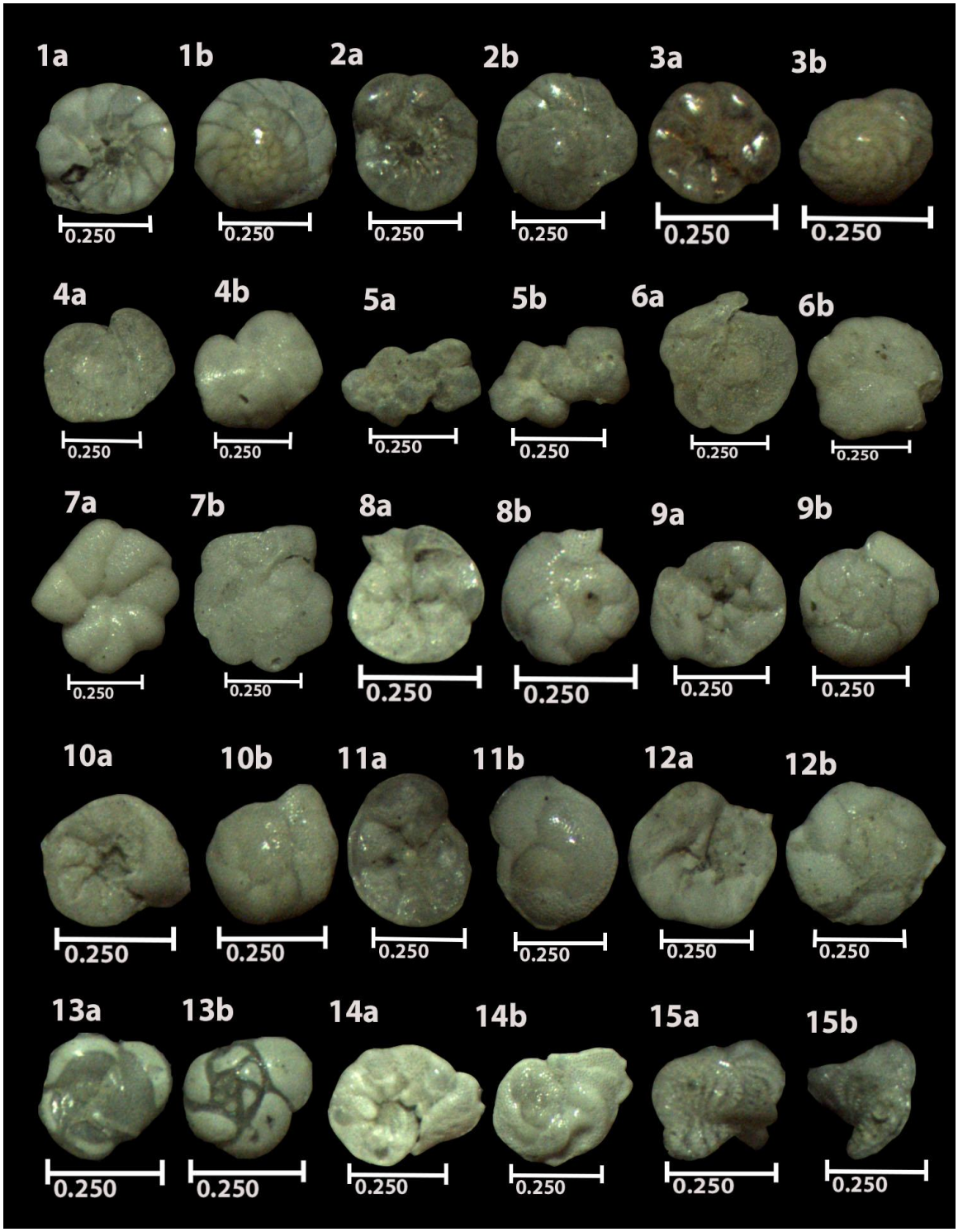

Figure 9. *1 a,b: Ammonia parkinsoniana (Avcılar-b), 2a,b: Ammonia parkinsoniana (Maltepe-a), 3 a,b: Ammonia parkinsoniana (Zeytiburnu-a), 4 a,b: Lobatula lobatula (Avcılar-b), 5 a,b: Lobatula lobatula (Kadıköy-a), 6 a,b: Lobatula lobatula (Maltepe-a), 7 a,b: Lobatula lobatula (Yenikapı-1-c), 8 a,b: Rosalina globularis (Avcılar-b), 9 a,b: Rosalina globularis (Kadıköy-a), 10 a,b: Rosalina globularis (Selimpaşa-c), 11 a,b: Rosalina globularis (Yenikapı-1b), 12 a,b; Rosalina globularis (Yanikapı-1c), 13 a,b: Eponides concameratus (Zeytinburna-a), 14 a,b; Lobatula lobatula (Üsküdar-d), 15 a,b: Elphidium crispum (Selimpaşa-d). (scale lengths are mm)*

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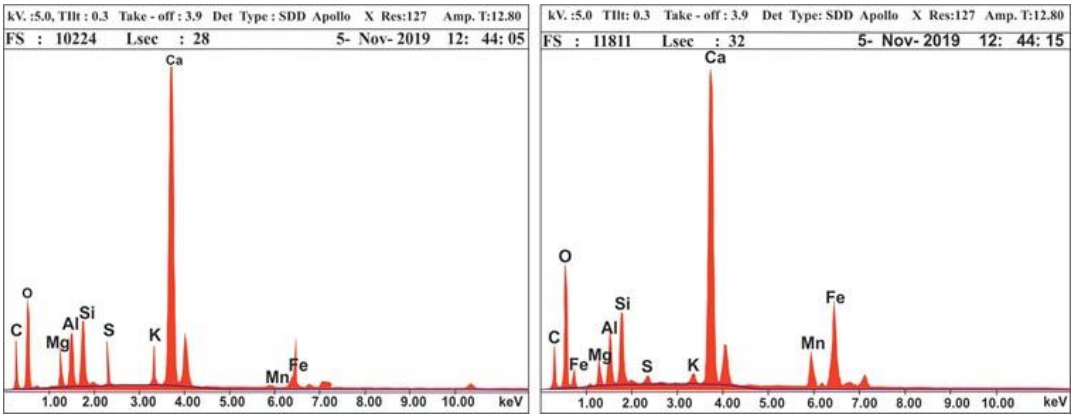

Figure 10. *Graphs of surface element analysis*

Conclusion

In this study, the effects of heavy metals on the sediments of the Istanbul part of Marmara Sea and the effects of pollution on benthic foraminifers were assessed. Benthic foraminifer assemblages and geochemical properties of the samples were determined in the study.

Elemental analysis of (Fe, Zn, Al, Mn, As, B, Co, Cr, Cu, Ni, Sb, Na, Mg, K, Ca, P, Pb, Hg, Cd, Ag, Bi, Cd, Mo, Pb, Pt, Sn, Se, and Hg) sediments from the sea was conducted using ICP-OES. The concentrations of some elements (especially for Fe, Mn, Ti, Zn, and Cr) were found to be very high in locations where ship traffic and domestic and industrial discharges are high.

Enrichment Factor (EF), Contamination factor (*Cfi*) (Hakanson, 1980), Pollution Load Index (PLI) and Pollutin Index (PI) (Yümün, 2017) were applied to the geochemical analysis results of sediment samples taken in the study area. According to enrichment factor (EF) and Contamination factor (Cfi) methods, enrichment and contamination are observed in terms of some elements. According to the enrichment factor, Cu (3.1), Ni (2.4) in Büyükçekmece, EFCu (7.6), EFZn (5.9) in Zeytinburnu, EFCu (6.0), EFZn(3.4) in Kumkapı-1, EFCu(7.5) ve EFZn(4.5) in Kumkapı-2 values are > 3, according to this Cu and Zn are high and very high enrichment. In the analysis made according to the contamination factor, the values are determined of Kumkapı-1 CfCu (1.90) and CfZn (1.12), Kumkapı-2 CfCu (2.80) and CfZn (1.60); Zeytinburnu CfCu (3.0) and CfZn (2.3); It was obtained as CfCu (1.80) and Cf Ni (1.30) in Büyükçekmece. According to these results, the locations appear to be contaminated in terms of Cu, Zn and Ni. According to four analysis methods, medium-high level of impurities were detected in these 3 (Büyükçekmece, Zeytinburnu and Kumkapı) locations.

In the Pollution Load Index (PLI) calculations, both the Shale values defined by Turekian et al. (1961) and Krauskopf et al. (1985), and the drilling concentration samples compiled from Yümün (2017) and Yümün and Kam (2019) were used as background elements. Since the shale values do not represent the Marmara region, no pollution was observed in this method. In the PLI calculations made using the Marmara Sea element concentration averages, pollution is observed in some locations. These second calculation values are correlated with the Pollution Index (PI) values in *Table 9,* and common dirty points (Büyükçekmece, Zeytinburnu and Kumkapı) are determined according to both methods. The Pollution Index (PI) values of these locations (Selimpaşa (1.131), Zeytinburnu (1.319), Yenikapı (1.044), Kumkapı (1.270), and

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Haydarpaşa Port (1.152) were also high. A contamination zone map was prepared using the Kriging method on the PI values, summarizing heavy metal concentrations of each sampling point of the study area. This pollution zone map has been useful in terms of visualizing and interpreting the pollution capacity of the study area.

In locations where heavy metal concentrations were high, the number of foraminifer individuals was very low. In addition, color changes and morphological defects were detected in most of the locations.

Here, while Pollution Load Index (PLI) and Pollution Index (PI) analyze the final impurities formed by the contribution of all elements, Enrichment factor (EF) and Contamination factor (Cfi) determine the polluting elements in the environment. The application and interpretation of these methods together in environmental analysis will yield useful results.

In addition, in order to identify possible lithological units that may cause metal accumulation in the bottom sediments, the geological map of the Northeast Marmara Drainage Area was compiled and the lithological units that surfaced in the coastal areas adjacent to these areas were examined. Summaries of the results of the Pollution Index, Enrichment Factor, Contamination Factor and Pollution Load Index were given in *Table 12*. Dirty locations (such as Zeytinburnu) in all indexes are marked in bold in the table.

Table 12. *The correlations of pollution index, enrichment factor, contamination factor and pollution load index*

Core sample No	Core sample location	Pollution index		Enrichment factor		Contamination factor		Pollution load index	
		(PI)	Exp.	EF	Exp.			PLI	
C-1	Silivri-1	0.898	CD	EF_{Ni} = 3.10	VE	C_f Ni = 1.50	MC	0.61	NC
C-2	Silivri-2	0.947	CD	EF_{Ni} = 2.90	LE	C_f Ni = 1.50	MC	0.58	NC
C-3	Selimpaşa-1	1.049	P	EF_{Ni} = 2.30	LE	C_f Ni = 1.10	MC	0.52	NC
C-4	Selimpaşa-2	1.131	P	EF_{Ni} = 2.80	LE	C_f Cu = 1.80	MC	0.66	NC
C-5	Kumburgaz	0.881	CD	EF_{Cu} = 3.10	VE	C_f Cu = 1.70	MC	0.56	NC
C-6	Büyükçekmece	0.996	CD	EF_{Cu} = 3.10	VE	C_f Cu = 1.80	MC	0.79	NC
C-7	Gürpınar	0.655	C	EF_{Cu} = 5.30	HE	C_f Cu = 1.80	MC	0.41	NC
C-8	Ambarlı	0.754	C	**EF_{Cu} = 3.7**	**VE**	**C_f Cu = 1.15**	**MC**	0.49	NC
C-9	Avcılar	0.631	C	EF_{Cu} = 3.50	VE	C_f Cu = 0.93	LC	0.43	NC
C-10	Küçükçekmece	0.696	C	EF_{Zn} = 4.38	VE	C_f Cu = 0.98	LC	0.46	NC
C-11	Yeşilköy	0.741	C	EF_{Cu} = 7.90	HE	C_f Cu = 1.91	MC	0.43	NC
C-12	**Zeytinburnu**	**1.319**	**HP**	**EF_{Cu} = 7.60**	**HE**	**C_f Cu = 3.00**	**MC**	0.90	NC
C-13	Yenikapı-1	1.044	P	EF_{Cu} = 4.90	VE	C_f Cu = 0.89	LC	0.62	NC
C-14	Yenikapı-2	0.741	C	EF_{Cu} = 2.50	LE	C_f Cu = 0.73	LC	0.48	NC
C-15	Kumkapı-1	0.954	CD	EF_{Cu} = 6.00	HE	C_f Cu = 1.90	MC	0.67	NC
C-16	**Kumkapı-2**	**1.270**	**HP**	**EF_{Cu} = 7.50**	**HE**	**C_f Cu = 2.80**	**MC**	0.84	NC
C-17	Boğaziçi	0.613	C	EF_{Cu} = 4.10	VE	C_f Cu = 1.20	MC	0.41	NC
C-18	Haydarpaşa	1.152	HP	EF_{Cu} = 2.00	LE	C_f Cu = 0.58	LC	0.36	NC
C-19	Üsküdar	0.791	C	EF_{Cu} = 2.90	LE	C_f Cu = 1.30	MC	0.60	NC
C-20	Kadıköy	0.718	C	EF_{Cu} = 3.10	VE	C_f Cu = 1.20	MC	0.53	NC
C-21	Kınalı Island	0.326	HC					0.29	NC
C-25	M. Ereğlisi	0.729	C					0.53	NC

LE: little enrichment; VE: very enrichment, HE: highly enrichment (definitely not of shell origin); LC: little contamination, MC: middle contamination; PLI value > 1 indicates the presence of contamination (C), PLI < 1 indicates no contamination (NC); PI: 0–0.50 (max. clean (MC)), 0.50–0.85 (clean (C)), 0.50–0.85 (max. clean (MC)), PI: 0.85-1.00 clean-dirty transition (CD), PI: 1.00-1.15 polluted (P), PI > 1.15 (high polluted zone (HP)

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A rich foraminifera group was observed and a total of 15 genera and 30 species were identified. Color changes were observed in *Ammonia compacta*. The changes in the foraminifer shells were assessed by examining the concentrations of toxic elements at the levels where morphological changes occurred in the foraminifer shells. The colour changes seen in the dirty zones were more common, especially in *Ammonia compacta*. Foraminifer shells were found to have different shades, ranging from yellowish brown to black. In order to determine the causes of these discolorations, shells were subjected to surface element analysis in an SEM. In places where the S values were high, dark grey-black colors were dominant. At places where the Fe and Mn values were high, the yellow-yellowish brown color was dominant. In areas where heavy metal concentrations are high, PI values are higher than critical values (PI = 1) and are defined as dirty areas. The number of genera, species and individuals of foraminifera is quite low in the places where pollution is high. It is thought that the low number of foraminifera samples in samples taken from Küçükçekmece, Büyükçekmece, Ambarlı and Avcılar regions are due to ship traffic and discharge of domestic and industrial wastewater into the sea.

This study, scientifically compared with the studies conducted in the West Marmara Sea (Yümün, 2017) and in the Gemlik region (Meriç et al., 2009; Yümün et al., 2021). In all three studies, it is seen that the pollution increases at the ship roads, ports and at the points where the streams carrying industrial wastes flow into the sea. However, heavy metal pollution caused by agricultural activities and geological formations creates differences according to the agricultural and geological structure of each region.

In future studies, a comparison should be made with the data of this study and previous studies. In this way, time-dependent pollution changes on the sea floor will be followed over time. In addition to these, an evaluation should be made by taking samples from the rivers pouring into the sea from the land.

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ENDOPHYTIC INFECTION PROGRAMS THE ASCORBATE-GLUTATHIONE CYCLE IN RICE (*ORYZA SATIVA* L.) UNDER Na_2CO_3 STRESS

REN, X. N. – SHAN, Y. – LI, X. – WANG, L. L. – LI, Y. Y. – MA, L. J. – LI, X. M.*

College of Life Science, Shenyang Normal University, No. 253 Huanghe North Street, Shenyang, Liaoning 110034, China

**Corresponding author*
e-mail: lxmls132@163.com; phone: +86-024-8657-8977; fax: +86-024-8659-2548

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Abstract. Soil salinization and alkalization have been identified as one of the principal causes of impaired productivity of rice plants worldwide. Endophytes reprogram plant metabolism and elicit beneficial effects on rice growth under salt-alkali stress. This work investigates the potential effects of endophytic infection on the key players in specifically the metabolism, ascorbate-glutathione cycle, in rice seedlings (*Oryza sativa* L.) under Na_2CO_3 stress. Compared to uninfected controls, endophytic infection significantly increased the activities of glutathione reductase and ascorbate peroxidase, the contents of total ascorbic acid, reduced ascorbic acid, total glutathione and reduced glutathione, and the ratios of reduced ascorbic acid/dehydroascorbic acid and reduced glutathione/oxidized glutathione, but decreased the dehydroascorbic acid and oxidized glutathione contents in rice seedlings under Na_2CO_3 stress. In addition, H_2O_2 and proline contents in rice seedlings were significantly decreased by endophytic infection. Taken together, endophytic infection holds great promise to improve the resistance to Na_2CO_3 stress for rice plants and the underlying mechanisms involves modulation of the metabolic intensity of ascorbate-glutathione cycle.
Keywords: *oxidative stress, endophytic fungus, salt-alkali stress, proline*

Introduction

Soil salinization and alkalization have emerged as a significant problem for about 932 million hectares of land worldwide and 100 million hectares in Asia (Wang et al., 2018). The phenomenon of soil salinity and alkalinity often appears spontaneously in nature (Chuamnakthong et al., 2019), and significantly suppresses plant growth leading to a substantial reduction of crop production (Zhang et al., 2017; Wang et al., 2018). Therefore, it is imperative to develop more effective approaches to improve the salt-alkali stress tolerance of plants.

Salt-alkali stress generally correlates with diverse pathophysiological processes such as physiological aridity, ion toxicity, pH homeostasis as well as ionic balance of intracellular in stressed plants (Zhang et al., 2020). In addition to the well-identified processes, there is a growing recognition that the salt-alkali stress often triggers abnormal generation of reactive oxygen species (ROS) in plants. Under favorable conditions, ROS at basal levels performs very essential physiological functions in multiple cell processes (Goraya and Asthir, 2016). However, plants under the salt-alkali stress undergo excessive ROS, which has shown to be detrimental to cell survival and might cause impaired plant growth or even eventual death (Gill and Tuteja, 2010; Koffler et al., 2015). These insights imply that ROS scavenging is critical to increase plant tolerance to salt-alkali stress.

Plants utilize the intrinsic antioxidant defense mechanisms to encounter aberrant ROS (Imahori et al., 2016). Increased activities of antioxidant enzymes have been found in

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plants suffering from the salt-alkali stress, which significantly minimize the untoward effects of excessive ROS on plants (Kasim et al., 2012; Mishra et al., 2013; Chumyam et al., 2017). Indeed, multiple antioxidant metabolic pathways act pivotal parts in the adaptation of plants to environmental stresses. Specifically, the ascorbate-glutathione (AsA-GSH) cycle is well identified to represent the most critical player of antioxidant metabolic systems which can scavenge abnormal ROS (Chumyam et al., 2017). This cycle strongly correlates with the anti-oxidative defense mechanism, and its metabolism intensity has a direct association with the plant tolerance (Ma et al., 2008).

Rice represents an important source of food globally. It exhibits poor salt-alkali tolerance especially in the early seedling period (Li et al., 2014; Sun et al., 2019). As such, soil salinization and alkalization emerge as one of the most common abiotic stress for rice plants, which have largely limited its productivity (Rao et al., 2013; Liang et al., 2014). Our previous studies have established that endophytic infection served as an effective biological tool to improve plant tolerance to the salt-alkali stress, and that multiple key functions were involved in the beneficial effects of endophytes on rice seedlings under Na_2CO_3 stress, such as improved nutrient uptake, photosynthesis and organic acid accumulation (Bu et al., 2012; Li et al., 2017). Since the AsA-GSH cycle has been well identified as the key players of ROS scavenging systems, the aim of our study was to explore whether endophyte-induced benefits involve the AsA-GSH cycle in rice seedlings under salt-alkali stress. This work documented that endophytic infection could modulate AsA-GSH cycle, thereby improving the resistance to salt-alkali stress in rice seedlings. The present results provide an encouraging tool to alleviate Na_2CO_3 stress in salt-alkali sensitive plants.

Materials and methods

All experiments in this study were performed in the Laboratory of Biochemistry and Molecular Biology, School of Life Sciences, Shenyang Normal University, China. Furthermore, all the measurements were determined by UV-visible spectrophotometer (UV-6000PC, Shanghai, China).

Preparation of microorganisms and rice seedlings

The endophytic fungus EF0801 from *Suaeda salsa* that is congeneric to *Sordariomycetes* sp. (99% similarity), was obtained and prepared as our previous studies (Bu et al., 2012; Li et al., 2017). Briefly, EF0801 endophytic strain was cultured at 24 ± 1°C in a 150-mL shaker flask at 180 rpm for 12 days. The fermentation broth generated from this procedure was employed for the treatments of rice seedlings.

Rice seeds (*Oryza sativa* L.) were subjected to sterilization with 2.65% sodium hypochlorite for 10 min, thorough rinse with distilled water and then germination. Following transferring to a 500-mL beaker which contained Hoagland solution, the germinating seeds (100 grains) were cultivated in the growth chamber (80% relative humidity, 16 h/8 h light/dark period, 28°C /26°C day/night, and 3000 lux). After 3 days of growth, rice seedlings were subjected to the treatments of Na_2CO_3 and endophytic infection.

Rice seedlings were randomly assigned into 2 groups: 1) endophyte-uninfected seedlings (E-) and 2) endophyte-infected ones (E+) inoculated with 5% fermentation broth. The seedlings of each group were grown in Hoagland solution containing the following concentrations of Na_2CO_3: 0 mM, 5 mM, 10 mM, 15 mM or 20 mM. In E+

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groups, the endophyte which colonized in the rice seedling roots was more than 90%, but none of colonization in E- groups. Diluted Hoagland solution was replenished to each beaker every day. For further analysis, the seedlings were collected and sampled on the 6th day of the treatments.

Determination of the activities of antioxidant enzymes

Fresh leaves (0.3 g) were homogenized with 50 mM phosphate buffer (pH 7.5, 3 mL) which containing 0.1 mM EDTA and TritonX-100 and 4% (w/v) polyvinylpyrrolidone (PVP-40). Following a 25 min centrifugation at 13,000 × g at 4°C, the supernatants were harvested to analyze the activities of glutathione reductase (GR) and ascorbate peroxidase (APX).

GR activity was monitored at 340 nm in reaction mixture (3 mL) containing 0.1 M Tris-HCl (pH 8.0), 1 mM oxidized glutathione (GSSG), 0.1 mL supernatant and 0.2 mM NADPH. The reaction was initiated by adding NADPH.

APX activity was determined by monitoring the decrease in absorbance at 290 nm. The assay mixture (3 mL) contained 50 mM Hepes-KOH (pH 7.6), 1.0 mM H_2O_2, 0.5 mM reduced AsA and 0.05 mL supernatant. The reaction was initiated by adding H_2O_2.

Determination of the antioxidant contents

Fresh leaves (0.4 g) were homogenized in a pre-cooled mortar and pestle containing ice-cold 5% TCA (4 mL). After a 20 min centrifugation at 13,000 × g at 4°C, the supernatants were harvested for analysing the contents of total ascorbic acid (TAsA), reduced ascorbic acid (AsA), total glutathione (TGSH) and reduced glutathione (GSH).

To determine the TAsA content, 1 mL supernatant was mixed with the reaction solution with the addition of 0.1 M phosphate buffer (pH 7.7, 0.25 mL) and 2 mM dithiothreitol (0.25 mL). After 10 min reaction at room temperature, 10% TCA solution (0.4 mL), 44% phosphoric acid (0.4 mL), 4% 2,2-bipyridine (prepared in 70% ethanol, 0.4 mL) and 3% Fe_2Cl_3 (0.2 mL) were mixed with the reaction solution. After 60 min incubation at 37°C, the absorbance was determined at 525 nm.

To quantify the AsA content, 1 mL supernatant was mixed with 0.1 M phosphate buffer (pH 7.7, 0.5 mL). Following 30 s reaction at room temperature, the next procedure was performed as described in the assays of TAsA content.

To quantify the TGSH content, 1 mL supernatant was mixed with 1 U GR, 0.1 M phosphate buffer (pH 7.7, 0.5 mL), 0.15 mM NADPH (1 mL). After 2 min reaction at room temperature, 0.6 mM DTNB (0.6 mL) was mixed with the reaction buffer. After 5 min incubation in a constant temperature bath (37°C), the absorbance was determined at 412 nm.

To quantify the GSH content, 1 mL supernatant was mixed with 0.1 M phosphate buffer (pH 7.7, 2.5 mL) and 0.6 mM DTNB (0.6 mL). After 5 min incubation in a constant temperature bath (37°C), the absorbance was then measured at 412 nm.

Determination of H_2O_2 content

Fresh leaves (0.5 g) were homogenized with 3 mL acetone. After centrifugation at 13,000 × g for 20 min at 4°C, the supernatants were harvested. Afterwards, 5% sulfuric acid (0.1 mL) and ammonia water (0.2 mL) were added to the 1 mL enzyme solution. Centrifugated at 4000 r/min for 10 min, the precipitation was harvested and washed with

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precooled acetone for 2-3 times. Following dissolution by 2 M sulfuric acid (4 mL), the H_2O_2 content in the precipitation was monitored at 520 nm.

Estimation of proline content

Leaves were (0.5 g) homogenized with 3% sulfosalicylic acid (5 mL), and then heated at 100°C for 10 min. After centrifugation at 3,000 × g for 15 min, the supernatant (2 mL) was mixed with distilled water (2 mL), acetic acid (2 mL) and acid ninhydrin (4 mL), boiled for 1 h and the reaction was stopped by cooling the tubes in ice bath for 5 min. The chromophore formed was extracted with toluene by vigorous shaking. Absorbance of the resulting organic layer was measured at 520 nm by spectrophotometer.

Statistical analysis

In this study, each experiment was independently repeated three times and results from the duplicate experiments were combined for statistical analysis. The data were shown as mean ± standard deviation (SD) from three independent experiments. The statistical analysis between the two groups were performed by two tailed Student's t-test or Mann-Whitney test (Czobor et al., 2017). The statistical difference between multiple groups were conducted using one-way ANOVA or Kruskal-Wallis test (Cheng et al., 2020; Liu and Wang, 2021). All statistical analysis was performed using GraphPad Prism (Version 5) and SPSS 16.0. and an alpha of 0.05 was employed for all tests.

Results

Variations in the growth of rice seedling

Na_2CO_3 stress elicited an obvious growth inhibition of endophyte-uninfected rice seedlings in a concentration-dependent manner. In contrast, endophytic infection effectively blocked the inhibition of growth of rice seedlings under Na_2CO_3 (*Fig. 1*).

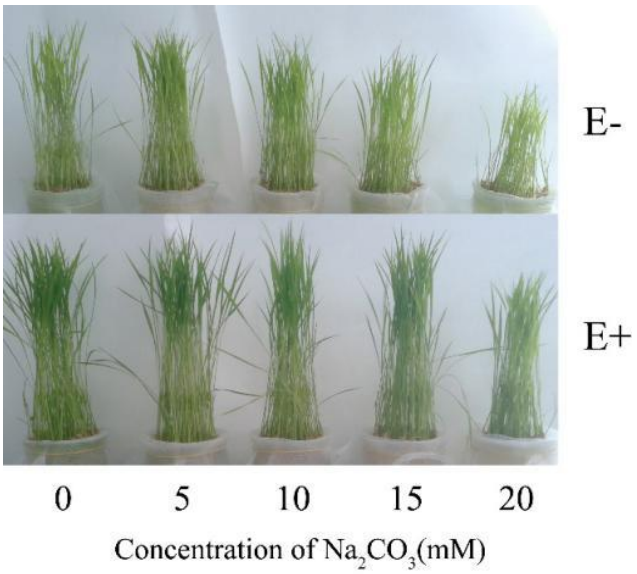

Figure 1. *Variations in the growth of endophyte-uninfected (E-) and -infected (E+) rice seedlings under Na_2CO_3 treatment*

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Variations in the activities of antioxidant enzymes

GR activity in the leaves of endophyte-infected and -uninfected rice seedlings first increased and then decreased with the increasing concentrations of Na_2CO_3 (*Fig. 2a*). When there was no Na_2CO_3 stress, endophytic infection significantly increased the GR activities in the rice seedlings. Rice seedlings with endophytic infection exhibited an obvious increase of GR activity under 5, 10 and 15 mM Na_2CO_3 stress in comparison with uninfected controls, whereas no significant influence under 20 mM Na_2CO_3 stress.

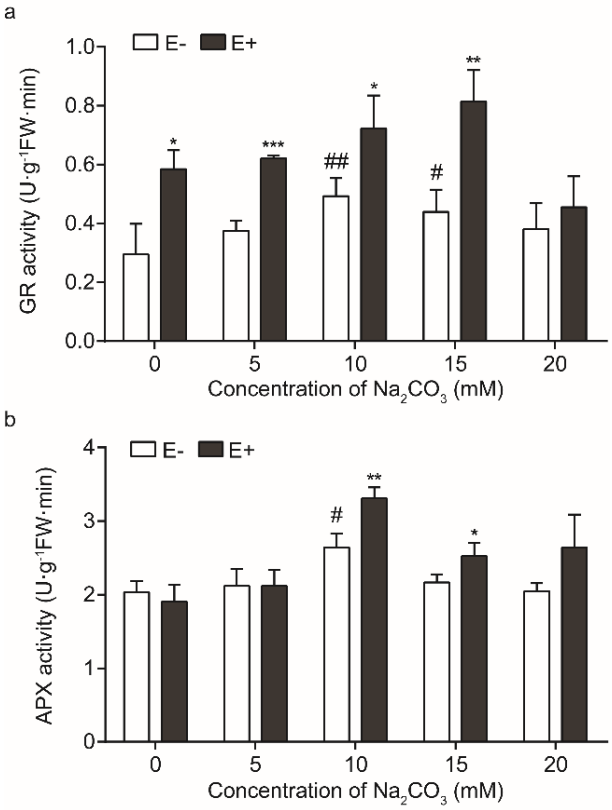

Figure 2. *Variations in the activity of antioxidant enzymes in the AsA-GSH cycle of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ *compared with E- 0 mM group by one-way ANOVA (a, b);* $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *and* $^{***}P \leq 0.001$ *compared with E- group by two-tailed Student's t-test*

APX activity showed a 30.1% increase upon 10 mM Na_2CO_3 stress in comparison with unstressed controls, while endophytic infection had little effects on APX activity in rice seedlings without Na_2CO_3 stress (*Fig. 2b*). Endophytic infection significantly increased APX activities under 10 and 15 mM Na_2CO_3 stress.

Variations in the contents of antioxidants

The effects of Na_2CO_3 stress on the contents of TAsA, TGSH and AsA exhibited a similar pattern (*Fig. 3a, b and c*). The contents of TAsA, AsA and TGSH in the leaves of both endophyte-infected and -uninfected seedlings firstly increased and then declined with the increasing concentrations of Na_2CO_3. Under no Na_2CO_3 stress, infection with the endophyte markedly increased the contents of AsA and TGSH, while TAsA content

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exhibited no significant change. Endophytic infection resulted in a marked increase in AsA and TGSH contents under Na_2CO_3 stress (except AsA under 15 mM Na_2CO_3), as well as the content of TAsA under 5 and 10 mM Na_2CO_3 stress.

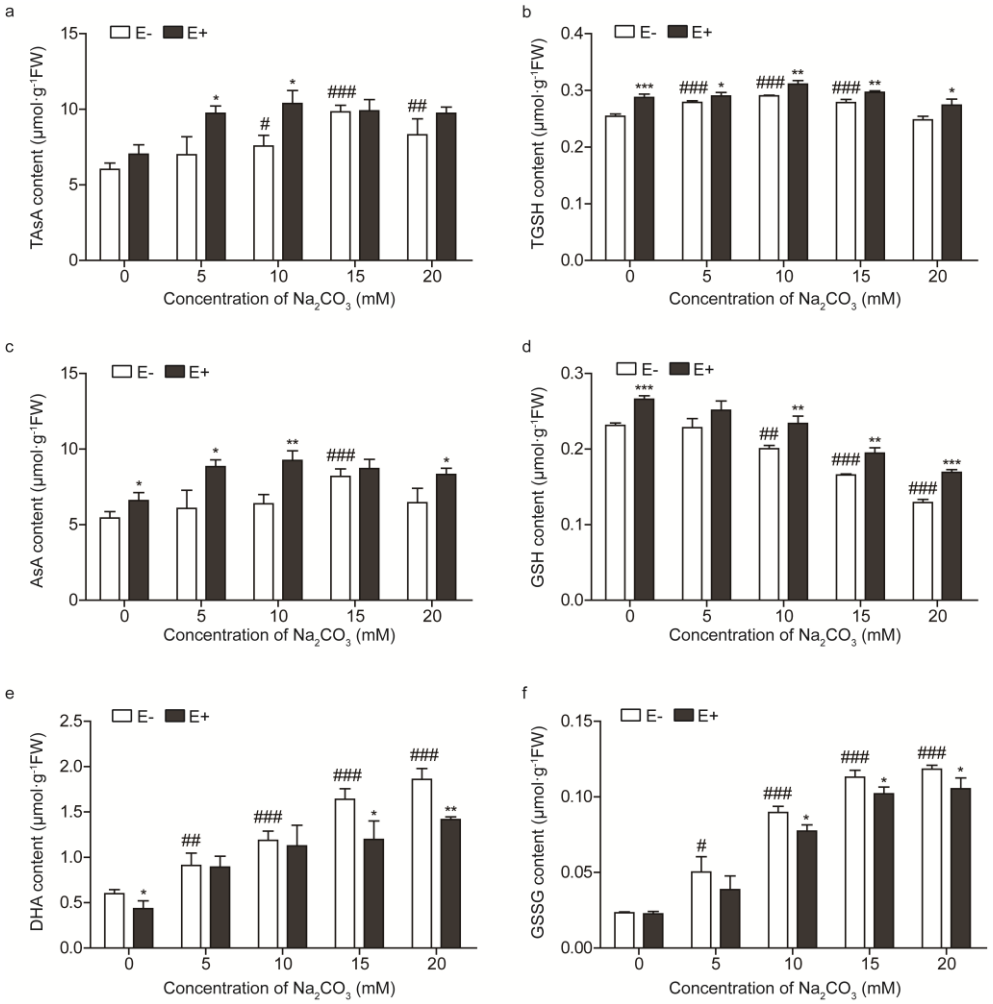

Figure 3. *Variations in the content of antioxidants in the AsA-GSH cycle of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ *and* $^{\#\#\#}P \leq 0.001$ *compared with E- 0 mM group by one-way ANOVA (a, b, c, e) or Kruskal-wallis test (d, f);* $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *and* $^{***}P \leq 0.001$ *compared with E- group by -two-tailed Student's t-test*

The change pattern of the contents of DHA and GSSG under Na_2CO_3 stress was opposite to that of GSH content (*Fig. 3d, e and f*). In both endophyte-infected and -uninfected seedlings, DHA and GSSG contents substantially increased with the increasing concentrations of Na_2CO_3, while GSH content decreased (except under 5 mM Na_2CO_3). Endophyte-infected seedlings displayed a marked increase in GSH contents under 0, 10, 15 and 20 mM Na_2CO_3 stress. When there was no Na_2CO_3 stress, DHA content was decreased by 23.9% with endophytic infection, but no significant difference for GSSG contents were found. Moreover, endophytic infection led to an obvious decrease of DHA contents under 15 and 20 mM Na_2CO_3 stress, as well as the GSSG contents under 10, 15 and 20 mM Na_2CO_3 stress.

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In both endophyte-infected and -uninfected seedlings, AsA/DHA ratio significantly decreased with the increasing concentrations of Na_2CO_3 (*Fig. 4a*). Endophytic infection significantly increased AsA/DHA ratio under 0, 10, 15 and 20 mM Na_2CO_3 stress. A similar pattern was observed for GSH/GSSG ratio (*Fig. 4b)*. Furthermore, endophytic infection resulted in a marked increase in GSH/GSSG ratio under 10, 15 and 20 mM Na_2CO_3 stress.

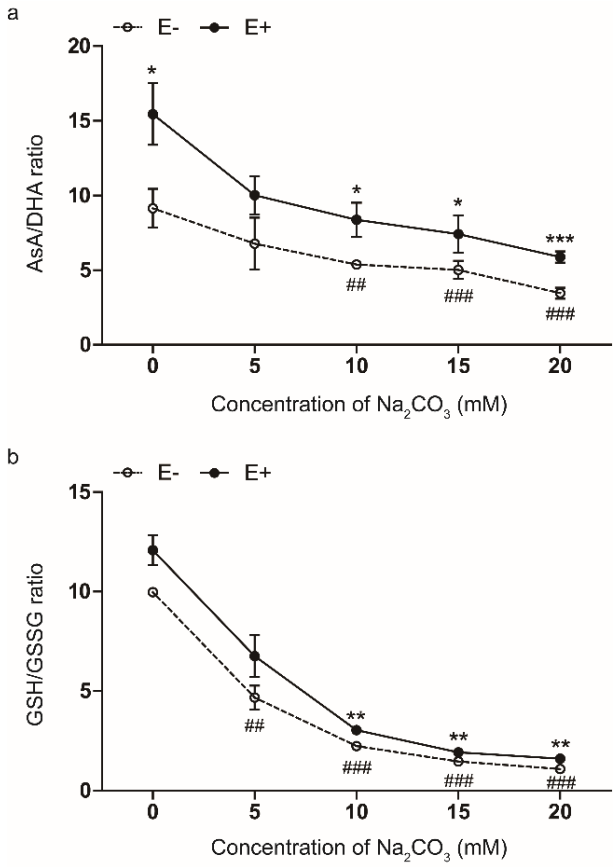

Figure 4. *Variations in the ratios of AsA/DHA and GSH/GSSG of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{\#\#}P \leq 0.01$ *and* $^{\#\#\#}P \leq 0.001$ *compared with E- 0 mM group by Kruskal-wallis test (a, b);* $^{*}P \leq 0.05$，$^{**}P \leq 0.01$ *and* $^{***}P \leq 0.001$ *compared with E- group by two-tailed Student's t-test*

Taken together, these findings indicated that the benefit of endophytic infection to plant Na_2CO_3 resistance strongly correlated with the increased metabolic intensity of antioxidant systems (AsA-GSH cycle) (*Fig. 5*).

Variations of H_2O_2 content

The contents of H_2O_2 increased under Na_2CO_3 stress in a concentration-dependent manner in the leaves of both endophyte-infected and -uninfected rice seedlings (*Fig. 6*). Crucially, endophytic infection markedly decreased H_2O_2 contents under 10 and 15 mM Na_2CO_3 stress.

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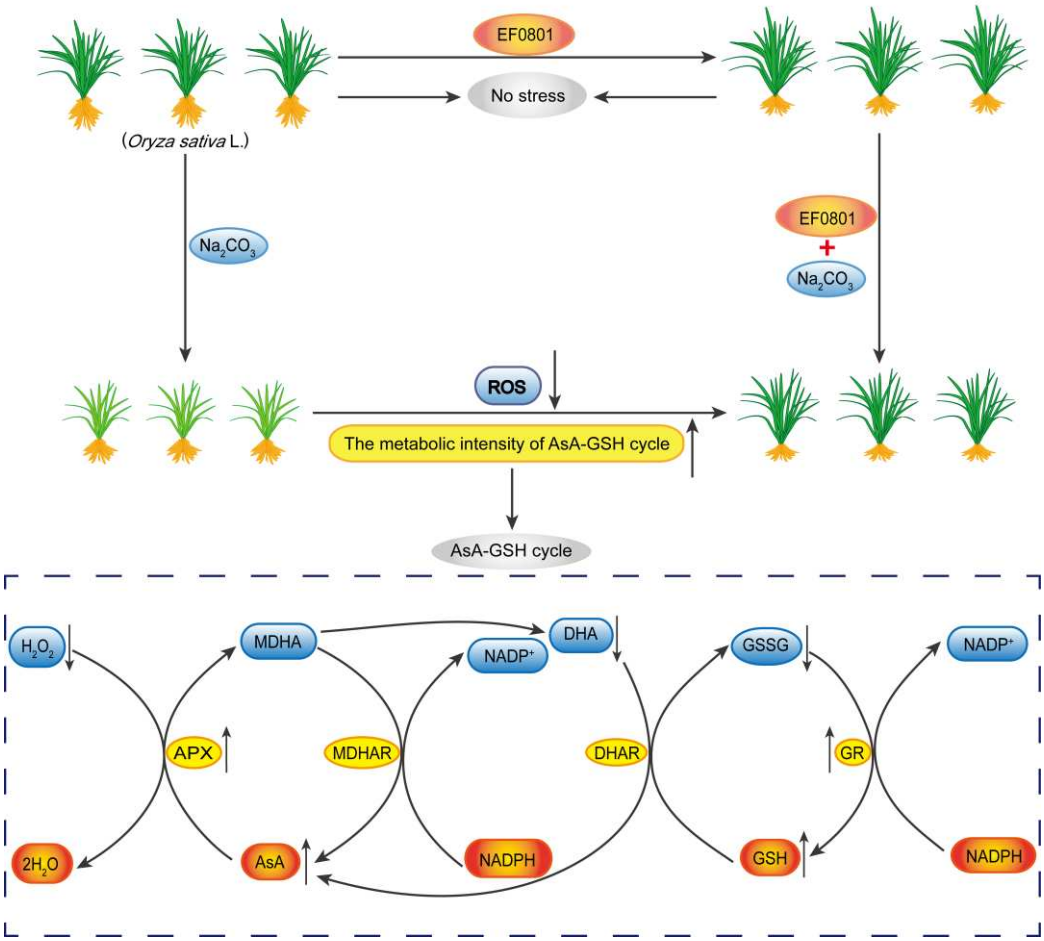

Figure 5. *Proposed model. ROS, Reactive oxygen species; AsA-GSH, Ascorbate-glutathione; H_2O_2, hydrogen peroxide; MDHA, Monodehydroascorbate; DHA, Dehydroascorbic acid; GSSG, Oxidized glutathione; APX, Ascorbate peroxidase; MDHAR, Monodehydroascorbate reductase; DHAR, Dehydroascorbate reductase; GR, Glutathione reductase; AsA, Reduced ascorbic acid; GSH, Reduced glutathione*

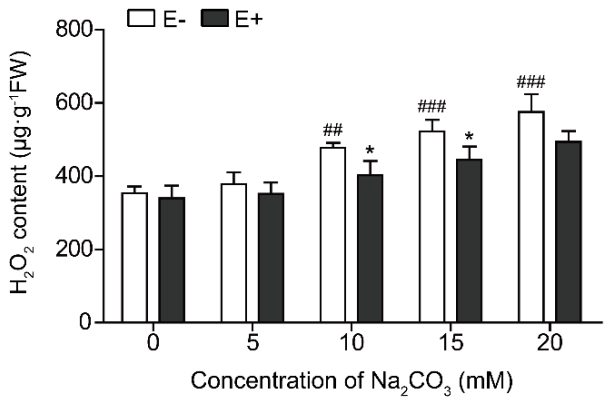

Figure 6. *Variations in the content of H_2O_2 of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3). $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ compared with E- 0 mM group by one-way ANOVA; $^{*}P \leq 0.05$ compared with E- group by two-tailed Student's t-test*

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Variations of proline content

With the increasing concentrations of Na_2CO_3, proline content in both endophyte-infected and -uninfected rice seedlings significantly increased (*Fig. 7*). Endophytic infection significantly decreased the proline content under 10 and 20 mM Na_2CO_3 stress. Thus, we concluded that modulation of proline accumulation seemed to represent another possible defense mechanism of endophyte for the plants against salinity.

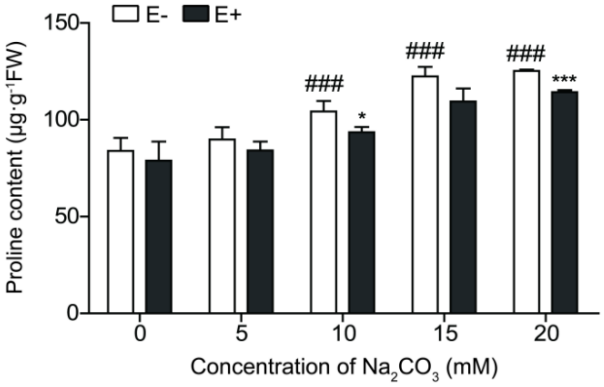

Figure 7. *Variations in the content of proline of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{###}P \leq 0.001$ *compared with E- 0 mM group by one-way ANOVA;* $^{*}P \leq 0.05$ *and* $^{***}P \leq 0.001$ *compared with E- group by two-tailed Student's t-test*

Discussion

Salt-alkali represents a main abiotic stress and largely limits crop production worldwide (Zhang et al., 2020). Therefore, designing optimal strategies to enhance plant resistance has been the subject of intense investigation. Our previous studies (Bu et al., 2012; Li et al., 2017) have demonstrated that Na_2CO_3 stress severely inhibited rice growth as evidenced by decreased shoot/root length and dry weight. Crucially, infection with endophyte EF0801 strain in rice seedlings significantly alleviated the growth-inhibition induced by Na_2CO_3 stress. In the present study, we further demonstrated that endophytic infection significantly raised the metabolic power of anti-oxidative defense systems by modulation of AsA-GSH cycle, thereby improving the resistance of rice seedlings to salt-alkali stress (*Fig. 5*).

Endophyte emerges as a fungus or bacterium which frequently lives within plants in their native environments (Gladieux, 2018). Analysis of endophyte-host interactions suggested that fungal endophytes could offer superior advantages of plant growth promotion and stress homeostasis regulation to host plants especially in stress conditions (Mirzahossini et al., 2015). In agreement, endophytic infection in plants had significant capacity to enhance host resistance to multiple stress environments, such as heat (Ismail et al., 2018), drought and salt (Moghaddam et al., 2021), nickel (Mirzahossini et al., 2015) or zinc (Li et al., 2012) or cadmium (Zhang et al., 2010; Ma et al., 2019). Our present findings further demonstrated that endophyte EF0801 strain was able to confer effective resistance to salt-alkali stress.

Excessive ROS represents a frequent event in plants under the salt-alkali stress. The present study demonstrated that Na_2CO_3 stress induced a significant increase of H_2O_2 content in rice seedlings. The anti-oxidative defense system has the abilities to mitigate

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unfavorable impacts of abnormal ROS on plants themselves. The functional activities of antioxidant enzymes GR and APX often serve as credible indicators of the power of anti-oxidative defense systems in plant cells (Ma et al., 2008). Aravind and Prasad (2005) found that the increased activities of GR and APX exerted protective roles in combating Cadmium-induced oxidative stress. Baltruschat et al. (2008) demonstrated that the significant enhancement of APX and GR activities was involved in *P. indica*-induced salt tolerance in roots of salt-stressed barley. In accord with this, our present results showed that increased activities of GR and APX were observed in rice seedlings under Na_2CO_3 stress. After endophyte infection, the activities of GR and APX were further increased. In addition, endophyte-induced tolerance to the salt-alkali stress in rice seedlings might involve modulation of the contents of TAsA, AsA, DHA, TGSH, GSH and GSSG, the main components in the AsA-GSH cycle. Since the ASA-GSH cycle represents key player in ROS scavenging systems, the endophyte-induced increase of metabolic intensity of this cycle may contribute to greater protection from oxidative injury caused by salt-alkali stress.

The AsA/DHA ratio is considered as a well-recognized index to assess the levels of AsA (Li et al., 2010). Higher AsA/DHA ratio generally indicate higher AsA contents, and correlate with the preventation of oxidative injury in plants (Mishra et al., 2013). The increased contents of AsA could effectively reduce ROS induced by salt stress, thereby protecting plants from oxidative attacks (Hasanuzzaman et al., 2014). Shalata et al. (2001) demonstrated that salt-sensitive tomato displayed a significant decrease of the reduced AsA content and an increase of DHA content in response to salt-stress, whereas the salt-resistant plants exhibited the opposite pattern. Furthermore, Baltruschat et al. (2008) demonstrated that salt stress decreased AsA/DHA ratio in endophyte-uninfected barley, while endophytic infection significantly enhanced the ratio of AsA/DHA in plants under saline exposure. In agreement, we observed that Na_2CO_3 stress led to a significant decrease of AsA/DHA ratio, which were effectively restored by endophytic infection in rice seedlings. Na_2CO_3 increased contents of AsA and DHA, but endophyte infection further increased the contents of AsA and decreased the DHA contents.

GSH is shown to be converted from GSSG which could increase the tolerance of plant cells to stress environments (Ma et al., 2019). Aravind and Prasad (2005) reported that Cd-10 μM stress resulted in a decrease of GSH, as well as an increase of GSSG in *Ceratophyllum demersum*. Our results showed that rice plants displayed the similar changes of GSH and GSSG in response to Na_2CO_3 stress. GSH/GSSG ratio represents a crucial index weighing the AsA-GSH cycle metabolism intensity, and has a direct relation with plant tolerance. Selote and Khanna-Chopra (2006) demonstrated that drought stress led to a significant decrease in GSH/GSSG ratio of wheat leaves. Likewise, we found that Na_2CO_3 stress significantly reduced GSH/GSSG ratio of rice leaves. Endophytic infection induced a significant increase of GSH but a decrease of GSSG, thereby effectively restoring GSH/GSSG ratio.

As the most water-soluble amino acid, proline has been shown to accumulate in plants under abiotic stress. However, the physiological significance of proline accumulation still remains a controversial subject. Lutts et al. (1999) found that lower levels of free proline were accumulated in salt-resistant cultivars than salt-sensitive ones in response to salt stress. Furthermore, Garcia et al. (1997) demonstrated that exogenous administration of proline led to exacerbated damages induced by the salt. In the present study, we found that Na_2CO_3 stress induced significant accumulation of proline in rice seedlings, and that endophytic infection led to an obvious reduction of proline contents. Our findings support

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the notion that the accumulation of proline serves as an indicator for stress damage, rather than a component of salt tolerance. Modulation of proline accumulation may represent another possible defense mechanism of endophyte for the plants against Na_2CO_3 stress.

Conclusion

Based on the present study, it is concluded that endophytic infection with the EF0801 strain was capable to protect rice seedlings from Na_2CO_3 stress, and that the benefits of endophyte to the resistance to Na_2CO_3 stress strongly correlated with the increase of the metabolic intensity of AsA-GSH cycle. Yet the molecular basis mediating the effects of endophytic infection on the AsA-GSH cycle requires future efforts. Once its roles and related mechanisms are well evaluated, the EF0801 endophyte will provide new perspectives for alleviating Na_2CO_3 stress in plants with high sensitivity to salt-alkali.

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RESPONSES OF BARLEY SEEDLINGS TO SALINITY AND DROUGHT UNDER FREEZE-THAW CONDITIONS

TANG, W.[1] – BAO, G.[1*] – YAN, B.[2] – QU, Y.[1] – GUO, J.[1] – ZHU, S.[1] – ZHAO, H.[3]

[1]*Key Laboratory of Groundwater Resources and Environment of the Ministry of Education, Jilin Provincial Key Laboratory of Water Resources and Environment, College of New Energy and Environment, Jilin University, Changchun 130012, China*

[2]*Environmental Monitoring Center Station of Jilin Province, Changchun 130011, China*

[3]*The Administration of Jingyu Water Conservation, Jingyu 135200, Jilin Province, China*

[*]*Corresponding author*
e-mail: baogz@jlu.edu.cn; fax: +86-0431-8850-2606

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Abstract. The Qinghai-Tibet Plateau is known for its high altitude, low rainfall and varying temperature, and the crops in this area are susceptible to abiotic stresses induced by drought, salinity and freeze-thaw conditions that cause damages to different properties such as the permeability of biological membrane, osmotic adjustment, and antioxidant enzyme system. Barley (*Hordeum vulgare* L.) is an indispensable crop on the plateau and plays an important role in agricultural ecosystem as well. In this study, Beiqing 3 was used as experimental material and physiological characteristics, including soluble protein (SP) content, malondialdehyde (MDA) content, antioxidant enzyme activity and relative water content (RWC) of seedlings were examined under freeze-thaw conditions combined with drought and alkali stress. Research results indicated that under the combined stresses of salinity and drought, barley seedlings were damaged by lipid peroxidation, weakened superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities, while osmotic adjustment ability in plants cell was enhanced. We suggested that, in agricultural management, the simultaneous occurrence of two stresses, salinity and drought, should be avoided in the early stage of barley planting to reduce the physiological stress on plants.
Keywords: *abiotic stresses, antioxidant enzyme activity, soluble protein, physiological effects, malondialdehyde*

Introduction

Lying in the Southern Qinghai-Tibet Plateau, the Brahmaputra Valley, where altitude varies from 2600 m to 3500 m, was known for the complex and changeable terrain, forming a unique plateau climate characteristic (Zhang et al., 2019). The fact that, during early spring, the freeze-thaw often occurs due to the long sunshine hours and the changeable temperature between day and night has various effects on the external morphology and internal physiological metabolism of plants (Arfan et al., 2019; Wang et al., 2019; Xu et al., 2019). It has been reported that the average precipitation of barley planting region is less than 400 mm, besides, 90% of the annual precipitation occurs in June to September, which tends to cause spring drought and affect spring sowing and early crop growth (Hou et al., 2018). The seasonal uneven precipitation and the exceeding evaporation on the Brahmaputra Valley make drought stress prone to occur during crop growth (Bibi et al., 2019), which often causes oxidative stress by the accumulation of reactive oxygen species in plants (Souza et al., 2004), affecting the structure and growth of plant (Duan et al., 2007). Attributing to current climate, the content of sodium bicarbonate ($NaHCO_3$) and sodium chloride (NaCl) in soil has increased on the

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Brahmaputra Valley, where salinization becomes increasingly serious. It has been confirmed that alkali stress imposed more harm to crops than salt stress does (Alvarez-Acosta et al., 2019). Besides, one paper has previously reported that high pH of alkali stress can short root length and seedlings height of rye, reduce the content of water and chlorophyll and decrease the relative transpiration rate (Guo et al., 2012).

Barley, a cereal crop of the genus Gramineae, can grow at an altitude of 3000 ~ 3400 m. Among them, Beiqing 3 has good resistance to cold and drought (Ahmed et al., 2015; He et al., 2015), is the main crop in Tibet and Qinghai. In this experiment, as materials, the Beiqing 3 seedlings were treated with salinity, drought and freeze-thaw stress to artificially simulate the growing environment of plants. The relative water content (RWC), antioxidant enzyme activity, contents of malondialdehyde (MDA) and soluble protein (SP) were examined in order to study the response characteristics of plants to drought, salinity and freeze-thaw.

Materials and methods

Seeds cultivation and salinity treatment

The study was taken out in Northeast China. The full-grained seeds were selected and soaked with 0.1% $KMnO_4$ solution for 2 h for disinfection, after which the seeds were rinsed with deionized water until the water becoming clear, then we spread 120 seeds evenly on each of 8 culture dishes randomly named FSD, FS, FD, F, SD, S, D and C (*Table 1*). 1/2 of Hoagland nutrient solution was used to prepare 60 m*M* $NaHCO_3$ mixed solution (pH = 8.06), 500 ml of which was added to the cultivated dishes of FSD, FS, SD and S at the same time, 500 ml 1/2 Hoagland nutrient solution was added to the others (FD, F, D and C). 8 dishes of seeds were placed in MGC-450BP light incubator (Shanghai Yiheng Scientific Instruments Co., Ltd) for germination (*Fig. 1a*), of which the cultivated conditions were set as 12 h light (25 ℃) and 12 h non-light (15 ℃). Daily watering (50 ml) was necessary during the cultivation.

Table 1. *Experimental design of groups under salinity (S), drought (D) and freeze-thaw (F) stress*

	FSD	FS	FD	F	SD	S	D	C
Salinity	+	+	-	-	+	+	-	-
Drought	+	-	+	-	+	-	+	-
Freeze-thaw	+	+	+	+	-	-	-	-

+ add stress, - no stress

Drought treatment

After seedlings were cultivated to 15 cm high with 2 or 3 leaves (around 1 week), they were treated with drought stress. $NaHCO_3$ mixed solution was used to prepare 20% PEG-6000 mixed solution for combined treatment of salinity and drought stress, and 1/2 Hoagland nutrient solution was used to prepare 20% PEG-6000 solution. The solution in the cultivated dishes of Group FSD and Group SD was replaced with 500 ml PEG-$NaHCO_3$ mixed solution, in the cultivated dishes of Group FD and Group D replaced with 500 ml PEG solution, in the cultivated dishes of Group FS and Group S

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replaced with 500 ml $NaHCO_3$ solution, in the cultivated dishes of Group F and Group C replaced with 500 ml 1/2 Hoagland nutrient solution. The drought treatment lasted for 48 h without watering.

Figure 1. *Photos of experimental equipment. (a): MGC-450BP light incubator (Shanghai Yiheng Scientific Instruments Co., Ltd). (b): BPHJ-120A high-low-temperature test chamber (Shanghai Yiheng Scientific Instruments Co., Ltd)*

Freezing and thawing stress treatment and sampling

After drought treatment, the cultivated dishes of Groups FSD, FS, FD and F were put into BPHJ-120A high-low-temperature test chamber (Shanghai Yiheng Scientific Instruments Co., Ltd) to carry out a freeze-thaw cycle for a period of 14 h (*Fig. 1b*), with the temperature curve being set as 15, 10, 5, 0, − 5, 0, 5 and 10 ℃, while other cultivated dishes of Groups SD, S, D and C were maintained in light incubator under previous culture conditions (*Fig. 2a*). Initially, the cultivated dishes were placed in the chamber at 15 ℃ that closed to room temperature at night. Controlled precisely by program, the temperature decreased to − 5 ℃ steadily at a speed around 0.04 ℃/min, and then the temperature increased from − 5 to 10 ℃ at a speed around 0.04 ℃/min. After the freeze-thaw cycle being started, five parallel samples were taken every 2 hours from 8 cultivated dishes at random according to the required amount of the measurement, the corresponding sampling temperature was 10, 5, 0, − 5, 0, 5, 10 ℃ respectively (Gong et al., 2020). All the samples were firstly wrapped up with tin foil paper, secondly fixed in liquid nitrogen immediately for 50 s and finally put into the ultra-low-temperature freezer at − 80 ℃ for storage in order to measure the content of MDA and soluble protein, SOD, POD and CAT activity. At the same time, fresh leaves were taken to determinate RWC.

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Figure 2. *Photos of experimental culture and equipment. (a): 9-day barley seedlings in light incubator. (b): UV-6100 UV–visible spectrophotometer (Metash Co. Ltd)*

Analysis

Relative water content (RWC)

The relative water content of seedlings was determined by the oven drying method (Colom and Vazzana, 2001). For each sample (around 0.1 g), fresh weight supposed to be measured and recorded as F_W after drying the surface of leaves with filter paper. Completely being immersed in distilled water until the weight of leaves being constant, the leaves were taken out and wiped up with filter paper. The saturated fresh weight of the leaves at this time was measured and recorded as the T_W. Finally, the leaves were de-enzymed for 15 min in oven that was heated up to 105 ℃, and then dried to a constant weight in 80 ℃. The dry weight was measured and recorded as D_W. The RWC of leaves is calculated by formula *Eq.1*:

$$RWC = (T_w - D_W)/(F_W - D_W) \times 100\% \quad \text{(Eq.1)}$$

Soluble protein (SP) content

The soluble protein content in seedlings was determined by the Coomassie brilliant blue method (Kong and Yi, 2008). 0.1 g leaves were selected randomly and shredded into a mortar, and then ground until homogenized with 5 ml distilled water, which next was

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centrifuged with a TDL-40B centrifuge (Anting Scientific Instrument Factory, Shanghai) at a speed of 3000 r/min for 10 min. 1 ml of the supernatant was diluted in 5 times with 4 ml distilled water, of which 1 ml diluted supernatant was taken into a test tube with 5 ml of Coomassie brilliant blue solution being added. After the mixed solution being shaken and placed for 2 min, the absorbance of the solution was measured at 595 nm with a UV-6100 UV–visible spectrophotometer (Metash Co. Ltd) (*Fig. 2b*). The soluble protein content was calculated by standard curves.

Malondialdehyde (MDA) content

Malondialdehyde (MDA) content in seedlings was determined by the thiobarbituric acid method (Kong and Yi, 2008). 0.5 g leaves were selected randomly and shredded into a mortar, and then ground into a homogenate with 5 ml 10% trichloroacetic acid (TCA) solution, which next was centrifuged at a speed of 4000 r/min for 10 min. Then 2 ml of the supernatant into was taken and fixed with 2 ml 0.6% thiobarbituric acid (TBA) solution. Mixtures was bathed in 99 ℃ water for 15 min, then cooled quickly in 5 min and centrifuged again at a speed of 4000 r/min for 10 min with a TDL-40B centrifuge (Anting Scientific Instrument Factory, Shanghai). The absorbance of supernatant was measured at 532 nm, 600 nm, and 450 nm with a UV-6100 UV–visible spectrophotometer (Metash Co. Ltd). The MDA concentration and MDA content were calculated according to formulas *Eq.2* and *Eq.3*.

$$MDA\ concentration\ (\mu mol/L) = 6.45 \times (D532 - D600) - 0.56 \times D450 \quad \text{(Eq.2)}$$

$$MDA\ content\ (\mu mol/g) = cMDA \times V_T/F_W \quad \text{(Eq.3)}$$

where:

- D450, D532, D600 are the absorbance at 450nm, 532nm and 600nm, respectively.
- cMDA is MDA concentration (μmol/L);
- V_T is the volume of TCA solution (ml);
- F_W is the fresh weight of seedlings (g).

Catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) activities

The activities of CAT, SOD and POD were determined with the CAT, SOD and POD kits provided by Nanjing Jiancheng Biological Institute (Bao et al., 2017). A parallel sample (around 0.25 g) were randomly taken and ground to a homogenate with 5 ml phosphate buffer on ice. After centrifugations at a speed of 2500 r/min, 3500 r/min and 3500 r/min with a TDL-40B centrifuge (Anting Scientific Instrument Factory, Shanghai), respectively for 10 min, the supernatant was used for following measurements according to instructions of kits.

Data processing

The experiments were repeated five times, and the data were expressed as mean ± standard error (SE) (n=5), which statistically performed with R 3.3.1 statistical software (R Foundation for Statistical Computing, Vienna, Austria) for one-way analysis of variance (ANOVA). When the variables were uniform, the significance analysis of data was analyzed using Duncan model, otherwise using Games-Howell model (Warner 2007). Pearson correlation coefficient was used to describe the correlation between

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variables. All results were shown in bars in figures plotted by Origin 8.0 software. Different letters presented in figures indicated significant differences between different treatment groups at the same time.

Results

Effect on relative water content of seedlings

In this experiment, the relative water content (RWC) in of 8 treatment groups decreased during freeze-thaw cycle. As shown from figure *Fig. 3*, RWC in barley seedlings had a maximum decrease under combined stresses of salinity and drought. The RWC in seedlings of Groups FS, FD and F had no significant differences compared to that in Groups S, D and C, respectively. However, in thawing stage, RWC in seedlings of Group FSD showed significant differences compared with that of Group SD. Notably, the RWC in barley seedlings of 4 freeze-thaw groups showed a sequence as F > FS > FD > FSD. Consistently, a similar order of RWC in seedlings can be observed among 4 non-freeze-thaw groups as well, that is, C > S > D > SD.

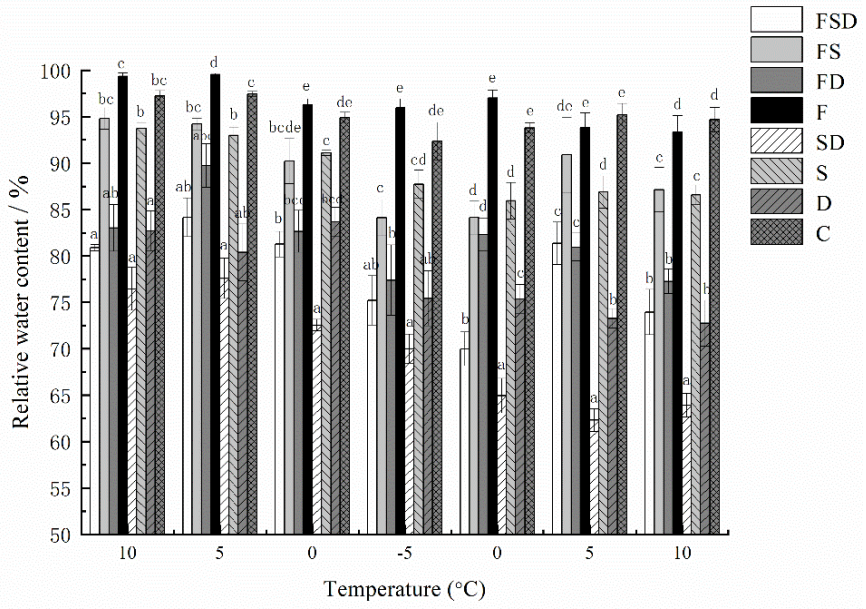

Figure 3. *The relative water content in barley seedlings under different treatment. The letter F, S, D and C represent freeze-thaw stress, salinity stress, drought stress and control, respectively. The temperature 10, 5, 0, -5, 0, 5 and 10 ℃ means the corresponding sampling temperature. The different low-case letters mean the significant difference at the same temperature ($P < 0.05$)*

Effect on soluble protein content of seedlings

It can be observed that the soluble protein (SP) content in barley seedlings of 4 treatment groups under freeze-thaw stress (FSD, FS, FD and F) was higher than that of 4 treatment groups without freeze-thaw stress (SD, S, D and C), respectively (*Fig. 4*). The SP content in seedlings of Group SD and Group S was significantly higher than that of Group D and Group C ($P < 0.05$), which indicated that under non-freeze-thaw conditions, the SP content in seedlings increased due to the occurrence of salinity stress.

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Nevertheless, the SP content in seedlings of groups under either single freeze-thaw stress or single drought stress had no significant difference compared with that of control group ($P > 0.05$). In the case of freeze-thaw stress, the SP content in seedlings of Groups FSD, FS, FD and F increased during the period of freeze-thaw stage. Among them, Group FSD reached the maximum at 0℃ (thawing stage), which exhibited a further 41.2% increase than the minimum 0℃ (freezing stage). Somewhat differently, the other 3 groups (FS, FD and F) all reached the maximum value at 5℃ (thawing stage), and were 46.4%, 86.6% and 72.7% higher than the minimum value, respectively.

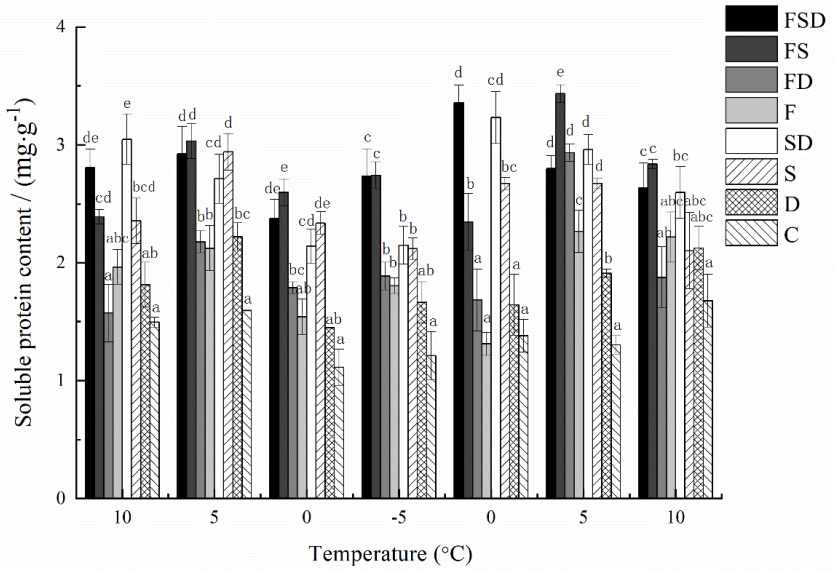

Figure 4. *The soluble protein content in barley seedlings under different treatment. The letter F, S, D and C represent freeze-thaw stress, salinity stress, drought stress and control, respectively. The temperature 10, 5, 0, -5, 0, 5 and 10 ℃ means the corresponding sampling temperature. The different low-case letters mean the significant difference at the same temperature ($P < 0.05$)*

Effect on MDA content of seedlings

Figure 5 shows that the malondialdehyde (MDA) content in barley seedlings of all experimental groups was higher than that of the control group. The non-freeze-thaw groups (SD, S, D and C) fluctuated little during a 14-hour freeze-thaw period, however the MDA content in barley seedlings of Group SD and Group S was significantly higher than that of Group D and C ($P < 0.05$). Besides, we have noticed that MDA content in barley seedlings in response to Group D was significantly higher than that in response to blank treatment ($P < 0.05$). Under the freeze-thaw stress, the MDA content in barley seedlings of single freeze-thaw group (F) was significantly lower than that of Groups FSD and FD ($P < 0.05$).

Effect on SOD activity

The SOD activity in barley seedlings of Groups FSD and SD had no significant difference compared with control group (C) (*Fig. 6*). The SOD activity significantly enhanced owing to the occurrence of drought stress in barley seedlings ($P < 0.05$), while significantly weakened due to the salinity stress in seedlings ($P < 0.05$). Under freeze-

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thaw stress, the SOD activity in barley seedlings of Groups FSD, FS, FD and F decreased at first and then increased. During the freeze-thaw cycle, except for 5℃, the SOD activity in barley seedlings of Group FD was significantly higher than that of Groups FSD, FS and F ($P < 0.05$).

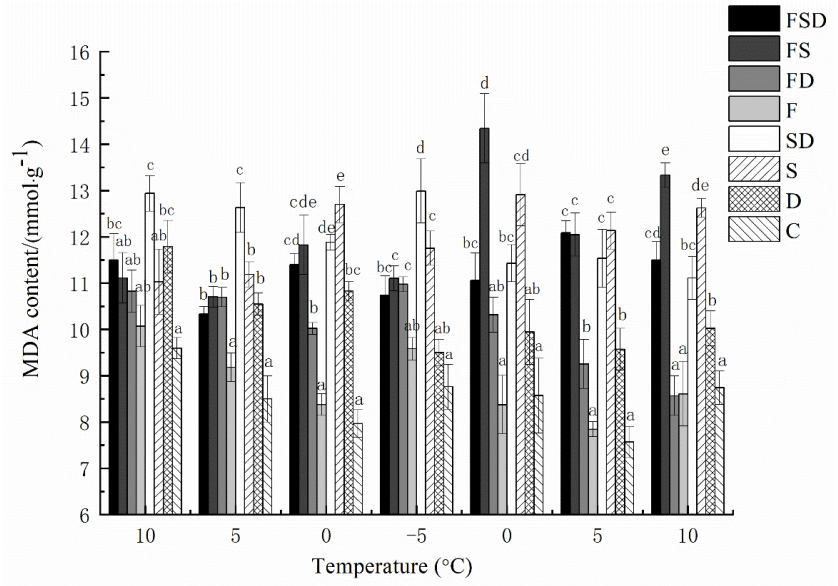

Figure 5. *The malondialdehyde (MDA) content in barley seedlings under different treatment. The letter F, S, D and C represent freeze-thaw stress, salinity stress, drought stress and control, respectively. The temperature 10, 5, 0, -5, 0, 5 and 10 ℃ means the corresponding sampling temperature. The different low-case letters mean the significant difference at the same temperature (P < 0.05)*

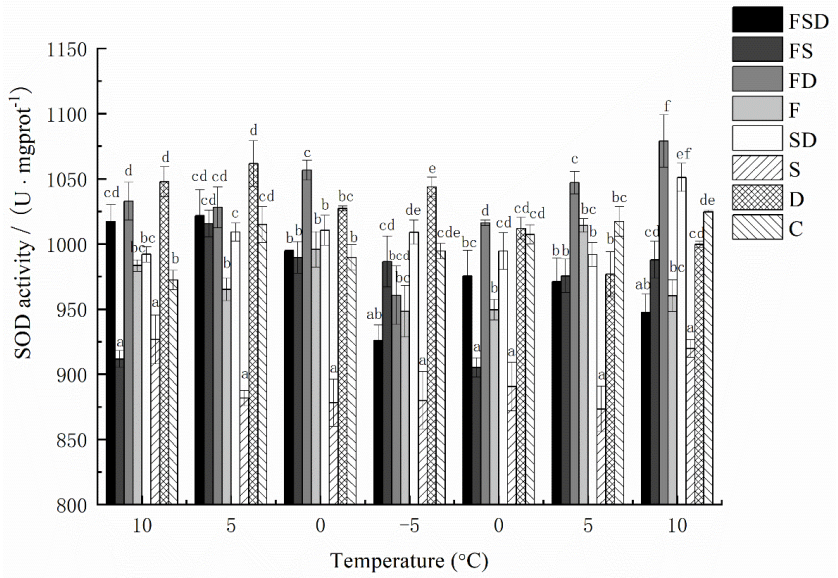

Figure 6. *The SOD activity of barley seedlings under different treatment. The letter F, S, D and C represent freeze-thaw stress, salinity stress, drought stress and control, respectively. The temperature 10, 5, 0, -5, 0, 5 and 10 ℃ means the corresponding sampling temperature. The different low-case letters mean the significant difference at the same temperature (P < 0.05)*

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Effect on CAT activity

It can be seen from *Figure 7* that, under non-freeze-thaw conditions, the CAT activity in barley seedlings of Groups SD, S and D was significantly lower than that of the control group ($P < 0.05$). CAT activity in seedlings of either Group F or Group FS showed a trend of initially increasing and then decreasing, while that of Group FD showing a general downward trend, and that of Group FSD showing an upward trend. Take if further, the CAT activity in seedlings of Group FSD was significantly lower than that of Group F ($P < 0.05$), and that of the Groups FS and FD was significantly lower than that of Group F only in the latter thawing case ($P < 0.05$).

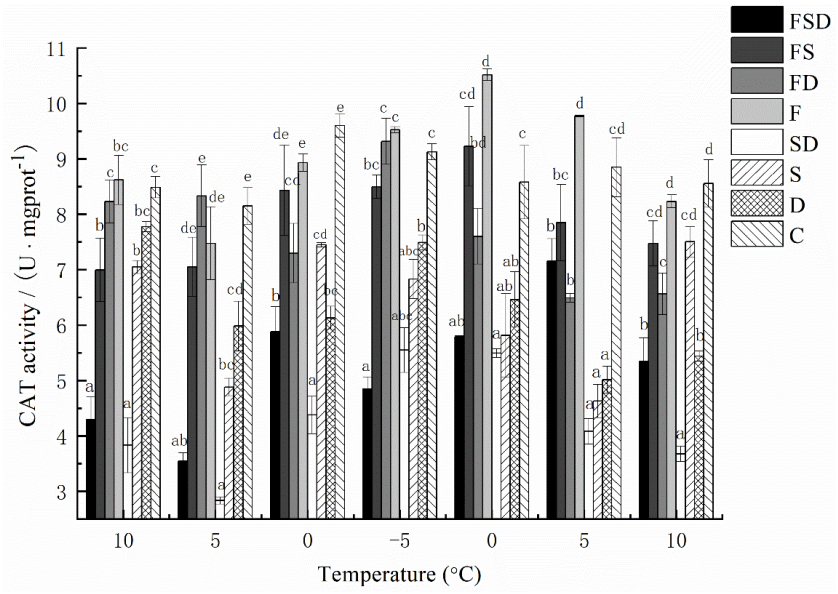

Figure 7. *The CAT activity in barley seedlings under different treatment. The letter F, S, D and C represent freeze-thaw stress, salinity stress, drought stress and control, respectively. The temperature 10, 5, 0, -5, 0, 5 and 10 ℃ means the corresponding sampling temperature. The different low-case letters mean the significant difference at the same temperature ($P < 0.05$)*

Effect on POD activity

In this experiment, during freeze-thaw cycle, the POD activity in barley of Groups FD and F showed a trend of initially increasing and then decreasing, while that of Groups FSD and FS showing an increasing trend (*Fig. 8*). When the temperature dropped to 10 °C (freezing stage), the POD activity in barley seedlings of Groups F, S and D were significantly lower than that of control group ($P < 0.05$). Under salinity stress, the POD activity in seedlings significantly decreased within a freeze-thaw cycle ($P < 0.05$). Nevertheless, no significant difference was observed in POD activity between Groups FD and D. Accordingly, under freeze-thaw stress, there was a significant reducing of POD activity of groups subjected to salinity treatment, but no effect on that of groups subjected to drought treatment.

Correlation analysis

It can be observed from *Table 2* that the content of SP and MDA in seedlings were significantly positively correlated under freeze-thaw conditions ($P < 0.01$). There was a

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significant negative correlation between SP content and antioxidant enzyme activity ($P < 0.01$). MDA was significantly negatively correlated with antioxidant enzyme activity and RWC, while CAT and POD were positively correlated ($P < 0.01$).

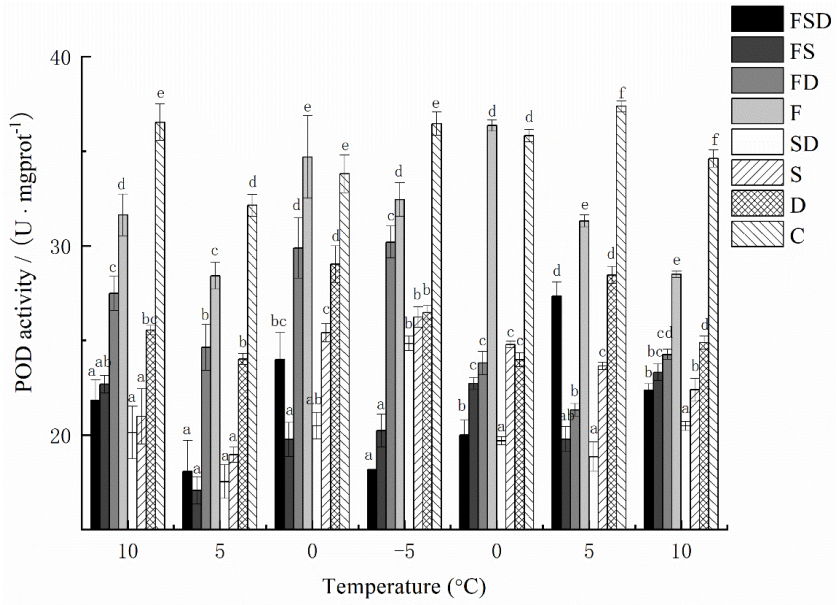

Figure 8. *The POD activity in barley seedlings under different treatment. The letter F, S, D and C represent freeze-thaw stress, salinity stress, drought stress and control, respectively. The temperature 10, 5, 0, -5, 0, 5 and 10 ℃ means the corresponding sampling temperature. The different low-case letters mean the significant difference at the same temperature ($P < 0.05$)*

Table 2. *Pearson correlation analysis between relative water content (RWC), soluble protein (SP) content, malondialdehyde (MDA) content, SOD, CAT and SOD activity in barley seedlings of freeze-thaw treatment groups*

	RWC	SP	MDA	SOD	CAT	POD
RWC	1	-0.524**	-0.427*	-0.341	0.726**	0.640**
SP		1	0.750**	-0.351	-0.799**	-0.868**
MDA			1	-0.407*	-0.663**	-0.798**
SOD				1	0.052	0.213
CAT					1	0.833**
POD						1

* Significant correlation at 0.05 level (both sides). ** Significant correlation at the 0.01 level (both sides)

Discussion

Either drought or alkaline salt can lead to a large amount of water loss in seedlings by reducing the osmotic pressure of plant cells (Shereen et al., 2019). It is one important conclusion from Alexander's research that the RWC of leaves is positively related to plant's stress resistance (Alexander et al., 2019). It has been reported that under the combined effects of water loss and high temperature, the reduction of RWC in Australian durum is greater than that under single stress (Liu et al., 2019). Consistently, in this

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experiment, the combined effects on RWC in barley seedling of salinity and drought were severer than the additive of salinity and drought alone; thus, interactions between the two stress factors were synergistic for RWC. Under freeze-thaw conditions, a decrease in temperature can not only ice the water in plant cells, but also caused a dehydration, resulting in a decrease in RWC (Iseri et al., 2013). As have shown that at the freezing stage, the decreased RWC contributed to alleviate the damage caused at freezing stage to plants and maintain the osmotic balance of plant cells (Hao et al., 2009).

Most of the soluble proteins in plants are enzymes involved in metabolism (Bao et al., 2019). An increase of soluble proteins can maintain the cell's higher osmotic potential, enhance the capacity of water absorption and holding, maintaining plant growth and improving resistance to stress (Yin et al., 2004). Here we observed from experiments that the SP content of barley seedlings increased under either drought or freeze-thaw stress (Groups F and D), while a higher accumulation of SP was measured in the groups subjected to basic-salt stress (FSD, FS, SD and S). These observations may attribute to the expression of resistant proteins in plant cells stimulated by alkaline stress, increasing the content of SP participating in osmotic adjustment in cells, thus making plants adapt to the external environment (Hazman et al., 2016). Under freeze-thaw stress, the SP content in seedlings of groups subjected to freeze-thaw stress decreased with the dropping temperature, which may be due to the accelerated decomposition of soluble proteins in cells, providing plants with energy to relieve the damage caused by stresses (Bae et al., 2006). At thawing stage, with the temperature rising to 10 ℃, the SP content in seedlings increased. These findings are similar to the results of Lee's research, in which they examined the proteomic changes of rice roots under low temperature stress and found that the expressions of 27 proteins were up-regulated at 10 ℃ (Lee et al., 2009).

The activity changes of antioxidant enzyme in plants caused by abiotic environmental stresses may have an effect on physiological characteristics to reduce damage (Ahsan et al., 2007). In a previous study, Zeng et al. (2019), using methods of indoor cultivating of soybean seedlings and experiments, disclosed that a large amount of CAT transcription and significant enhancement of enzyme activity were observed under high aluminum stress. Researches have shown that the antioxidant enzyme activities in leaves of *Pyracantha fortuneana* and *Rosa cymosa* are significantly enhanced under severe drought stress, indicating the strong resistance to drought stress of these species, however the antioxidant enzyme activities are greatly weakened in leaves of *Broussonetia papyrifera* and *Cinnamomum bodinieri* under same conditions, indicating the weaker resistance to drought stress (Liu et al., 2011). Here, our experiment showed that CAT and POD activities in barley seedlings significantly weakened under non-freeze-thaw stress. The results suggested a possible reason of lipid peroxidation on the cell membrane affected by stresses duration, leading to the damage to cells and the effect on the synthesis of substances like proteins in cells, at last reducing the antioxidant enzyme activity. This is consistent with the study by Gao et al. (2012). Moreover, an observed decrease in SOD activity in the leaves of *Camptotheca acuminata* seedlings accompanied low temperature stress, which was found by Feng et al. (2002). It was worth noting that CAT and POD activities increased, while SOD activity decreased with a decrease of temperature, which could be explained by the role played by SOD as the first line in defensing and eliminating reactive oxygen species (ROS). A large consumption of SOD in the process of eliminating ROS and an inefficient synthesis of enzymes in the case of low temperature were confirmed in the research results of Bao et al. (2019).

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Environmental stress can disrupt the homeostasis of cells and the dynamic balance between production and clearance of ROS, leading to excessive accumulation of ROS in cells, causing the oxidative damage to biomolecules such as lipids (Mano, 2012), proteins (Dean et al., 1997) and nucleic acids (Cadet et al., 2003), and the disruption of osmotic balance in plants (Bian et al., 2018), which were discussed in detail in numerous studies. MDA is the end product of lipid peroxidation and can be induced by stress in plants organ, e.g. leaves, shoots or roots (Iseri et al., 2013; Karagoz et al., 2018). In this paper, MDA content in barley seedlings increased under the single or combined stress of salinity and drought, importantly, MDA content accumulated more under single salinity stress than that under combined stress. In addition to osmotic stress on seedlings, salinity stress, compared with drought, is accompanied by high pH stress as well, causing damage to plant cell membranes and eventually leading to the accumulation of MDA, which has been confirmed in a study by Ali et al. (2011). The freeze-thaw manipulation treatments decreased the MDA content of barley seedlings, during which, the activities-enhanced CAT played a key regulatory role (Wu et al., 2018).

Conclusion

In summary, as an important crop on the Qinghai-Tibet Plateau, barley has equipped with great resistance to the freeze-thaw environment within the long-term evolution. Considering current global warming, soil salinization and drought have become increasingly serious, resulting in physiological responses like the accumulation of MDA and the changes in antioxidant enzymes activity. Herein we showed that either single or compound stresses of drought, salinity and freeze-thaw could make MDA accumulated excessively in seedlings because of the imbalance between oxygen free radical reaction and lipid peroxidation reaction, which caused oxidative stress on plants, affecting the stability of plant cells. Moreover, the contents of MDA and SP in barley seedlings increased significantly under the combined stresses of salinity and drought, while the RWC significantly reduced. As a conclusion, to avoid simultaneous occurrence of drought and salinity stress, the intensity of spring irrigation is supposed to increase in areas with severe drought stress. Though the resistance characteristics of plants under one freeze-thaw cycle were studied in this paper, in view of multiple freeze-thaw cycles in nature, one important future direction of physiological responses to freeze-thaw stress is studying the different resistance characteristics of plant between under one freeze-thaw cycle and under multiple freeze-thaw cycles.

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SEASONAL VARIATION OF PLANKTONIC FUNGAL COMMUNITY STRUCTURE IN THE XIJIANG RIVER, CHINA

LIU, Q. F.[1,2] – LAI, Z. N.[1,2*] – WANG, C.[1,2] – ZHANG, D. F.[1,2] – MAI, Y. Z.[1,2] – DU, H.[3]

[1]*Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510380, China*
(first author: liuqfwh@163.com)

[2]*Fishery Ecological Environment Monitoring Center of Pearl River Basin, Ministry of Agriculture and Rural Affairs, Guangzhou 510380, China*

[3]*Key Lab of Freshwater Biodiversity Conservation Ministry of Agriculture and Rural Affairs, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan 430223, China*

[*]*Corresponding author*
e-mail: znlai01@163.com

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Abstract. To investigate the composition variation of planktonic fungal communities, and their relationship with water physical and chemical parameters in the Xijiang River in China, the compositions of planktonic fungal communities in the high-water and low-water period of the Xijiang River were studied in this paper by high-throughput sequencing of fungal internal transcribed spacer amplicons. Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Rozellomycota, and Zygomycota were detected from the planktonic fungal communities. However, most high-quality sequences (59.58 ± 0.03%) could not accurately identify fungal phyla. Alpha-diversity indexes of the fungal communities and the relative abundances of *Phoma brasiliensis*, *Pseudozyma* sp., *Psathyrella* sp., *Haematonectria haematococca*, *Podoscypha* sp. in the high-water period were significantly higher than those in the low-water period, while the relative abundances of *Basidiobolus* sp., *Rhizophydium littoreum*, *Teratosphaeria jonkershoekensis*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia* sp., *Alternaria eichhorniae*, *Knufia epidermidis*, and *Scedosporium prolificans* were significantly lower. No significant correlation has been found between the fungal community distance and geographical distance along the river. Water temperature and dissolved oxygen significantly influenced the fungal community structure in the high-water period. These results provide information for us to understand the composition and influencing factors of the planktonic fungal community in rivers.
Keywords: *subtropical fungal community, riverine, water ecosystem, high-throughput sequencing, internal transcribed spacer, physical and chemical parameters*

Introduction

Fungal community plays an important role in maintaining the normal operation of water ecosystems (Cai et al., 2006). Aquatic fungi decompose organic matter and promote nutrient migration, which cooperate with protozoa to utilize aquatic nutrients and promote the flow of material and energy in water ecosystems (Grattan and Suberkropp, 2001; Gulis and Suberkropp, 2003; Chung and Suberkropp, 2008; Röske et al., 2012; Zhang et al., 2013). These roles have been demonstrated for planktonic fungal communities (Gao et al., 2010; Gutierrez et al., 2011; Kagami et al., 2012). However, despite the fact that the composition and function of fungal communities in soil (Broeckling et al., 2008; Mueller et al., 2015; Carson et al., 2019; Lance et al., 2020), sediment (Wu et al., 2013; Sui et al., 2016; Wang et al., 2018a), ocean (Gao et al., 2010; Gutierrez et al., 2011), lake

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(Kagami et al., 2012), reservoir (Shang et al., 2018; Chen et al., 2018) and other ecosystems have been widely studied, the research on fungal communities in river water ecosystems is very scarce (Bärlocher and Boddy, 2016).

The river ecosystem is one of the most important ecosystems on earth, and its ecological function is of great concern (Hopkins et al., 2011). Rivers are the primary conduits for land-to-ocean transfer of materials including terrestrial organic matter, nutrients, and anthropogenic pollutants. Microbial communities in rivers, estuaries, and plumes regulate the nutrient concentrations and biogeochemistry of these river-borne materials and mediate their impact on carbon cycling. Although the compositions of prokaryotic communities in rivers and their variation with physical and chemical factors and seasons were investigated (Maksimenko et al., 2008; Kent and Bayne, 2010; Kaevska et al., 2016; Wang et al., 2018b), the composition of fungal communities in river ecosystems is rarely studied.

The Xijiang River is the mainstream of the Zhujiang (Pearl) River, which lies in a subtropical monsoon area, South China. It is 353,120 km^2 of area and 230×10^9 m^3 of annual runoff. The period from April to September is the flood season (Gao et al., 2002). The Xijiang River is rich in river water and bait resources, and the ecological environment is suitable for the growth of fish, shrimp, crab, and shellfish. It is rich in fish resources, with 136 fish species. It is an important habitat and breeding ground of aquatic organisms in subtropical areas (Li et al., 2009, 2010). To investigate the composition and variation of planktonic fungal communities between the high-water and low-water periods, and their relationship with water physical and chemical parameters, in the present study, the compositions of planktonic fungal communities in the high-water and low-water period of Xijiang River in China were studied by high-throughput sequencing of fungal ITS gene amplicons on the Illumina HiMeq platform.

Materials and Methods

Sampling collection

Surface water samples were collected in March (low-water period) and June (high-water period) of 2017 from 13 sites in the Xijiang River (*Fig. 1*) using previously described methods with minor modifications (Yu et al., 2015). Briefly, triplicate water surface samples (approximately 0.5 m below water surface) were collected from each site using a 5 L Niskin bottle, mixed, and immediately stored in EPR-5590 sterile sampling bags (LABPLAS, Canada). The water samples were subsequently transferred to the laboratory on ice. 500 ml water from each sample was filtered with 0.22 μm pore size polycarbonate membranes (Millipore, USA), and the filters were stored at −80 °C until DNA extraction. Other water samples were used to determine water physical and chemical parameters.

Determination of water physical and chemical parameters

Water transparency was field measured according to a standard method (Huang, 2000; Ni et al., 2010). Water temperature (WT, °C), pH, dissolved oxygen (DO, mg/L), oxidation-reduction potential (ORP, mv), conductivity (Cond, μS/m), and total dissolvable solid (TDS, g/L) were field measured using a ProQuatro smart portable multi-parameter water quality analyzer (YSI, USA). Approximately 500 ml water was filtered by WHATMAN GF/C filter membrane and used to measure the chlorophyll-a content

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(Chla, μg/L) according to a previously described method (The State Environmental Protection Administration, 2002). Concentrations of NH_4^+-N, NO_3^--N, NO_2^--N, TN, TP, and SiO_3^{2-}-Si were determined using a SKALAR flow injection water quality analyzer (SKALAR Analytical, Netherlands) according to the manufacturer's instructions. Concentration of un-ionized ammonia (NH_3, mg/L) was calculated by NH_4^+-N, pH, and WT according to a previously described method (Zou and Cheng, 2002).

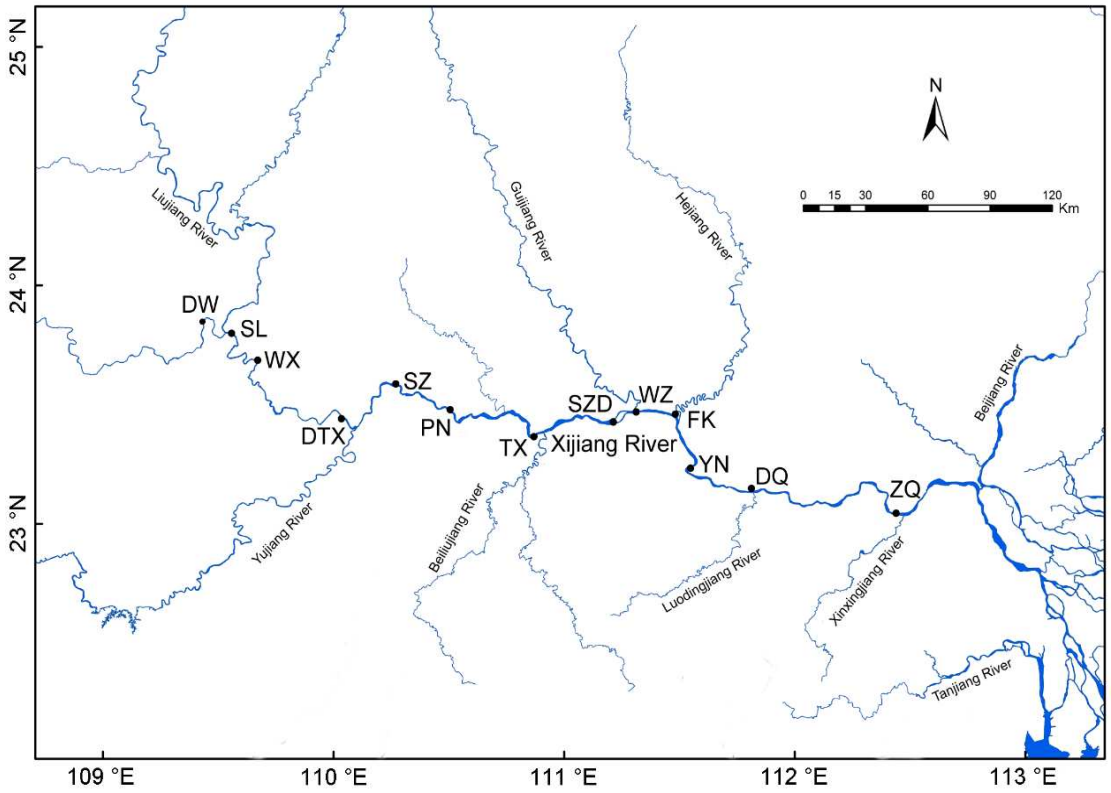

Figure 1. *Distribution of sampling sites. The blue lines indicate the Xijiang River and its tributaries. The black spots are the sampling sites. DW, Dawan Town (109.469 E 23.867 N); SL, Shilong Town (109.547 E 23.780 N); WX, Wuxuan County (109.675 E 23.677 N); DTX, Datengxia (110.050 E 23.454 N); SZ, Shizui Town (110.267 E 23.586 N); PN, Pingnan County (110.498 E 23.492 N); TX, Tengxian County (110.883 E 23.374 N); SZD, Sizhoudao Island (111.214 E 23.435N); WZ, Wuzhou City (111.338 E 23.476 N); FK, Fengkai County (111.492 E 23.453 N); YN, Yunan County (111.551 E 23.240 N); DQ, Deqing County (111.789 E 23.139 N); ZQ, Zhaoqing City (112.433 E 23.047 N)*

DNA extraction and sequencing

Water DNA was extracted using the MicroElute Genomic DNA kit (Omega, USA) according to the manufacturer's instructions. The total DNA was eluted in 50 μl of Elution buffer by a modification of the procedure described by manufacturer (QIAGEN, Germany) and stored at -80 °C until used for the PCR amplification by Vazyme Biotech Co., Ltd, Nanjing, China.

The internal transcribed spacer 2 (ITS2) region of the fungus was amplified using the extracted fungal DNA as a template and the primer pair fITS7 (5'-GTGARTCATCGAATCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-

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3') (Honkanen et al., 2020). The reaction was carried out in 25 μl mixtures containing approximately 25 ng of water genomic DNA, 12.5 μl PCR Premix, 2.5 μl of each primer (10 nmol/L), and PCR-grade water to adjust the volume. PCR reaction was performed in a Master cycler gradient thermocycler (Eppendorf, Hamburg, Germany) set to the following conditions: initial denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s; and then final extension at 72 °C for 10 min. The PCR products were confirmed with 2% agarose gel electrophoresis. Ultrapure water, instead of a sample solution, was used as a negative control to exclude the possibility of false-positive PCR results. The PCR products were normalized by AxyPrep^{TM} Mag PCR Normalizer (Axygen, CA, USA). The amplicon pools were prepared for sequencing with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and the size and quantity of the amplicon library were assessed on the LabChip GX (Perkin Elmer, Waltham, MA, USA). PhiX control library (v3) (Illumina, USA) was combined with the amplicon library (expected at 30%). The libraries were sequenced on MiSeq platform (Illumina, USA).

The reads were filtered by quantitative insights into microbial ecology (QIIME) quality filters. The CD-HIT pipeline was used for picking operational taxonomic units (OTUs) with 97% sequence similarity through making OTU table. Representative sequences were chosen for each OTU, and taxonomic information were assigned to each representative sequence using the Ribosomal Database Project classifier. Chao1 index (Chao, 1984), number of observed species, Shannon index, and Simpson index were the commonly used alpha-diversity indexes to measure the biodiversity of microbiota (Bucci et al., 2014; Ni et al., 2019; Xu et al., 2020). These alpha-diversity indexes were calculated using the R vegan package (Dixon, 2003).

Data analysis

Wilcoxon rank-sum tests were conducted using R software (R Core Team, 2017) to compare the differences of water physical and chemical parameters, and alpha diversity indexes between the samples collected in March and those in June. Linear discriminant analysis effect size (LEfSe) was conducted through the Galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/). The boxplots were plotted using R ggpubr package. RDA was conducted using R vegan package. Bray-Curtis distances of the planktonic fungal communities were calculated based on OTU tables using R vegan package (Dixon, 2003), and Pearson's product-moment correlation analysis was conducted using R basicTrendline package. Results with P-values of less than 0.05 were considered significant differences.

Results

Seasonal variation of water physical and chemical parameters in the Xijiang River

Among the 16 common water physical and chemical parameters, only WT, pH, transparency, TP, and NH_4-N concentration were significantly different (Wilcoxon rank-sum test, $p < 0.05$). The WT, pH, and TP in June were significantly higher than those in March, while the transparency and NH_4-N concentration were significantly lower than those in March (*Fig. 2*).

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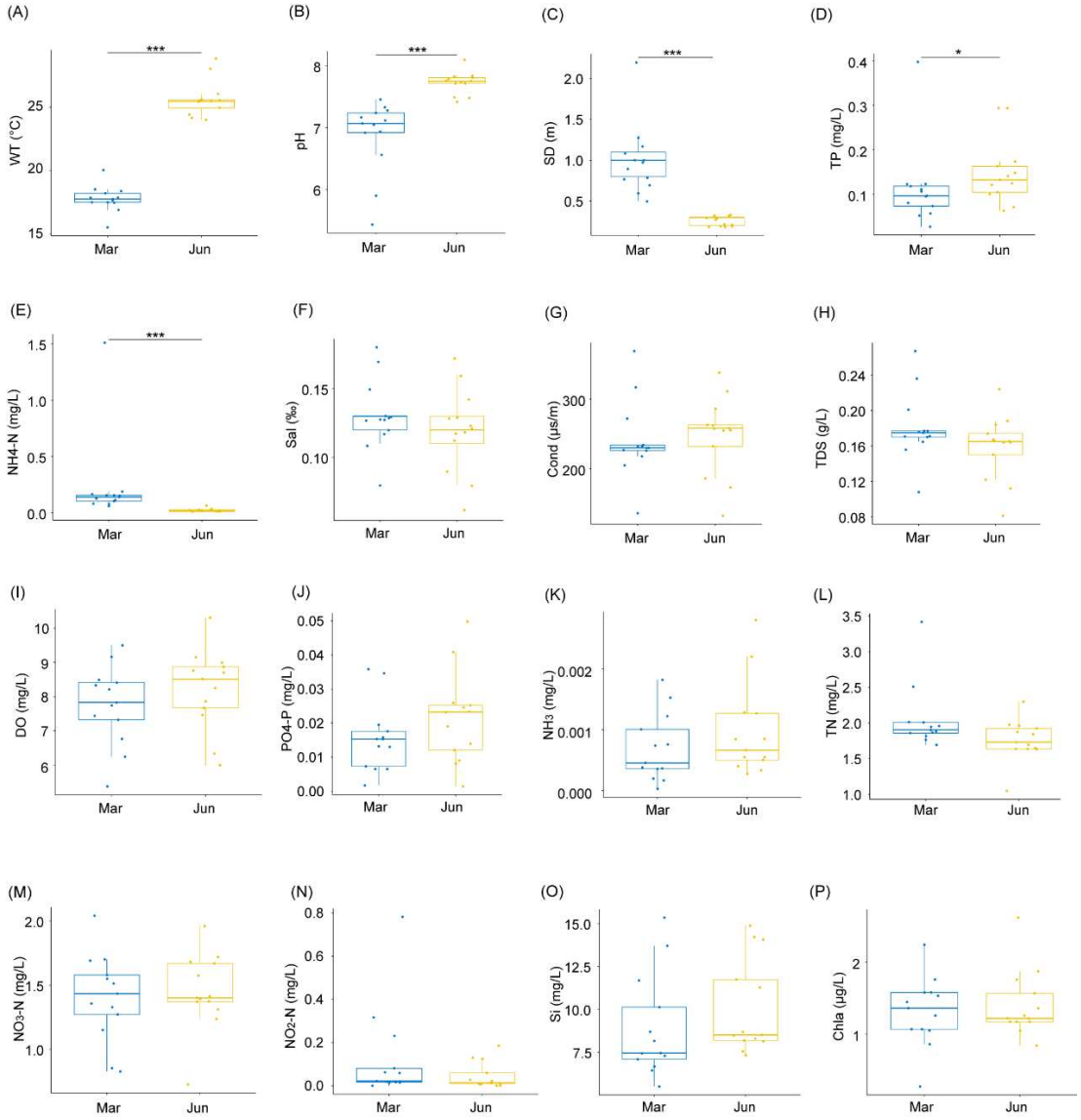

Figure 2. *Differences of water physical and chemical parameters between March and June in the Xijiang River. WT, water temperature; SD, transparency; TP, total phosphorus; Sal, salinity; Cond, conductivity; TDS, total dissolved solids; DO, dissolved oxygen; TN, total nitrogen; Chla, chlorophyll-a*

Seasonal variation of fungal community structure in the Xijiang River

Total 570,573 high-quality sequences were obtained from 26 samples collected in March and June. Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Rozellomycota, and Zygomycota were detected from the planktonic fungal communities (*Fig. 3A-F*). However, the number of their sequences only accounted for 40.42 ± 0.03% of the total high-quality sequences, and most high-quality sequences (59.58 ± 0.03%) could not identify accurately fungal phyla (*Fig. 3G*). Except for Glomeromycota and Rozellomycota, the relative abundances of other phyla fluctuated more in Marth than in June (*Fig. 3*). Moreover, despite the existence of fluctuation between sampling sites, the relative abundance of Ascomycota increased gradually from upstream to downstream, while that of Zygomycota decreased from upstream to downstream in March (*Fig. 3*).

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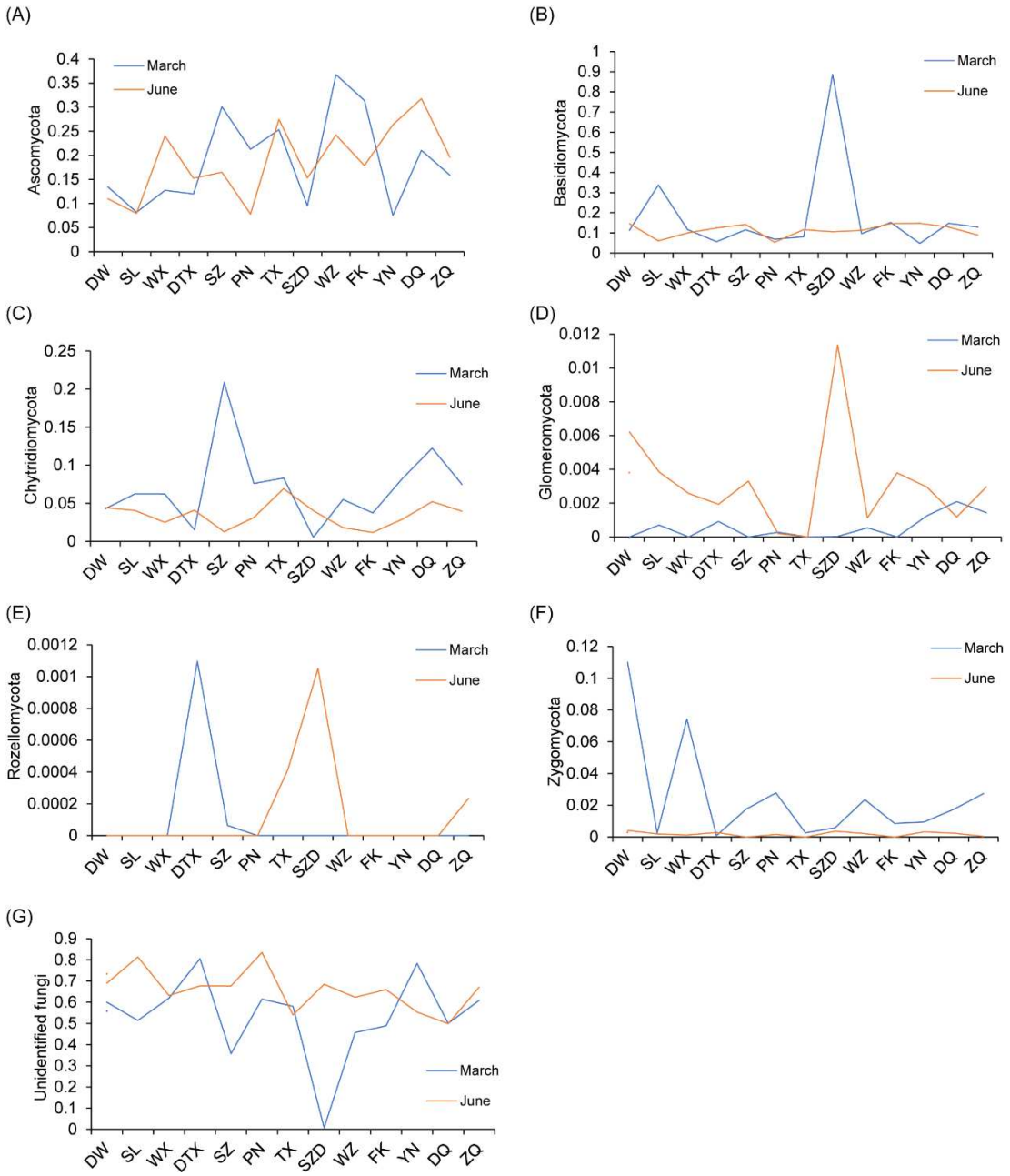

Figure 3. *Relative abundance changes of fungal phyla in the water of the Xijiang River. DW, Dawan Town (109.469 E 23.867 N); SL, Shilong Town (109.547 E 23.780 N); WX, Wuxuan County (109.675 E 23.677 N); DTX, Datengxia (110.050 E 23.454 N); SZ, Shizui Town (110.267 E 23.586 N); PN, Pingnan County (110.498 E 23.492 N); TX, Tengxian County (110.883 E 23.374 N); SZD, Sizhoudao Island (111.214 E 23.435N); WZ, Wuzhou City (111.338 E 23.476 N); FK, Fengkai County (111.492 E 23.453 N); YN, Yunan County (111.551 E 23.240 N); DQ, Deqing County (111.789 E 23.139 N); ZQ, Zhaoqing City (112.433 E 23.047 N)*

Although there was no significant difference in the number of OTUs detected in the planktonic fungal communities in March and June, the Shannon, Simpson, and Chao1 indexes of the fungal communities in June were significantly higher than those in March (Wilcoxon rank-sum test, $p < 0.05$; *Fig. 4*).

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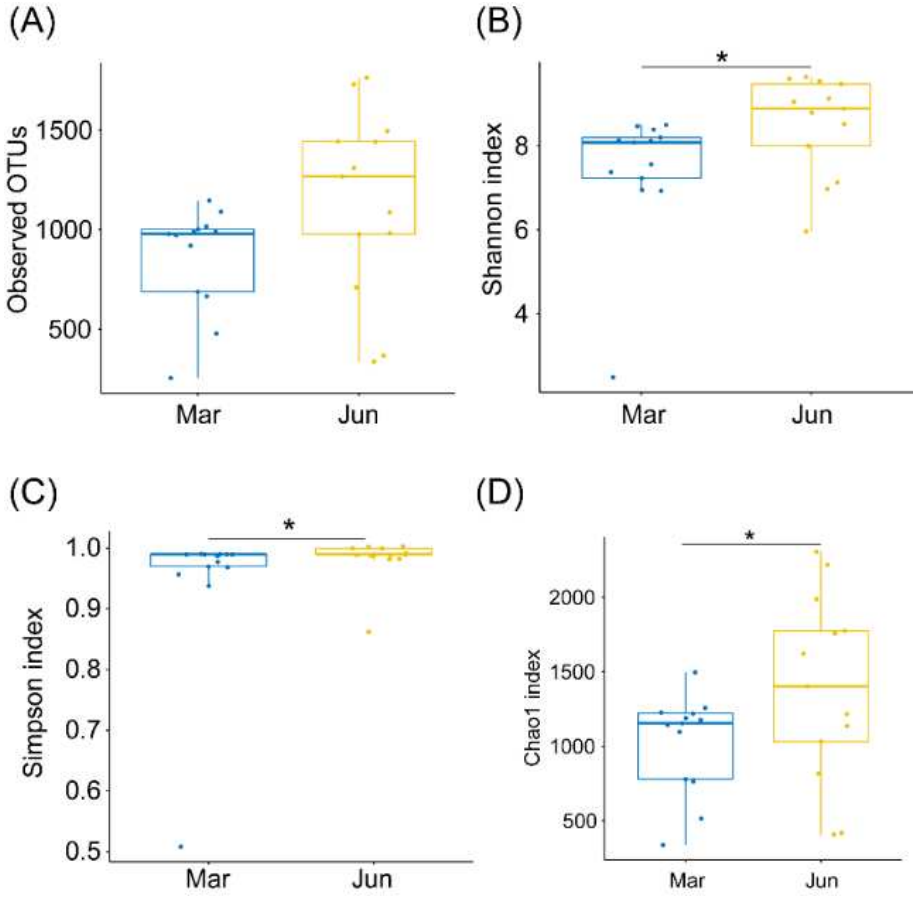

Figure 4. *Comparison of α-diversity indexes of planktonic fungal communities between March and June in the Xijiang River*

In the dominant fungi that were identified to species level, the relative abundances of *Phoma brasiliensis*, *Pseudozyma* sp., *Psathyrella* sp., *Haematonectria haematococca*, *Podoscypha* sp. in the fungal in June were significantly higher than those in March, while the relative abundances of *Basidiobolus* sp., *Rhizophydium littoreum*, *Teratosphaeria jonkershoekensis*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia* sp., *Alternaria eichhorniae*, *Knufia epidermidis*, and *Scedosporium prolificans* were significantly lower (LEfSe, LDA > 2; *Fig. 5*).

To analyze the dispersal limitation of planktonic fungal communities in the river and analyze the impact of water environmental parameters on the planktonic fungal community structure, we analyzed the relationship between the Bray-Curtis distances of planktonic fungal communities and geographical distances by correlation analysis, and the relationship between water environmental parameters and planktonic fungal community structure using RDA. Our results showed that no significant correlation between fungal community distance and geographical distance along the river (Pearson's product-moment correlation, $p > 0.05$; *Fig. 6A and B*). Although no water environmental parameter significantly influenced the fungal community structure in March, WT and DO significantly influenced the fungal community structure in June (*Fig. 6C and 6D*).

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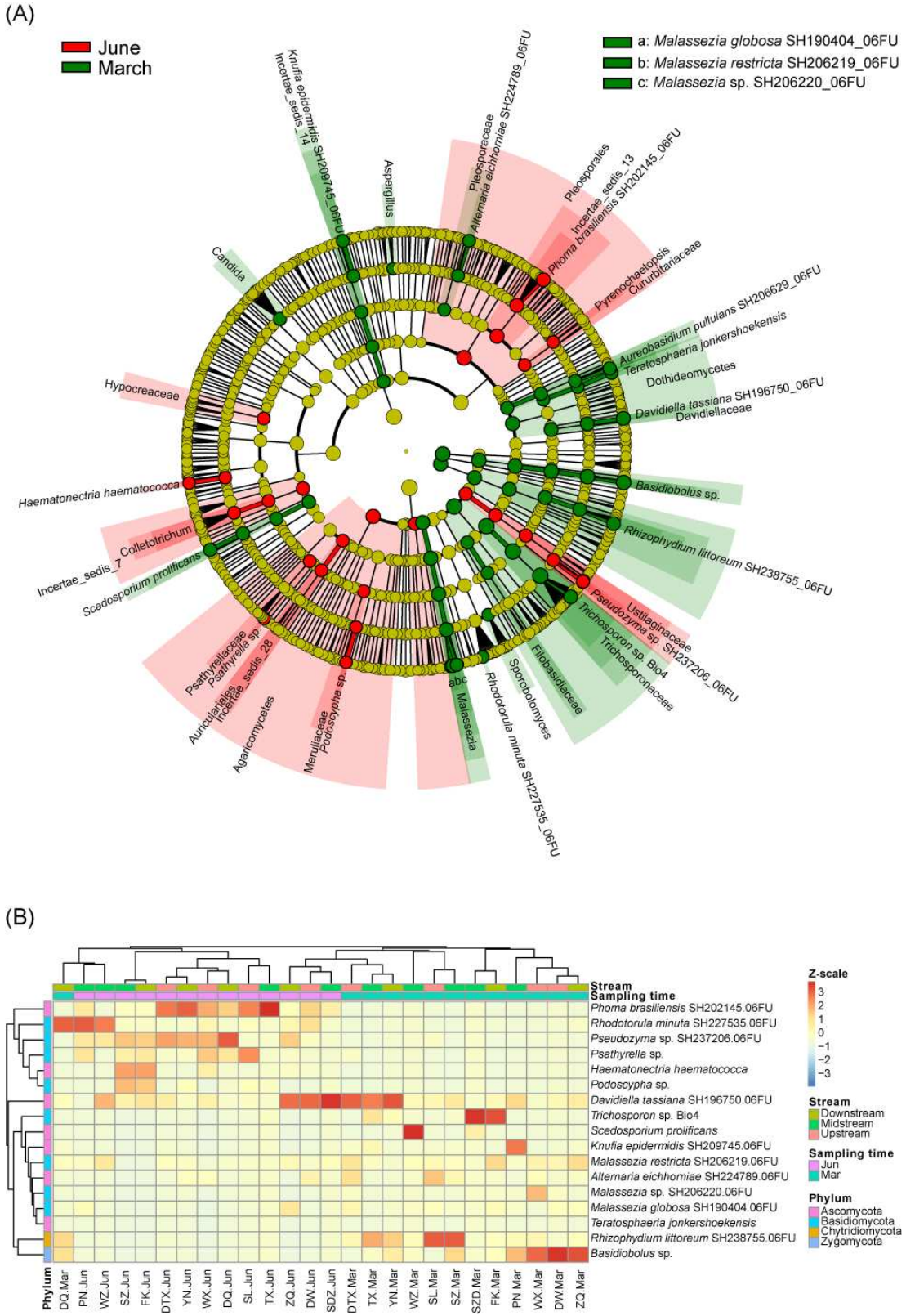

Figure 5. *Cladogram plot of Linear discriminant analysis effect size (LEfSe) (A) and heatmap profile of significantly different fungal species (B) between the samples collected in March and June*

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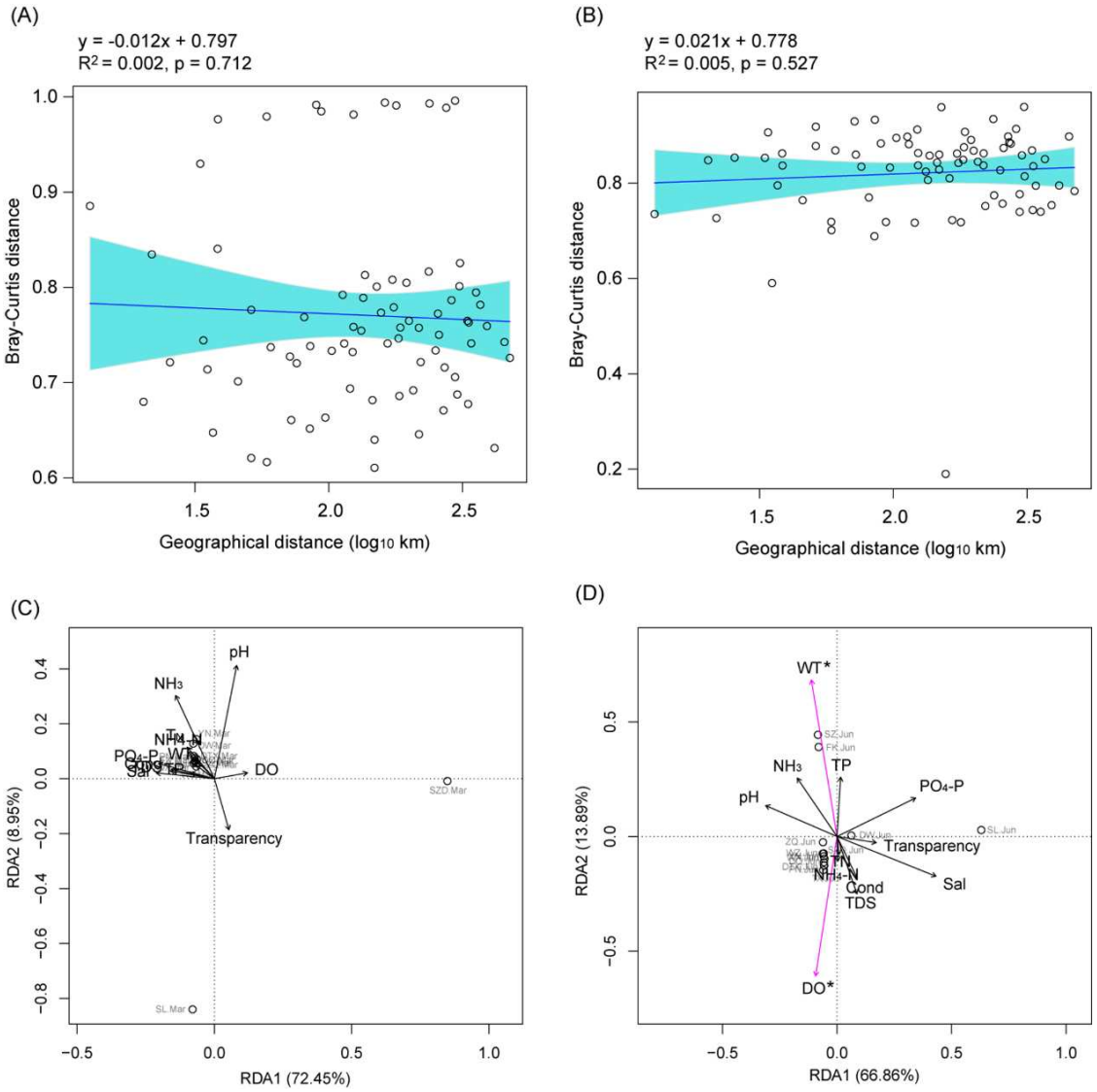

Figure 6. *Correlation between fungal community distance and geographical distance along the river in March (A) and June (B), and RDA profiles showed the relationship of water physical and chemical parameters and planktonic fungal community structures in the samples collected in March (C) and June (D). WT, water temperature; TP, total phosphorus; Sal, salinity; Cond, conductivity; TDS, total dissolved solids; DO, dissolved oxygen; TN, total nitrogen; Chla, chlorophyll-a. *, $p < 0.05$*

Discussion

Panzer et al. (2015) summarized the research on environmental fungal communities based on 18S rRNA gene, and found that Ascomycota, Basidiomycota, and Chytridiomycota are the main components of freshwater fungal communities. In this study, we found that although Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Rozellomycota, and Zygomycota were detected from the planktonic fungal communities, the number of their sequences only accounted for 40.42 ± 0.03% of the total high-quality sequences. This result implied that there were a large number of fungal species that had not been studied in detail. Moreover, compared with bacterial community compositions of freshwater and sediment, the results of our analysis on the

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compositions of fungal communities in the Xijiang River were similar to those in the sediment of a lake - Ascomycota, Basidiomycota, and Chytridiomycota had the highest abundance (Wang et al., 2018a). Among them, Chytridiomycota mainly feeds on dead aquatic plants or other detritus in water, and the released zoospores are preyed by zooplankton, which plays an important role in the food web of water (Kagami et al., 2014).

The community structure of planktonic fungi has been shown to be affected by pH, WT, and conductivity, as well as the physical and chemical properties of the organic matter, such as nitrogen and phosphorus (Cudowski et al., 2015; Reich et al., 2017). However, our results showed that only WT and DO had significant effects on the community structure of planktonic fungi in the high-water period (*Fig. 6C and D*). This might be because the physical and chemical indicators of various sampling sites in the river had little difference (*Fig. 2*), thus eliminating the impact of physical and chemical parameters on the composition of river fungal communities. Therefore, it is necessary to investigate the community compositions of phytoplankton fungi in river at a larger time and space scale with wider range of differences in physical and chemical parameters in the future.

Dispersal limitation is considered an important factor that influences the β-diversity of microbiota, which is mainly reflected in the fact that the distance of microbiota increases with the increase in geographical distance (Ni et al., 2014; Cao et al., 2016; Logares et al., 2020). Due to the weak active dispersal ability of microorganisms, it usually presents a trend of dispersal limitation (Shurin, 2000; Peay et al., 2010; Chytrý et al., 2012; Ni et al., 2014; Beaton et al., 2016). However, our results showed no significant correlation between fungal community distance and geographical distance along the river. These results indicated that planktonic fungal communities eliminated the deficiency in active dispersal through passive dispersal of river water flow, which eliminated the dispersal limitation of the planktonic fungal communities in rivers.

Conclusion

Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Rozellomycota, and Zygomycota were detected from the planktonic fungal communities. However, the number of their sequences only accounted for 40.42 ± 0.03% of the total high-quality sequences. Shannon, Simpson, and Chao1 indexes of the fungal communities in the high-water period were significantly higher than those in the low-water period. The relative abundances of *Phoma brasiliensis*, *Pseudozyma* sp., *Psathyrella* sp., *Haematonectria haematococca*, *Podoscypha* sp. in the high-water period were significantly higher than those in the low-water period, while the relative abundances of *Basidiobolus* sp., *Rhizophydium littoreum*, *Teratosphaeria jonkershoekensis*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia* sp., *Alternaria eichhorniae*, *Knufia epidermidis*, and *Scedosporium prolificans* were significantly lower. No significant correlation between the fungal community distance and geographical distance along the river. Water temperature and dissolved oxygen significantly influenced the fungal community structure in the high-water period. However, it is necessary to investigate the community compositions of phytoplankton fungi in river at a larger time and space scale in the future, and more freshwater phytoplankton fungi were needed to study through culturing method and their genomic database was needed to supplement. In addition, the relationship among planktonic fungi, bacteria, and multicellular organisms still needs to further study.

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HABITAT CHARACTERISTICS AND POPULATION STRUCTURE OF *DIPTERIS CHINENSIS*, A RELICT PLANT IN CHINA

OUYANG, K. T.[1,2] – REN, H.[1,2*] – XU, Z. H.[1,2] – WANG, F. G.[1] – LIU, S. Z.[1] – ZHANG, Q. M.[1] – HU, M. F.[3] – ZHANG, Y. J.[3] – LIU, Z. J.[3] – GUO, Q. F.[4]

[1]*Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China*

[2]*University of Chinese Academy of Sciences, Beijing 100049, China*

[3]*Nanling National Nature Reserve Administration, Shaoguan 512727, China*

[4]*Eastern Forest Environmental Threat Assessment Center, USDA Forest Service, Research Triangle Park NC27709, USA*

[*]*Corresponding author*
e-mail: renhai@scbg.ac.cn

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Abstract. *Dipteris chinensis* Christ is endemic to China and is at risk of extinction due to anthropogenic disturbance and climate change. Our understanding of *D. chinensis* population structure and habitat is poor. Here, we investigated the habitat characteristics and population structure of a *D. chinensis* community at Chenjia in the Nanling National Nature Reserve, southern China. We conducted a principal component analysis followed by correlation analysis to identify the main habitat factors associated with the abundance of young *D. chinensis* plants. We found that the species grew in a warm and humid environment under evergreen coniferous and broad-leaved mixed forests. Young *D. chinensis* abundance was positively correlated with the litter standing crop, soil organic matter content, and soil total nitrogen. However, the production of new *D. chinensis* plants appeared to be limited by a low reproduction rate (as evidenced by a population age structure skewed toward mature plants) and interspecific competition. These findings are useful for directing and prioritizing future conservation efforts.
Keywords: *conservation, fern, community structure, habitat factors, correlation analysis*

Introduction

Habitat loss and fragmentation caused by human disturbance coupled with global climate change threaten plant diversity worldwide (Ren et al., 2019). Many countries have made direct efforts for the conservation of flowering plant diversity, especially the protection of rare and endangered plants (Thomas and Stohlgren, 2013; Dong et al., 2017) but other smaller plant groups such as ferns are often left under-protected. Rare and endangered ferns (pteridophytes) play a key role in maintaining overall plant diversity and ecosystem stability, and are critical for understanding species origin, evolution, and classification (Murakami et al., 2005). Pteridophytes also have economic values as food and medicine and as ornamental plants (Lu, 2007). In addition, because ferns are sensitive to changes in the climate and other environment, they may serve as indicators of environment change (Lu, 2007). Nevertheless, few studies have examined the population structure and habitat characteristics of rare and endangered pteridophytes.

Dipteris chinensis Christ (Cheng, 2005) is a perennial herb in the family Dipteriaceae. Its Chinese common name, octopus fern, reflects the unique shape of its leaves (*Fig. 1*). Like many other pteridophytes, *D. chinensis* is a relict species and is endemic to China

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with limited distributions in Chongqing, Guizhou, Guangxi, Hunan, and Guangdong Provinces. Its rhizome is long and transverse, woody, with one leaf. Plant height is mostly between 0.6 and 2.0 m. Its leaves are papery and double fan-shaped, i.e., the middle part of each leaf is divided to form two symmetrical fans. Each fan is further divided into 4-5 unequal lobes, and the leaf width is 40-60 cm (Lu, 2007). Pteridophytes include only eight species belonging to one genus (*Dipteris*) and have narrow geographical distributions and quite limited genetic diversity (Zhou, 2015; Chen, 2016). Studies of pteridophytes include taxonomic revision and phylogenetic evaluation of the Dipteridaceae (Lu and Tian, 2011; Choo and Escapa, 2018), morphology of the genus *Dipteris* (Chandra and Kaur, 1993), and extraction and application of pteridophyte-derived compounds (Wang, 2010). This species has not been protected except for individuals growing in the natural reserve. No reports are available, however, on the population structure and habitat of *D. chinensis*.

Figure 1. *Dipteris chinensis age classes. (a) Young age class; (b) Middle age class; (c) Old age class; and (d) The abaxial surface of a mature sporophyll*

In this research, we studied the habitat characteristics and population structure of *D. chinensis* at the Chenjia management station of the Nanling National Nature Reserve. We attempted to answer the following questions: (1) Which habitat factors control the abundance of young *D. chinensis*? and (2) Does the age structure of the *D. chinensis* population indicate population trend (increasing or decreasing)? The results may contribute to the protection and sustainable utilization of *D. chinensis.*

Materials and Methods

Study site

This investigation was conducted at the Chengjia of Nanling National Nature Reserve of Yangshan County, Guangdong Province, China (24°42′–24°47′N, 112°51′–112°57′E) (*Fig. 2*). The study site is located in a mountainous area at the altitude of 800-900 m and has a subtropical monsoon climate (Wang et al., 2007). The mean annual temperature is 17.7℃, with the highest temperature of 34.4℃ in July and the lowest temperature of -4℃ in January (1954) (Liu et al., 2020). The mean annual relative humidity is 84%, and the mean annual precipitation is about 1705 mm. Frost and snow occur in winter. The area has a yellow-red soil (Wang et al., 2013). The main vegetation type at the nature reserve is subtropical mountain evergreen broadleaved forest.

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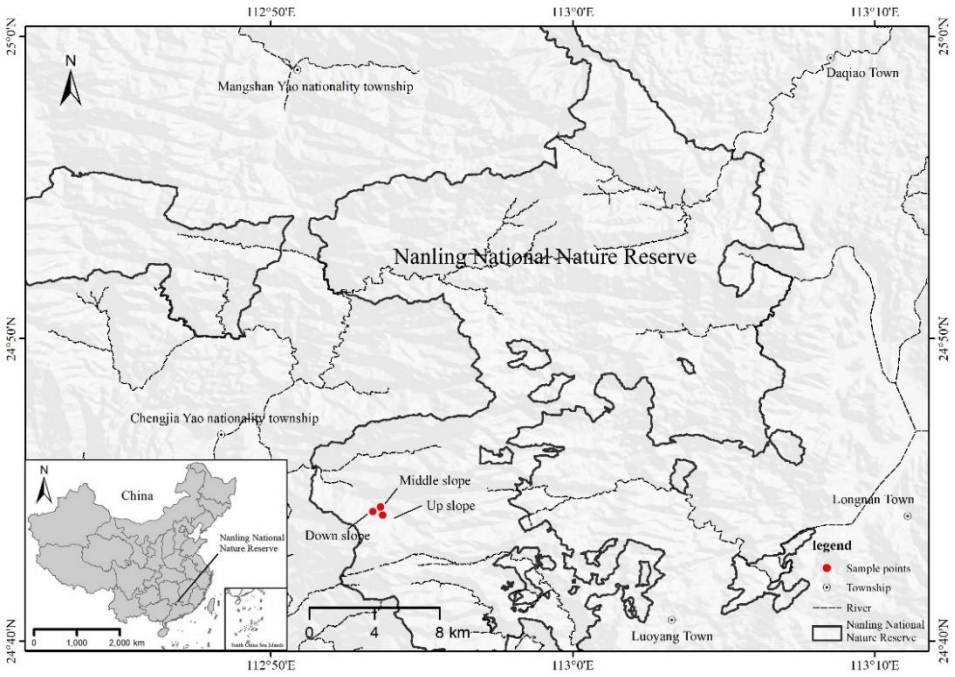

Figure 2. *The geographical location of the sampling sites of Dipteris chinensis at the Nanling National Nature Reserve, southern China. Red polygon in small map indicates the Nanling National Nature Reserve. Sample plots were set in red dots*

Field survey and laboratory analysis

In June 2020, we established three 20 m×20 m plots on slopes of the *D. chinensis* distribution area (*Table A1*), in addition to measuring the altitude and slope of each plot, we identified all tree species in 20 m×20 m plot and determined their numbers and the height, as well as trunk and crown diameters of each tree. We also identified all shrubs in one 5 m×5 m subplot (for shrubs) in the center of each plot, and identified all herbs in two 1 m×1 m quadrats (for herbs) in each subplot (Zhou, 2019). To investigate the age structure of the *D. chinensis* population, we counted the number of individuals in each age class (see *Fig. 1*; a young sporophyte has hair, light green leaf and stem, no notch and sporangium; the sporangia appeared in the middle age sporophyte than in the young stage; an old sporophyte has black or black brown stem and its leaf with deep notch is leathery, glabrous, green, and has many orange black spots attached on the abaxial surface) in each of the three subplots. To identify the dominant plant species in each plot, we calculated the importance values (*IV*) and niche breadth (*Bi*) for each plant species in each plot. We adopted the method by Li (2018) and Ge (2008), and calculated *IV* and *Bi* with *Eq.1*, *Eq.2* and *Eq.3*.

$$IV = \frac{Relative\ density + Relative\ coverage + Relative\ frequency}{3} \quad \text{(Eq.1)}$$

$$IV = \frac{Relative\ density + Relative\ dominance + Relative\ frequency}{3} \quad \text{(Eq.2)}$$

$$Bi = \frac{1}{\sum_j^r (nij/Ni)^2} \quad \text{(Eq.3)}$$

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where *Bi* is the niche breadth of species *i*, n_{ij} is the number of resource states *j* used by species *i* (in this paper, the *IV* of species in the plot), *Ni* is the total amount of species *i* (in this paper, the sum of *IV* of species in all plots), and *r* is the number of resource classes (number of plots).

Three species diversity indices of Shannon-wiener index (Magurran, 1988), Simpson index (Simpson, 1949), Pielou's index (Qian and Ma, 1994) were calculated with *Eq.4*, *Eq.5* and *Eq.6*.

$$H'_e = -\sum_{i=1}^{S} P_i \ln P_i , P_i = \frac{n_i}{N} \quad \text{(Eq.4)}$$

$$\mathrm{D} = 1 - \sum_{i=1}^{S} P_i^2 \quad \text{(Eq.5)}$$

$$J_e = \frac{H'e}{H'max} H'_{max} = \ln S \quad \text{(Eq.6)}$$

where P_i was the relative importance of species *i* in the quadrat, and *S* was the number of species.

To examine the relationship between the environmental factors and young *D. chinensis* abundance, we randomly collected soil samples from nine points to 10 cm depth, and three soil ring knife samples in each of the 20×20 m plots (Zhang, 2018); leaf litter was removed before soil samples were collected. The three soil samples from each plot were combined to yield one composite soil sample per plot, i.e., three composite soil samples (down slope, middle slope and upslope) and were transported to the laboratory for analyses. The contents of soil organic matter, soil total nitrogen, soil total phosphorus, and soil total potassium were measured by the dichromate method, the semi-micro Kjeldahl method, molybdenum antimony colorimetry, and atomic absorption spectrophotometry (ContrAA700, Analytik Jena, Germany), respectively. Soil pH (10 ml soil in 25 ml of water) was measured with a pH meter. Soil water content in the composite samples and bulk density for the ring knife samples were determined by measuring soil mass before and after oven-drying (Liu, 1996).

We collected litter samples from three randomly selected 1 m×1 m quadrats under *D. chinensis*. The masses of the samples were determined before and after drying at 65℃ (Liu et al., 2020). In September 2020, we measured the leaf area index (LAI; an indirect indication of light transmittance under the forest canopy) with an LAI-2200C instrument (LI-COR, USA) at 15 randomly selected points in each plot at 4:00 pm (Zhou, 2011).

Data analysis

To identify the life forms and the floristic phytogeography types of all plants in the studied community, we used the Raunkiaer lifestyle system (1934) and Floristics of Seed Plants from China (Li, 1996; Wu et al., 2010). We used the Vegan package in R language to analyze the species diversity (Zhang, 2010). We also performed two separate analyses to determine the relationship between habitat factors and young *D. chinensis* abundance. We then performed principal component analyses (PCA) in the Vegan package followed by Pearson correlation analysis in the Pysch package to identify the main habitat factors associated with the abundance of young *D. chinensis*. All statistical analyses were performed in R 3.6.3.

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Results

Community habitat characteristics

Species composition

A total of 52 species of vascular plants (28 families, 2 ferns, 1 lycophyte, 2 gymnosperms, and 47 angiosperms) were found in the *D. chinensis* community (*Table 1*). The species composition at the down slope and middle slope plots was similar (*Fig. 3*). The abundant families were Theaceae, Ericaceae, lauraceae, and Aquifoliaceae. Most species were represented by trees (88%), and others were herbs and vines (11%). Among vascular plants, more species were tropical than temperate, including 21 tropical families and 4 temperate families (Qin, 1978; Li, 1996; Zang, 1998; Wu et al., 2010). On the other hand, 33 genera were represented by 11 distribution types, most of which were tropical (*Table 2*).

Table 1. *Species composition of the Dipteris chinensis community*

Type	Number of families	Percentage of all families	Number of genera	Percentage of all genera	Number of species	Percentage of all species
Fern	2	7.14	2	6.06	2	3.85
Gymnosperm	2	7.14	2	6.06	2	3.85
Angiosperm	23	82.14	28	84.85	47	90.38
Lycophytes	1	3.57	1	3.03	1	1.92
Total	28	100.00	33	100.00	52	100.00

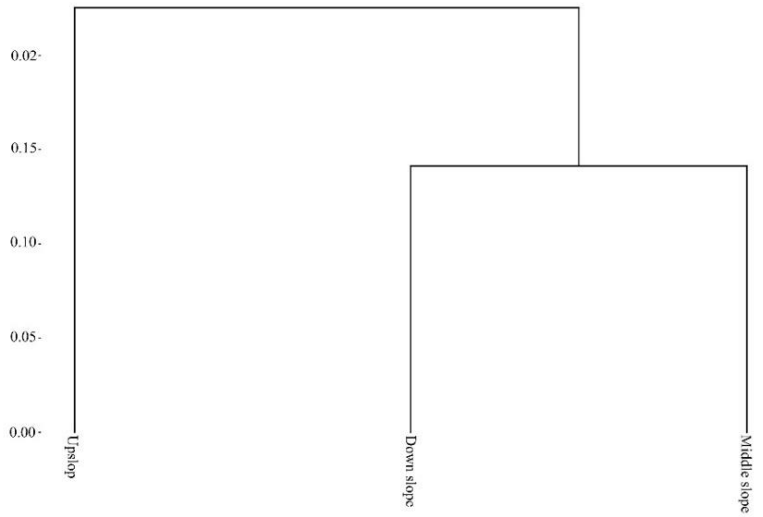

Figure 3. *The cluster diagram of sample plots of Dipteris chinensis communities at the Nanling National Nature Reserve, Southern China*

Composition of plant life form

Most of the vascular plants in the community were phanerophytes (*Fig. 4*). Microphanerophytes were the most abundant, followed by mesophanerophytes, nanophanerophytes, chamaephytes, and megaphanerophytes.

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Table 2. *Types of distribution of families and genera in the Dipteris chinensis community*

Distribution type	Number of families	Percentage of all families	Number of genera	Percentage of all genera
1. Cosmopolitan	3	–	0	-
2. Pantropic	16	57.14	5	15.15
3. Tropics & Subtropics East Asia & Tropical America Disjuncted	0	0.00	4	12.12
4. Old World Tropics	1	3.57	1	3.03
5. Tropics Asia to Tropics Australasia Oceania	1	3.57	4	12.12
6. Tropics Asia to Tropics Africa	0	0.00	2	6.06
7. Tropics Asia	3	10.71	6	18.18
Subtotal of tropical elements (2-7)	21	84.00	22	66.67
8. North Temperate	3	10.71	3	9.09
9. East Asia & North America Disjuncted	1	3.57	5	15.15
10. Old World Temperate	0	0.00	0	0.00
11. Temperate Asia	0	0.00	1	3.03
12. Mediterranean, West to Central Asia	0	0.00	0	0.00
13. Central Asia	0	0.00	0	0.00
14. East Asia	0	0.00	0	0.00
15. Endemic to China	0	0.00	2	6.06
Subtotal of temperate elements (8–15)	4	19.05	11	33.33

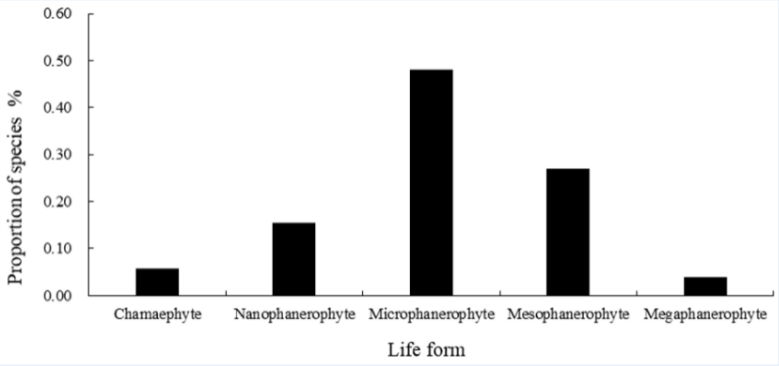

Figure 4. *Proportions of plant species in the Dipteris chinensis community at the Nanling National Nature Reserve, southern China represented by the indicated life forms*

Species diversity

In terms of diversity, the middle slope had the highest species richness, Shannon-Wiener diversity index, and Simpsons index, but the low Pielou index (*Table 3*). The down slope had the lowest species richness, Shannon-Wiener diversity index, and Simpsons index, but the highest Pielou index.

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Table 3. *Species diversity of the Dipteris chinensis community (Mean) at the Nanling National Nature Reserve, southern China*

Site	Species richness/plot	Shannon-Weinner index	Simpsons index	Pielou index
Down slope	21	2.58	0.89	0.85
Middle slope	39	2.93	0.91	0.80
Upslope	28	2.64	0.90	0.80
Total	52	3.03	0.91	0.78

Species importance and niche breadth

The dominant species in the *D. chinensis* community was *Cunninghamia lanceolata*, followed by *Rhododendron ovatum*, *Eurya macartneyi*, and *Machilus chinensis* (*Table 4*). The importance value for all other plant species was less than 0.10. The importance value and niche breadth were the same for *Dipteris chinensis*, *Diplopterygium laevissimum*, and *Indocalamus latifolia*.

Table 4. *Importance value and niche breadth of the top 20 species in Dipteris chinensis communities at the Nanling National Nature Reserve, southern China*

Number	Species	Importance value	Niche breadth
1	*Cunninghamia lanceolata*	0.57	2.67
2	*Rhododendron ovatum*	0.22	2.69
3	*Eurya macartneyi*	0.18	2.8
4	*Machilus chinensis*	0.11	2.54
5	*Adinandra glischroloma*	0.09	2.81
6	*Castanopsis fissa*	0.08	2.76
7	*Syzygium buxifolium*	0.07	2.47
8	*Machilus phoenicis*	0.07	2.84
9	*Castanopsis faberi*	0.07	1.72
10	*Castanopsis eyrei*	0.07	1.24
11	*Symplocos lancifolia*	0.06	1.85
12	*Daphniphyllum oldhamii*	0.06	2.85
13	*Vaccinium carlesii*	0.05	2.66
14	*Diplopterygium laevissimum*	0.05	2.99
15	*Indocalamus longiauritus*	0.05	2.99
16	*Dipteris chinensis*	0.05	2.99
17	*Rhododendron cavaleriei*	0.04	1.57
18	*Elaeocarpus chinensis*	0.04	2.65
19	*Dendropanax proteus*	0.03	2.91
20	*Symplocos stellaris*	0.03	2.91

Soil factor and leaf area index

The *D. chinensis* community was located on a slope of 15-30 degrees. Soil bulk density was 0.61 ± 0.31 g cm^{-3} (mean ± SD), and the mean soil moisture content was 29 ± 7%. Other means and standard deviations were 4.67 ± 0.09 for soil pH, 5.54 ± 0.74% for soil

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organic matter content, 4.09 ± 0.65 g kg^{-1} for soil nitrogen content, 0.19 ± 0.03 g kg^{-1} for soil phosphorus content, and 0.98 ± 0.22 g kg^{-1} for soil potassium content. The litter standing crop (dry mass) was 1.14 ± 0.25 kg m^{-2}. The mean leaf area index of the community was 1.75 ± 0.81.

Relationship between young D. chinensis abundance and habitat factors

Results from principal component analyses (PCA) using 12 selected habitat factors (*Table 5*) indicated that the first three axes (PCA1, 2 and 3) explained 29%, 25% and 22%, respectively, of the variation in the abundance of young *D. chinensis*. These three axes together explained 76% of the total variation. The first axis was significantly related to soil organic matter, soil total nitrogen and soil pH, we suspect the young *D. chinensis* tended to grow in habitats with high soil nutrient content. The second axis was significantly correlated with soil total potassium, population density and litter standing crop, which indicates the young fern prefers to live in these habitats with higher soil potassium and relative humidity. The third axis was significantly associated with leaf area index and population coverage, suggesting that *D. chinensis* adapted to more shade environment.

Table 5. *The loading matrix of habitat factors on the first two axes of PCA of young Dipteris chinensis community at Chenjia in the Nanling National Nature Reserve, southern China*

Habitat factor	Principal component		
	1	2	3
Soil organic matter	0.94	-0.10	-0.16
Soil total nitrogen	0.93	-0.09	0.17
Soil total phosphorus	0.2	0.26	-0.83
Soil total potassium	-0.35	0.87	-0.22
Soil pH	0.87	0.20	0.12
Soil water content	0.69	-0.50	0.08
Soil density	0.11	0.23	0.49
Litter standing crop	0.14	0.77	0.18
Leaf area index	0.27	-0.15	0.81
Slope	0.48	-0.83	0.20
Population density	0.12	0.71	0.29
Population coverage	-0.03	0.42	0.82
Eigenvalue	3.51	3.17	2.54
Percentage of variance (%)	29.00	25.00	22.00
Percentage of cumulative variance (%)	29.00	54.00	76.00

We then conducted a Pearson correlation analysis of the relationship between young *D. chinensis* abundance and those habitat factors with the large loading values on the first three axes of PCA. The results showed that there was a significant positive correlation between young *D. chinensis* abundance and the litter standing crop ($P = 0.05 \leq 0.05$), soil organic matter content ($P = 0.01 \leq 0.01$), soil total nitrogen ($P = 0.03 < 0.05$).

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Population structure of D. chinensis

The *D. chinensis* population was skewed toward the mature age class, and young plants represented only a small proportion of the population (*Fig. 5*).

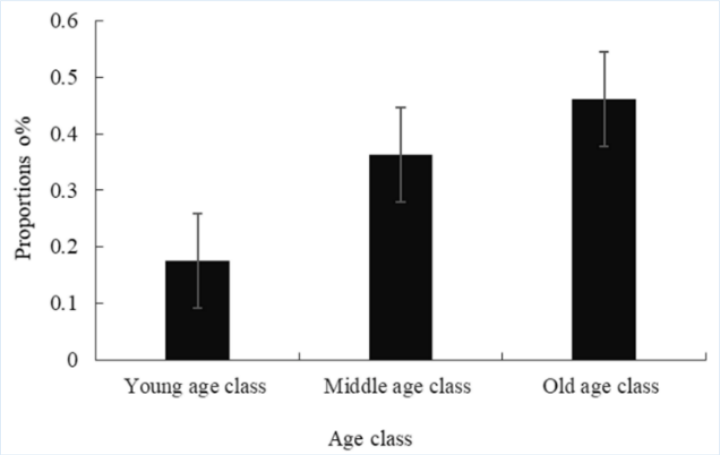

Figure 5. *Age structure of Dipteris chinensis populations at the Nanling National Nature Reserve, southern China*

Discussion

Relationship between the young D. chinenesis and habitat factors

Dipteris chinensis prefers to grow in a warm and humid environment (Zhou et al., 2015). In our study, consistent with previous reports that *D. chinensis* prefers to grow in a warm and humid environment (Chen, 2016), we found that the *D. chinensis* population in the current study was part of a tropical plant community, and the vascular flora in this community has tropical floristic characteristics (*Table 2*). At the Nanling National Nature Reserve, where the current research was conducted, *D. chinensis* is distributed on a northeast-facing hillside at 800 to 900 m. The area has a subtropical monsoon climate with frequent precipitation and high humidity, which promotes the rapid growth of phaenerophytes (Li et al., 2017). Consistent with the latter study, we found that the *D. chinensis* was a member of a plant community dominated by phanerophytes (*Fig. 4*), which are kinds of large shrubs and trees that support understory plants by maintaining a warm environment in the winter and a cool environment in the summer. By intercepting the strong light in summer and cold winds in the winter, the phanerophyte canopy provides an environment conducive for the survival of *D. chinensis* (Andivia et al., 2018).

We detected a positive correlation between the abundance of young *D. chinensis* and the litter standing crop. As noted earlier, *D. chinensis* prefer to grow in a warm and humid environment, and litter covering the soil surface can help maintain soil moisture and temperature (Boeken and Orenstein, 2001; Loydi et al., 2013) and can thereby provide a suitable environment for the clonal growth of *D. chinensis*. We also detected a positive correlation between young *D. chinensis* abundance and the content of soil organic matter as well as soil total nitrogen. As we found that *D. chinensis* in the study formed ramets by rhizomes, however, in the initial phase of pteridophyte clonal growth, the growth process of young sporophytes with poor resistance is dependent on the nutrient supply of the mother plant and the nutrient content originated from the habitat (Du et al., 2010;

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Zhang et al., 2019). Indeed, from mother plant to budding sporophyte, the mother plant needs to consume a massive of energy materials (Slade and Hutchings, 1987). Under a suitable environment, the mother plant will allocate more biomass to the rhizome to produce more clonal ramets, on the contrary, it inhibits the growth of rhizome in a barren environment (Huber-Sannwald et al., 1998). In addition, similar results have been found in other studies. Liu (2017) found that the young sporophytes of *Dicranopteris pedata* died due to insufficiency in nutrients. More importantly, Soil nitrogen is an important limiting factor for plants growth and development (Lars, 2004), which affects the plants distribution to some extent (Wang et al., 2021). Nitrogen plays a principal role in plant life history such as height, and leaf and root tiller growth (Pan, 2012). The clonal propagation of rhizomes increases the number of individuals, indicating the importance of soil nitrogen content for the growth of young *D. chinensis*.

Dipteris chinensis, I. longiauritus and *D. laevissimum* in the community have similar niche breadths (*Table 4*). Their rhizomes absorb more nutrients from soil when they are in the growth peak period, and the limited soil resources may cause interspecific competition. *Indocalamus longiauritus*, with the strong carbon capture capacity and high water and photosynthesis use efficiency (Montti et al., 2014; Fadrique et al., 2020), has the fastest growth rate of all plants (Pearson et al., 1994). It usually dominates the understory and shows strong competitive advantage compared with the seedlings of other species (Bai et al., 2011). The genus *Diplopterygium* usually consume large amounts of soil nitrogen during its vigorous period (Ai, 2010). These two herbs (*I. longiauritus* and *D. laevissimum*) not only occupy much soil spaces but also seize a tremendous amount of soil nutrients during their growth, therefore are not conducive to the population regeneration of *D. chinensis*.

A declining population of D. chinensis

The *D. chinensis* population is indeed in decline. One possible reason for the decline is that *D. chinensis* cannot generate juvenile sporophytes through spore reproduction due to low soil humidity, and mainly depends on clonal propagation via rhizomes (Sun et al., 2019). However, this regeneration process is affected by its self-conditions (initial costs of mother plants), as shown by Pearson correlation analysis that the amount of soil organic matter and litter standing crop are significantly affecting the young *D. chinensis individual*; therefore, the supplement of the young fern is reducing. Another explanation for the decline in *D. chinensis* is competition with *Indocalamus longiauritus* (a bamboo: Zhao and Yang, 1985: 232) and *Diplopterygium laevissimum* (Holttum, 1991: 11). The rhizomes of the bamboo spread rapidly on the soil surface to form a root network thus may slow the clonal spread of *D. chinensis*.

Conclusions

Our study indicates that the litter standing crop, soil organic matter, and soil nitrogen are significantly related to the abundance of young *D. chinensis.* The species shows a declining trend in population size at the Nanling National Nature Reserve in south China, perhaps because of competition with *Indocalamus longiauritus* and *Diplopterygium laevissimum* and lack of effective litter coverage on soil surface. In order to protect *D. chinensis* more effectively, we recommend removing *I. longiauritus* and adding litter to increase *D. chinensis* abundance. Research is also needed to enhance *D. chinensis's* sexual reproduction.

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APPENDIX

Table A1. *Overview of the sample plots of Dipteris chinensis at the Nanling National Nature Reserve, southern China*

Site	Latitude & longitude	Elevation(m)	Slope(°)	Aspect	Soil thickness(m)	Depth of the bedrock (m)
Down slope	24°44′23″N,112°53′34″E	889	15	Northeast	>0.5	>5.0
Middle slope	24°44′25″N,112°53′36″E	863	20	Northeast	>0.5	>5.0
Upslope	24°44′45″N,112°54′40″E	823	30	Northeast	>0.5	>5.0

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PHYSIOLOGICAL AND MOLECULAR MECHANISMS UNDERLYING SALT STRESS TOLERANCE IN JOJOBA (*SIMMONDSIA CHINENSIS*)

ALGHAMDI, B. A.[1*] – BAFEEL, S. O.[1] – EDRIS, S.[1,2,3] – ATEF, A.[1] – AL-MATARY, M.[1] – BAHIELDIN, A.[1,2]

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University (KAU), Jeddah, Saudi Arabia
(e-mail/ORCID: sbafil@kau.edu.sa/0000-0002-7912-1993 – O. Bafeel;
sedris@aucegypt.edu/0000-0002-2810-3736 – S. Edris;
ahmed_atefaig2@yahoo.com/0000-0001-7530-2060 – A. Atef;
m.matri777@gmail.com/0000-0002-8815-0489 – M. Al-Matary;
bahieldin55@gmail.com/0000-0002-6496-7881 – A. Bahieldin)

[2]Department of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

[3]Princess Al-Jawhara Al-Brahim Centre of Excellence in Research of Hereditary Disorders (PACER-HD), Faculty of Medicine, King Abdulaziz University (KAU), Jeddah, Saudi Arabia

**Corresponding author*
e-mail: Budouralghamdi@gmail.com; ORCID: 0000-0003-4048-6052

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Abstract. The study aims to characterize the leaves of Jojoba (*Simmondsia chinensis*) under salt stress (50, 100, and 200 mM NaCl) at physiological and molecular levels. Physiological analysis included photosynthetic pigments of chlorophylls-a, and -b as well as carotenoids. H_2O_2 content as well as two antioxidant enzymes, namely catalase and ascorbate peroxidase, were also analyzed. Overall results of physiological parameters indicated their differential response due to the stress. Molecular analysis, done via RNA-Seq and mapping highly expressed transcripts to KEGG pathways, resulted in a large number of regulated genes under salt stress, of which, 13 genes in six pathways were further studied referring to the studied physiological parameters. These genes included *ALDO* and *TKT* that promote both Calvin-Benson cycle and the production of erythrose-4-phosphate that acts upstream nine genes towards better growth under stress conditions. Other genes included *HAO* that mediates crosstalking of "carbon fixation in photosynthetic organisms" pathway and *APX* that participates in "Ascorbate and aldarate metabolism" pathway. Overall, the six pathways were proven to crosstalk under salt stress in Jojoba to provide salt stress tolerance. This information can help using this plant as a source of biodiesel in salinized soil or areas of water scarce.
Keywords: *Chl, carotenoids, H_2O_2, CAT, APX, ALDO, TKT, HAO, RNA-Seq, carbon fixation, tryptophan metabolism*

Introduction

Salt stress imposes a challenge in agriculture sector worldwide (Liang et al., 2018). It results in the alteration of some metabolic pathways in plants underlying many physiological and biochemical processes (Bafeel et al., 2016). Next-generation sequencing (NGS) technologies coupled with computational tools allowed the study of transcriptomes of model and non-model organisms, especially those with no available genome sequencing data like *Simmondsia chinensis* (Link) Schneider (Martin and Wang, 2011; Garber et al., 2011; Jain, 2012; Nejat et al., 2018). The high-throughput RNA-Seq technology allows the study of the whole plant transcriptomes, the dynamics of gene expression and regulation towards the adaptability to soil salinity (Wang et al., 2009; Sailaja et al., 2014; Cao et al., 2018; Nejat et al., 2018). Understanding the

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physiological and molecular dynamics of salt stress responses will allow the development of genotypes with enhanced salt tolerance. Studying the plant response to such adverse conditions at the molecular and physiological levels will provide a new avenue towards the improvement of the agricultural productivity.

Jojoba (*Simmondsia chinensis*) is known for its important applications in biodiesel industry. It has several other applications due to medicinal properties including cosmetics and personal care formulations (Passerini and Lombardo, 2000; Al-Obaidi et al., 2017). However, due to the scarce of information of transcriptome of Jojoba, it is a challenge to detect genes and transcription factors that might confer salt stress tolerance. Transcriptomic analysis of plant responses to salt stress based on RNA-Seq has been performed in various species including *Rhazya stricta* (Hajrah et al., 2017), Arabidopsis (Kawa and Testerink, 2017), barley and rice (Ueda et al., 2006). As a result of abiotic and biotic stresses, production and accumulation of toxic reactive oxygen species (ROS) have been recorded by many researchers. These conditions result in oxidative stress and cause membrane leakage through increased lipid peroxidation (Bowler and Fluhr, 2000; Shah et al., 2001; Mittler, 2002; Panda and Khan, 2004; Wu et al., 2014). ROS also affects other important macromolecules including proteins, nucleic acids, photosynthetic pigments and lipids (Mittler, 2002; Wu et al., 2014; Hend et al., 2015). Antioxidants include both enzymatic (Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), etc.) and non-enzymatic components that can reduce the chance of facing oxidative stress conditions by mediate scavenging of ROS (Mittler, 2002; Wu et al., 2014; Hend et al., 2015; Abd-Allah et al., 2015).

The present study aims at detecting transcriptome of Jojoba (*Simmondsia chinensis* (Link) Schneider) under salt stress in order to get better insights into some physiological mechanisms by which this plant species tolerates the stress. We will also try to get a better understanding of molecular processes complying to physiological data.

Materials and methods

Plant material sampling and watering regime

Experiment was conducted at the laboratories of the Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia. Jojoba seeds were grown in 10 pots in a growth chamber (9 cm, 3 seeds/pot) filled with soil mix (1 soil: 1 vermiculite) and watered with deionized double distilled water under the following growth conditions; e.g., 21 ± 2 °C (day/night) under light intensity of ~175 umoles m^{-2} s^{-1} and a 16 h-light/8 h-dark cycle. Experiment was designed as shown in *Figure 1*. Emerged plantlets were allowed first to grow for 19 days, then, they were morphologically screened for homozygosity and accordingly number of plantlets per pot was narrowed to one (Bahieldin et al., 2015). Then, pots were divided into two groups. The first group (five pots) was continued to be irrigated every five days with deionized double distilled water (control), while the other group was salt stressed (treatment). It was important to use distilled water to avoid impurities and indigenous microbes that can pose additional stress (biotic) to the plant. The plantlets were irrigated three times with intervals of five days. Salt stressed plantlets were first irrigated with 50 mM NaCl, followed by 50 and 100 mM NaCl incremental increases, respectively, of salt stress as recommended by Munns (2002). The samples of the salt stressed plantlets were harvested after plantlets were exposed to 50, 100 and 200 mM NaCl, respectively.

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Then, leaf samples of stressed and non-stressed plantlets were harvested the second day of irrigation at days 25, 30 and 35, respectively. Then, replicates of each group were divided for molecular and physiological analysis.

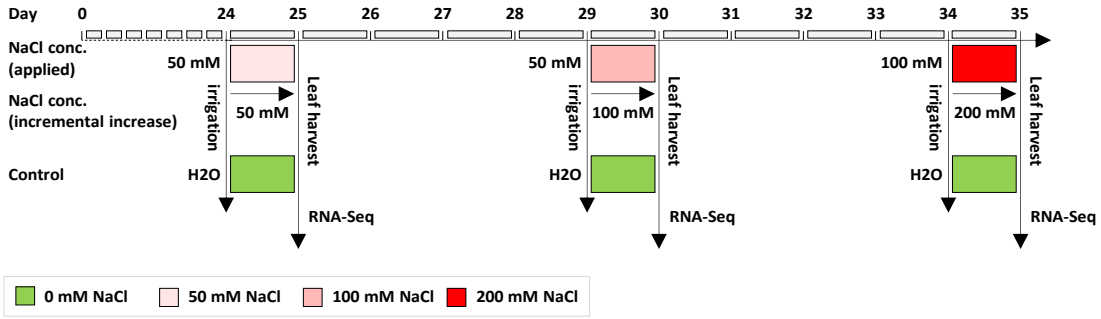

Figure 1. *Schematic representation of the salt stress experiment conducted starting day 24 at three NaCl concentrations increased incrementally with five-day intervals (50, 100, and 200 mM) in Jojoba plant. Leaf samples were harvested the second day of applying salt treatments (days 25, 30 and 35, respectively) for RNA-Seq analysis*

Physiological experiments

Determination of photosynthetic pigments

Chlorophylls and carotenoids were measured using UV-VIS Spectroscopy according to Hiscox and Israelstam (1979) with some modifications (Su et al., 2010). 0.1 g of each fresh green leaf sample (three replicates) was placed in a test tube. Then, 5 ml of ethanol (95%) was added to each tube at 60 °C. then (green solution) was homogenized with extraction and placed in a cuvette. The absorbance readings were determined spectrophotometry by Lambda 25 UV-Vis spectrometer at wavelengths 663, 644, and 452 nm. Two ml of ethanol (95%) was used as a blank. Chlorophylls and carotenoids concentrations were calculated using *Equations 1, 2* and *3,* respectively, according to Sumanta et al. (2014) as follows:

$$\text{Chlorophyll-a } (Chl_a) = (13.36, A664) - (5.19, A649) = \mu g/ml \quad \text{(Eq.1)}$$

$$\text{Chlorophyll-b } (Chl_b) = (27.43, A649) - (8.12, A664) = \mu g/ml \quad \text{(Eq.2)}$$

$$\text{Carotenoid (car)} = [1000, A470 - (2.13\ Chl_a - 97.63\ Chl_b)] / 209 = \mu g/ml \quad \text{(Eq.3)}$$

Determination of hydrogen peroxide (H_2O_2) content

H_2O_2 content was measured according to Mukherjee and Choudhuri (1983) with some modification. H_2O_2 was extracted from homogenized leaf tissue (0.5 g) eluted in 5 mL of cold acetone. The extract was centrifuged at 6000 g for 25 min, then, 5 ml of sulfuric acid was added to 0.5 g of titanium dioxide, and solution was heated gently until fumes of sulfuric acid are evolved. After cooling, water was gradually added to reach 100 ml. Then, 3 ml of supernatant was mixed with 1 ml of titanium sulfate in H_2SO_4 and the mixture was centrifuged at 6000 g for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm and H_2O_2 level was calculated using the extinction coefficient of 0.28 $\mu mol^{-1}\ cm^{-1}$.

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Antioxidant enzymes activity

Antioxidant enzymes extraction

The enzymes were extracted according to Cakmak and Marschner (1992) with some modifications. Leaves tissue (0.5 g) was grounded in liquid N_2 to fine powder. Then, it was homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) with 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 g polyvinyl pyrrolidone (PVP). The samples were then centrifuged at 18,000 rpm for 10 min at 4 °C, and the supernatants were collected to be used for enzymes activity assays.

Enzyme activities

(1) Catalase (CAT) (EC 1.11.1.6)

Determination of CAT activity was assayed according to the spectrophotometric modified method of Aebi (1984). The reaction medium contained 2600 ml of 50 mM potassium phosphate buffer (pH 7), and 100 µl of the enzyme. The reaction initiated by adding 300 µl of 10 mM H_2O_2. Three readings were scored, each after 3 min. The absorbance was measured in spectrophotometer at 240 nm. The catalase activity was calculated using the molar extinction coefficient 39.4 mmol/cm. Enzyme activity was then calculated using *Equation 4:*

$$\text{Enzyme activity} = \frac{\Delta\ \text{CAT} \times \text{extraction volume}}{\text{Fwt.} \times \text{analysis volume of enzyme extract}} \times \text{extinction coefficient} \quad \text{(Eq.4)}$$

(2) Ascorbate peroxidase (APX) (EC 1.11.1.11)

Determination of Ascorbate peroxidase activity was assayed according to the spectrophotometric method of Nakano and Asada (1981). The activity was determined by the decreased absorption at 290 nm at 3 min-intervals due to ascorbate oxidation. Then, 200 µl of enzyme extract was added in a cuvette and 2.5 ml of 50 mM potassium phosphate buffer (pH 7) was added. Then, 100 µl of 5 mM H_2O_2 and 50 µl of 0.1 mM Na_2-EDTA were added. Finally, 100 µl of 0.5 mM ascorbic acid was added to start the reaction. The absorbance was measured in spectrophotometer at 290 nm. The ascorbate peroxidase activity was calculated using the molar extinction coefficient 2.8/mmol × cm. Enzyme activity was then calculated using *Equation 5*:

$$\text{Enzyme activity} = \frac{\Delta\ \text{APX A} \times \text{extraction volume}}{\text{Fwt.} \times \text{analysis volume of enzyme extract}} \times \text{extinction coefficient} \quad \text{(Eq.5)}$$

Statistical analysis

Paired comparison of T-test was made between the control and treatment samples across each concentration. Threshold of p-value $\leq .05$ was considered statistically significant.

Molecular analysis

Flash-frozen leaf materials from individual Jojoba plant samples were crushed into a fine powder in a microcentrifuge tube using a sterilized metal rod. Total RNA was extracted from three similar-sized (10 mm^2) leaf discs per plant (approximately 50 mg tissue) using Trizol (Invitrogen) and treated with RNase-free DNase (Promega Inc.).

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The yield and quality of RNA were determined using a Nanodrop-8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). After RNAs were quantified, 30 µg (400 ng/µl) was used for RNA-Seq. To test for the presence of DNA contamination in RNA samples, the *actin* gene was amplified by PCR of the original RNA samples. RNA samples were then shipped to Beijing Genome Institute (BGI), Beijing, China for deep sequencing. Next-generation sequencing using illumina Miseq generated raw data in FASTQ format. Raw transcriptomic data of Jojoba were submitted to the NCBI for reviewing and receiving accession numbers.

Raw data was filtered and trimmed for low-quality score reads, then, adaptor and primer sequences were removed and reads less than 40 bp were removed using Trimmomatic v0.30 (Bolger et al., 2014). Sequencing data with Phred quality score $Q \geq 20$ was further used in assembly. Expected read counts were used as input to differential expression analysis by EdgeR package (version 3.0.0, Robinson et al., 2010). Analysis of the RNA-Seq datasets indicated the recovery of >5 million reads per sample. *De novo* assembly was done for the different samples using the Trinity RNA-Seq Assembly package (r2013-02-25) with optimized parameters and K-mer size set to 25. CLC Genomics workbench (CLC Bio, Boston, MA 02108 USA) was used to validate the assembled transcript contigs by mapping high-quality reads back to the assembled transcript contigs. To identify the coding DNA sequences (CDS) from assembled transcript contigs, an online tool ORF-Predictor (Min et al., 2005) (http://proteomics.ysu.edu/tools/OrfPredictor.html) with the default parameters was used. Then, differential expression and cluster analysis were done by EdgeR (version 3.0.0). Blastx was performed (with an E-value cut off of $1e^{-5}$) and fold change values of differentially expressed transcripts were measured against the *actin* used as the house-keeping gene.

Significant Pearson correlation was determined during permutation analysis. The generated clusters were analyzed for GO terms using Blast2GO (http://www.blast2go.org/). GO terms for all the BLASTX functionally annotated CDS was retrieved using GO mapping. CDS were categorized by WEGO analysis which involved sketching a WEGO plot based on GO hits. To retrieve GO terms for annotated CDS, the GO mapping used defined criteria. This included use of (i) BLASTX result accession IDs to retrieve gene names or symbols, (ii) UniProt IDs, and (iii) direct search in the dbxref table of the GO database. Gene names or symbols thus identified were then searched against the species-specific entries of the gene-product tables in GO database. To retrieve UniProt IDs, Protein Information Resource (PIR) was used. PIR includes protein sequence database (PSD), UniProt, SwissProt, TrEMBL, RefSeq, GenPept, and PDB databases. Using GO analysis, all the annotated nodes comprising GO functional groups were specified.

All predicted CDS were annotated against protein database in order to assign putative function of the transcriptome after translation into protein. The regulated genes under salt stress (200 mM NaCl) were mapped across its corresponding control to reference canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/).

Results

Leaf samples were harvested 1, 6 and 11 days after plantlets initially exposed to salt stress. Due to the incremental increase of salt stress pressure, the concentrations of salt stress at the latter time points were 50, 100 and 200 mM NaCl. This took place when

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plantlets were 25-, 30- and 35-days old, respectively, after seeds were put for germination. The reason for the incremental increase of salt stress is to allow plants to gradually adapt to salt stress.

Physiological parameters related to salt stress

Plant pigments

According to the results in *Figure 2a,* chlorophyll-a (Chl_a) slightly inhibited in salt (50 and 100 mM NaCl) treated plants compared to their respective control plants at days 25 and 30, respectively, while slightly increased in salt treated plants compared to its respective control plant at day 35 with salt concentration of 200 mM NaCl. *Table A1* (see *Appendix*) indicates that the mean Chl_a contents in control and salt (50 mM NaCl) treated plants at day 25 were 2.56 and 2.42 mg/g FW, respectively, while Chl_a contents in control and salt (100 mM NaCl) treated plants at day 30 were 1.95 and 1.70 mg/g FW, respectively, and decreased to 1.36 mg/g FW at day 35 in control plants, while not changed (e.g., 1.71 mg/g FW) in salt (200 mM NaCl) treated plants.

None of the three time points showed significant differences between means of control and respective salt treated plants (*Table A1*). *Figure 2b* illustrates slight increase of chlorophyll-b (Chl_b) content in salt (100 mM NaCl) treated plants at day 30 compared to their respective control plants, while significant decrease at day 35 in salt (200 mM NaCl) treated plants compared to its respective control plants. *Table A1* indicates that the mean Chl_b contents at day 25 in control and salt (50 mM NaCl) treated plants were 3.14 and 3.04 mg/g FW, respectively, while Chl_b contents at day 30 in control and salt (100 mM NaCl) treated plants increased to 5.05 and 5.85 mg/g FW, respectively, and slightly changed to 5.48 and 4.76 mg/g FW, respectively, at day 35 in control and salt (200 mM NaCl) treated plants. *Figure 2c* showed no significant difference in the carotenoid content at day 25 between salt (50 mM NaCl) treated plants compared to its respective control plant, while significant increase in the carotenoid content in salt (100 mM NaCl) treated plants at day 30 compared to the control plants, and a significant decrease in salt (200 mM NaCl) treated plants at day 35 compared to the control plants. *Table A1* indicates that the mean carotenoid contents in control and salt (50 mM NaCl) treated plants at day 25 were 8.67 and 7.96 mg/g FW, respectively, while carotenoid contents were increased to 12.92 and 14.78 mg/g FW at day 30 in control and salt (100 mM NaCl) treated plants, respectively, and almost not changed (e.g., 13.57 mg/g FW) at day 35 in control plants, while reduced to 10.18 in salt (200 mM NaCl) treated plants. Overall, there are no significant differences between control and salt treated plants at 50 mM NaCl in terms of the three types of pigments (*Fig. 2* and *Table A1*).

Hydrogen peroxide (H_2O_2)

The results in *Figure 3* indicates that H_2O_2 concentration ($\mu mol^{-1}\ cm^{-1}$) significantly decreased at 50 and 100 mM NaCl, while significantly increased at the salt concentration of 200 mM NaCl. *Table A2* indicates that the mean H_2O_2 contents in control and salt (50 mM NaCl) treated plants at day 25 were 13.12 and 9.32 $\mu mol^{-1}\ cm^{-1}$, respectively, while contents were 11.71 and 8.99 $\mu mol^{-1}\ cm^{-1}$, respectively, at day 30 in control and salt (100 mM NaCl) treated plants and became 12.34 and 15.22 $\mu mol^{-1}\ cm^{-1}$, respectively, at day 35 in control and salt (200 mM NaCl) treated plants. The overall results indicate that H_2O_2 is highly sensitive to salt stress as its concentration significantly changed at different levels of salt stress.

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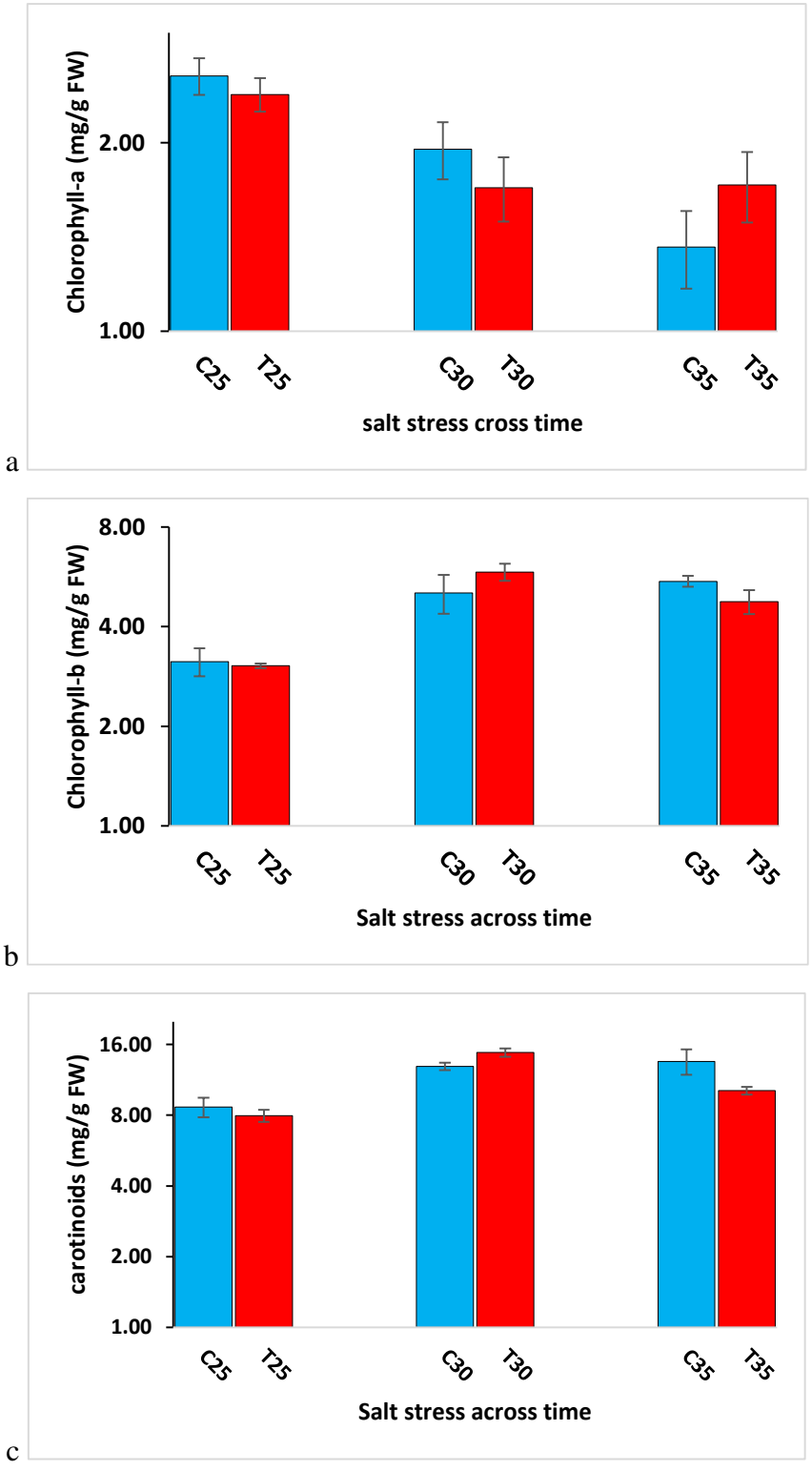

Figure 2. *Means of chlorophyll-a (a), chlorophyll-b (b) and carotenoids (c) (mg/g FW) in leaves of non-salinized (control) and salinized (treatment) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). Means were calculated for three replicates. Vertical bars indicate ± SE. Statistical analysis was done with P value of < 0.05. * = significant*

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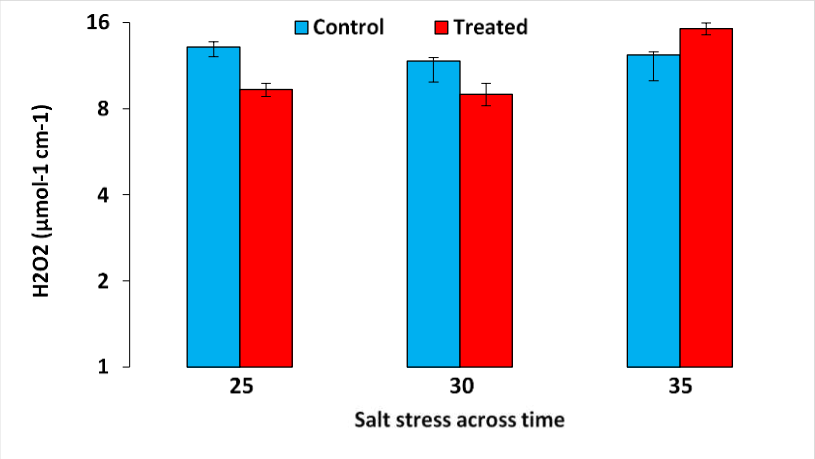

Figure 3. *Means of hydrogen peroxide (H_2O_2) concentration ($\mu mol^{-1}cm^{-1}$) in leaves of non-salinized (control) and salinized (treatment) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). Means were calculated for three replicates. Vertical bars indicate ± SE. Statistical analysis was done with P value of < 0.05. ** = very significant, *** = highly significant*

Enzyme activities

The results in *Figure 4* for catalase (CAT) activity (U/g FW) in plant leaves indicated significant increase at 50 and 100 mM NaCl, while non-significantly increased at 200 mM NaCl. *Table A2* indicates that the mean CAT activities in control and salt (50 mM NaCl) treated plants at day 25 were 13.11 and 27.16 U/g FW, respectively, while CAT activities were 41.88 and 168.53 U/g FW, respectively, at day 30 in control and salt (100 mM NaCl) treated plants and reduced to 35.35 and 45.93 U/g FW, respectively, at day 35 in control and salt (200 mM NaCl) treated plants. Aascorbate peroxidase (APX) activity (U/g FW), shown in *Figure 5,* in plant leaves significantly increased at 100 mM NaCl, while non-significantly increased at 50 and 200 mM NaCl. *Table A2* indicates that the mean APX activities in control and salt (50 mM NaCl) treated plants at day 25 were 4.58 and 5.98 U/g FW, respectively, while APX activities were 7.06 and 11.23 U/g FW, respectively, at day 30 in control and salt (100 mM NaCl) treated plants and became 14.84 and 15.63 U/g FW, respectively, at day 35 in control and salt (200 mM NaCl) treated plants. The overall results indicate that showed the same pattern of activity under salt stress as its level significantly increased at 100 mM NaCl salt stress, while no significant increases were observed at 50 and 200 mM NaCl. These results indicate that these two enzymes have no prolonged effect under salt stress.

Molecular genetic parameters related to salt stress

Assembly of transcripts resulted in a large number of genes regulated under the pressure of incremental increases of salt stress (*Table A3*). These genes were mapped to reference canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/). We have used the term enrichment referring to the increase of gene-encoded enzymes or metabolites in salt-stressed (200 mM NaCl) plants compared to the non-stressed. Enriched metabolite indicates that encoding gene is highly expressed under salt stress. The primary gene selection

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criterion was based on the high level (≥5 FC) of expression. In the present study, we focused on highly expressed genes in order to detect some molecular mechanisms used by Jojoba to respond positively to salt stress and probably confer tolerance. The secondary selection criterion involved genes complementing the detected physiological responses to salt stress. Expression levels of three of these genes (*ALDO*, *TKT* and *HAO*) were validated via qPCR and results complemented those of RNA-Seq (data provided upon request).

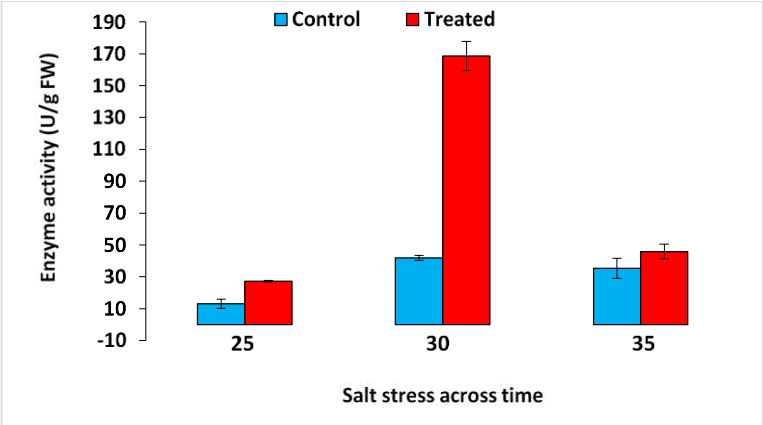

Figure 4. *Means of catalase (CAT) activity (U/g FW) in leaves of non-salinized (control) and salinized (treatment) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). Means were calculated for three replicates. Vertical bars indicate ± SE. Statistical analysis was done with P value of < 0.05. *** = highly significant*

KEGG analysis

Referring to the physiological parameters involved in the differential response to salt stress in Jojoba plant leaves, a number of 13 enzyme-coding genes in addition to five protein-coding genes were studied (*Table 1*). Among these pathways, "carbon fixation in photosynthetic organisms", "ascorbate and aldarate metabolism" and "glyoxylate and dicarboxylate" are major players (*Figs. A1-A3*, respectively). The "carbon fixation in photosynthetic organisms" pathway is the gate towards the opening of other downstream cross-talking pathways (*Figs. A4-A6*). The latter pathways are "phenylalanine, tyrosine and tryptophan biosynthesis" (*Fig. A4*), "tryptophan metabolism" (*Fig. A5*) and "plant hormone signal transduction" (*Fig. A6*). These pathways act in promoting plant's ability to stand harsh environmental conditions.

Carbon fixation in photosynthetic organisms

Physiological analysis in the present study indicated the involvement of the two chlorophyll (Chl_a and Chl_b) pigments as well as carotenoid pigment in the plant response to salt stress. These pigments are the main contributors to the process of carbon fixation during photosynthesis. Molecular analysis indicated the participation of two important upregulated genes in the "carbon fixation in photosynthetic organisms" pathway (*Fig. A1*). These two genes are *ALDO* that encodes fructose-bisphosphate aldolase (EC: 4.1.2.13) and the other gene is *TKT* that encodes transketolase (EC: 2.2.1.1) (*Table 1*). The two gene, e.g., *ALDO* and *TKT*, was upregulated under salt stress

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(50, 100 and 200 mM NaCl) (*Fig. 6*). *ALDO* gene participates in 11 pathways, while *TKT* gene participates in eight pathways (*Table 1*). In the "carbon fixation in photosynthetic organisms" pathway, fructose-bisphosphate aldolase is enriched under salt stress in Jojoba to participate in the conversion of glyceraldehyde-3-phosphate via two routes of the pathway (*Fig. A1*). The first route refers to the conversion of glyceraldehyde-3-phosphate to fructose-1,6-biphosphate, while the second route is a bypass through triosephosphate isomerase for the conversion of glyceraldehyde-3-phosphate to sedoheptulose-1,7-biphosphate. These two routes promote the occurrence of Calvin-Benson cycle, hence carbon fixation, which are important processes in providing energy required under salt stress.

Enrichment of the other enzyme transketolase makes the cell favors the first route in which glyceraldehyde-3-phosphate is converted via the three enzymes fructose-bisphosphate aldolase, fructose-1,6-bisphosphatase I and transketolase to erythrose-4-phosphate. This first route also indicates that glyceraldehyde-3-phosphate can directly be converted by transketolase to erythrose-4-phosphate as a bypass.

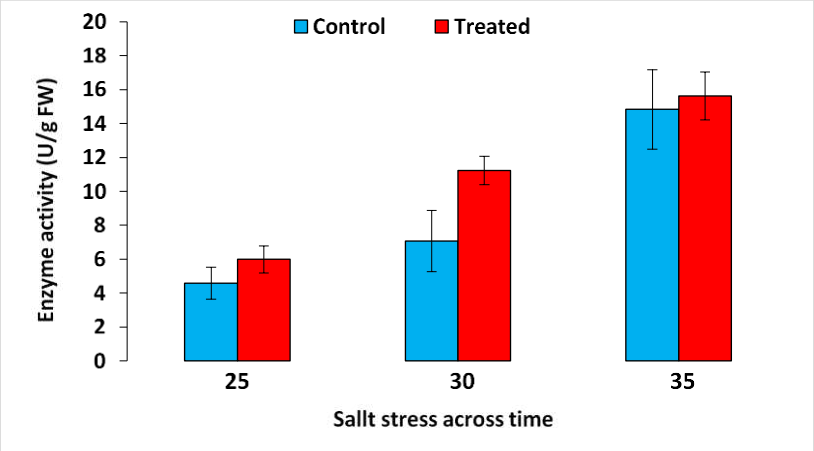

Figure 5. *Means of ascorbate peroxidase (APX) activity (U/g FW) in leaves of non-salinized (control) and salinized (treatment) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). Means were calculated for three replicates. Vertical bars indicate ± SE. Statistical analysis was done with P value of < 0.05. * = significant*

Table 1. *Enriched enzymes and pathways under salt stress in leaves of Jojoba plants*

Accession no.	Enzyme	Pathway
EC: 4.1.2.13	Fructose-bisphosphate aldolase	Biosynthesis of amino acids Biosynthesis of secondary metabolites **Carbon fixation in photosynthetic organisms** Carbon metabolism Fructose and mannose metabolism Glycolysis/Gluconeogenesis HIF-1 signaling Metabolic pathways Methane Metabolism Microbial metabolism in diverse environments Pentose phosphate

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EC: 2.2.1.1	Transketolase	Biosynthesis of amino acids Biosynthesis of ansamycins Biosynthesis of secondary metabolites **Carbon fixation in photosynthetic organisms** Carbon metabolism Metabolic pathways Microbial metabolism in diverse environments Pentose phosphate
EC: E2.5.1.54	3-deoxy-7-phosphoheptulonate synthase	**Phenylalanine, tyrosine and tryptophan biosynthesis** Metabolic pathways Biosynthesis of secondary metabolites Biosynthesis of amino acids Quorum sensing
EC: 4.2.1.10 EC: 2.4.2.18 EC: 4.1.1.48	3-dehydroquinate dehydratase I Anthranilate phosphoribosyl transferase Indole-3-glycerol phosphate synthase	**Phenylalanine, tyrosine and tryptophan biosynthesis** Metabolic pathways Biosynthesis of secondary metabolites
EC: 4.1.3.27	Anthranilate synthase	**Phenylalanine, tyrosine and tryptophan biosynthesis** Phenazine biosynthesis Metabolic pathways Biosynthesis of secondary metabolites
EC: 4.2.1.20	Tryptophan synthase	Glycine, serine and threonine metabolism **Phenylalanine, tyrosine and tryptophan biosynthesis** Metabolic pathways Biosynthesis of secondary metabolites
EC: 2.6.1.99 EC: 1.14.13.168	Tryptophan pyruvate aminotransferase Indole-3-pyruvate monooxygenase	**Tryptophan metabolism** Metabolic pathways
EC:3.5.1.4	Amidase	Arginine and proline metabolism Phenylalanine metabolism **Tryptophan metabolism** Aminobenzoate degradation Styrene degradation Metabolic pathways Microbial metabolism in diverse environments
EC: 1.1.3.15	(S)-2-hydroxy-acid oxidase	Biosynthesis of secondary metabolites **Glyoxylate and dicarboxylate metabolism** Metabolic pathways Microbial metabolism in diverse environments
EC: 1.11.1.11	L-ascorbate peroxidase	**Ascorbate and aldarate metabolism** Glutathione Metabolism Metabolic pathways

Pathways discussed are shown in bold letters

Downstream crosstalking pathways

Erythrose-4-phosphate is an important metabolite in the occurrence of carbon fixation via fructose-bisphosphate aldolase and transketolase as previously indicated. In the present study, erythrose-4-phosphate acted as a rate limiting metabolite that wired another important cross-talking pathway namely "phenylalanine, tyrosine and tryptophan

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biosynthesis" towards the production of tryptophan (*Fig. A4*). This has taken place via the upregulation under salt stress of as much as six genes encoding 3-deoxy-7-phosphoheptulonate synthase (EC: E2.5.1.54), 3-dehydroquinate dehydratase I (EC: 4.2.1.10), anthranilate synthase (EC: 4.1.3.27), anthranilate phosphoribosyl transferase (EC: 2.4.2.18), indole-3-glycerol phosphate synthase (EC: 4.1.1.48) and tryptophan synthase (EC: 4.2.1.20) (*Fig. 7*). Of which, the first enzyme (e.g., 3-deoxy-7-phosphoheptulonate synthase) acts as the rate-limiting step of the pathway. Then, tryptophan is converted to the important auxin namely indole acetic acid (IAA) via two routes, each containing two steps in "tryptophan metabolism" pathway (*Fig. A5*). The first two-step route involves the upregulation of genes encoding tryptophan pyruvate aminotransferase (EC: 2.6.1.99) and indole-3-pyruvate monooxygenase (EC: 1.14.13.168). The second route involves the upregulation in the second step involving amidase (EC:3.5.1.4) (*Fig. 8*). IAA is known as a rate limiting metabolite in "plant hormone signal transduction" pathway towards the production of many other metabolites (e.g., AUX, TIR, ARF, GH3, SAUR, etc.) that promote cell enlargement and overall high plant growth, thus, confers stress tolerance. Several of the genes encoding these important metabolites were upregulated under salt stress (*Figs. 9* and *A6*).

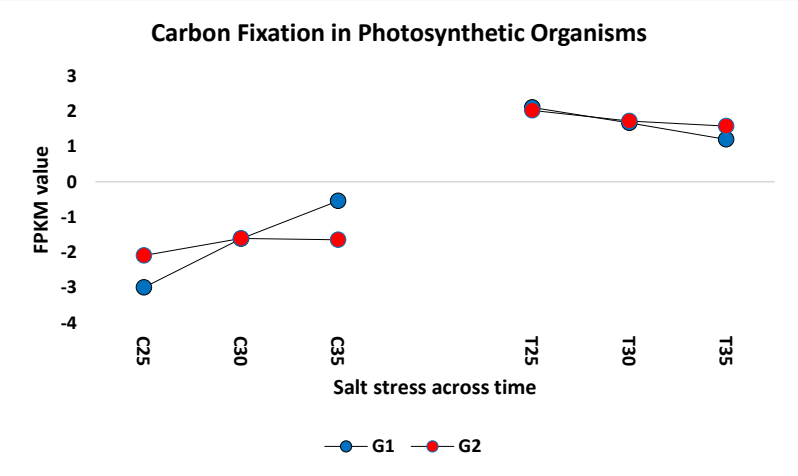

Figure 6. *The performance of fructose-bisphosphate aldolase (ALDO) (G1) and transketolase (TKT) (G2) genes of "Carbon fixation in photosynthetic organisms" pathway in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively)*

Glyoxylate and dicarboxylate metabolism

Physiological analysis indicated the involvement of hydrogen peroxide (H_2O_2) in the plant response to salt stress (*Fig. 3*). This molecule contributes to the cycle of mutual biosynthesis of (S)-2-hydroxy-acid oxidase//catalase of "glyoxylate and dicarboxylate metabolism" pathway (*Fig. A2*). The present study indicated the participation of one gene in this cycle namely *HAO* that encodes (S)-2-hydroxy-acid oxidase (EC: 1.1.3.15) (*Table 1*). This gene was upregulated at 50, 100 and 200 mM NaCl (*Fig. 10*). The encoded enzyme participates in four pathways (*Table 1*), but we focused on one pathway that complement the studied physiological parameter of H_2O_2. In this pathway, (S)-2-hydroxy-acid oxidase participates in the production of H_2O_2, thus, we expect that

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overproduction of the enzyme under salt stress likely results in the increased level of H_2O_2. The latter molecule represents an avenue towards the production of catalase enzyme. Physiological analysis indicated that the level of H_2O_2 significantly reduced under 50 and 100 mM NaCl (*Fig. 3*), while the level of catalase activity significantly increased under these two salt concentrations (*Fig. 4*). These contrary results indicate that H_2O_2 level is diminished towards the production of catalase enzyme (EC: 1.11.1.6) as shown in the pathway (*Fig. A2*).

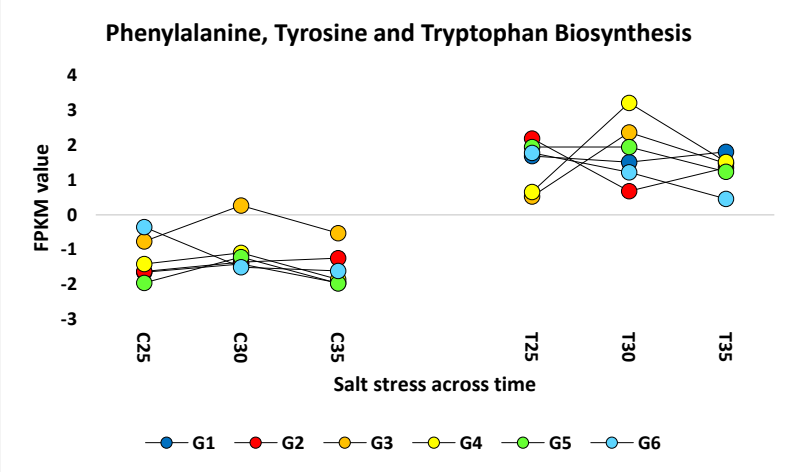

Figure 7. *The performance of genes encoding metabolites in the "Phenylalanine, tyrosine and tryptophan biosynthesis" pathway in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). G1 = 3-deoxy-7-phosphoheptulonate synthase, G2 = 3-dehydroquinate dehydratase I, G3 = anthranilate synthase, G4 = anthranilate phosphoribosyl transferase, G5 = indole-3-glycerol phosphate synthase, G6 = tryptophan synthase*

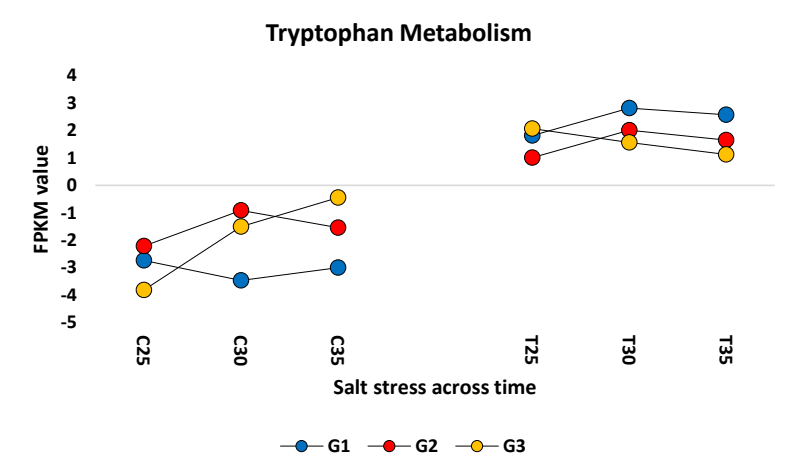

Figure 8. *The performance of genes encoding metabolites in the "Tryptophan metabolism" pathway in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). G1 = pyruvate aminotransferase, G2 = indole-3-pyruvate monooxygenase, G3 = amidase*

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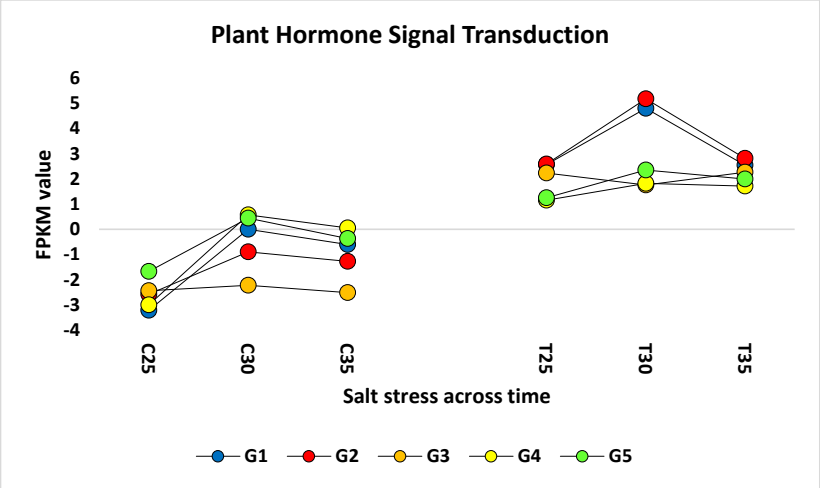

Figure 9. *The performance of genes encoding metabolites in the "Plant hormone signal transduction" pathway in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively). G1 = Auxin-responsive protein IAA1, G2 = Auxin response factor 1 (ARF1), G3 = transport inhibitor response 1 (TIR1), G4 = auxin responsive GH3.3, G5 = SAUR-like auxin-responsive SAUR2*

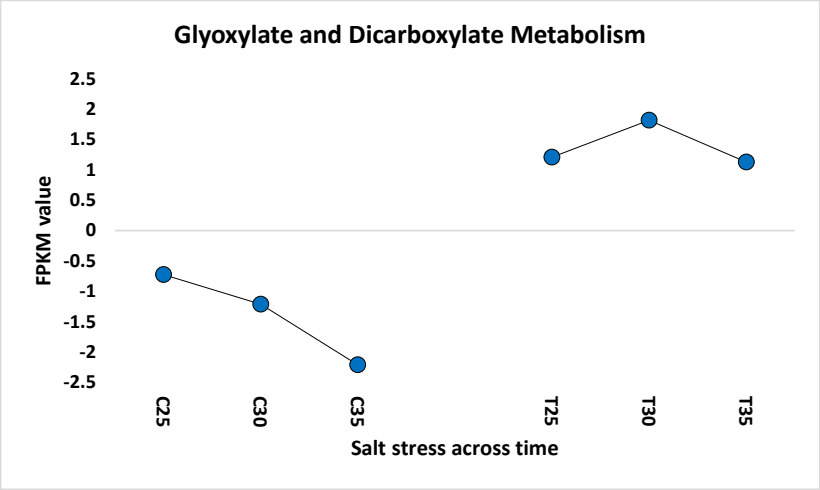

Figure 10. *The performance of (S)-2-hydroxy-acid oxidase gene (HAO) in "Glyoxylate and dicarboxylate metabolism" pathway leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively)*

The (S)-2-hydroxy-acid oxidase enzyme participates in two cycles within the "glyoxylate and dicarboxylate metabolism" pathway (*Fig. A2*). As indicated earlier, the first cycle involves mutual production of (S)-2-hydroxy-acid oxidase//catalase. The second cycle involves the mutual production of glycolate//(S)-2-hydroxy-acid oxidase//glyoxylate. It is well-known that glycolate oxidation is catalyzed either by glycolate dehydrogenase or a glycolate oxidase (EC: 1.1.99.14), while glyoxylate reduction is catalyzed by glyoxylate reductase (NADP+) (EC: 1.1.1.26) (*Fig. A7*).

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Glycolate oxidase catalyzes the chemical reaction (shown in *Fig. A7*) by oxidizing glycolate to glyoxylate as in the following equation: [(S)-2-hydroxy acid + O2 ⇌ 2-oxo acid + H_2O_2] (Kern et al., 2020). Interestingly, the two cycles that are connected to (S)-2-hydroxy-acid oxidase in the "glyoxylate and dicarboxylate metabolism" pathway crosstalk with "carbon fixation in photosynthetic organisms" pathway via the enzyme's substrate, e.g., glycolate. Crosstalking is mainly mediated by the action of transketolase (EC: 2.2.1.1), and the two downstream enzymes namely ribose-5-phosphate isomerase (EC: 5.3.1.6) and phosphoribulokinase (EC: 2.7.1.19) of "carbon fixation in photosynthetic organisms" pathway with ribulose-1,5-biphosphate exists as the intermediate metabolite (*Fig. A7*). The latter is converted by ribulose-bisphosphate carboxylase (EC: 4.1.1.39), then, by phosphoglycolate phosphatase (EC: 3.1.3.18) of "glyoxylate and dicarboxylate metabolism" pathway towards the production of glycolate, e.g., substrate of (S)-2-hydroxy-acid oxidase (EC: 1.1.3.15). Another route for the connection of the two pathways is mediated by the action of the two enzymes phosphoglycerate kinase (EC: 2.7.2.3) and glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.12) of "carbon fixation in photosynthetic organisms" pathway with glycerate-3-phosphate as an intermediate metabolite towards the production of glyceraldehyde-3-phosphate, e.g., substrate of fructose-bisphosphate aldolase (EC: 4.1.2.13). This indicates that expression of *HAO* gene under salt stress might promote entrance to "carbon fixation in photosynthetic organisms" pathway, while expression of *ALDO* and *TKT* genes promote entrance to the downstream pathways of "phenylalanine, tyrosine and tryptophan biosynthesis", "tryptophan metabolism" and "plant hormone signal transduction" to confer salt stress tolerance in plants.

Ascorbate and aldarate metabolism

Physiological analysis indicated the significant increase of ascorbate peroxidase (APX) (EC: 1.11.1.11) activity at 100 mM NaCl (*Fig. 5*), while molecular analysis indicated the upregulation of the gene encoding APX at 100 and 200 mM NaCl (*Fig. 11*). The gene encoding APX participates in three pathways in which only one pathway was considered for further analysis (*Table 1*). APX converts ascorbate to monodehydro ascorbate (*Fig. A3*). Interestingly, "glyoxylate and dicarboxylate metabolism" pathway crosstalks with "ascorbate and aldarate metabolism" pathway with tartronate semialdehyde as the connecting intermediate metabolite (*Figs. A2* and *A3*). This latter metabolite further interconnects with monodehydro ascorbate via APX and six preceding steps in the "ascorbate and aldarate metabolism" pathway (*Fig. A3*), while interconnects directly with glyoxylate via the action of tartronate-semialdehyde synthase (EC: 4.1.1.47) in the "glyoxylate and dicarboxylate metabolism" pathway (*Fig. A2*). Tartronate-semialdehyde is also a connecting metabolite between "glyoxylate and dicarboxylate metabolism" and "carbon fixation in photosynthetic organisms" pathways via the action of two enzymes namely 2-hydroxy-3-oxopropionate reductase (EC: 1.1.1.60) and glycerate-3-kinase (EC: 2.7.1.31).

Overall, the six pathways "carbon fixation in photosynthetic organisms", "phenylalanine, tyrosine and tryptophan biosynthesis" (*Fig. A4*), "tryptophan metabolism" (*Fig. A5*) and "plant hormone signal transduction", "ascorbate and aldarate metabolism" and "glyoxylate and dicarboxylate" were proven to crosstalk under salt stress in Jojoba to provide a mechanism by which this plant tolerates salt stress as schematically represented in *Figure 12*.

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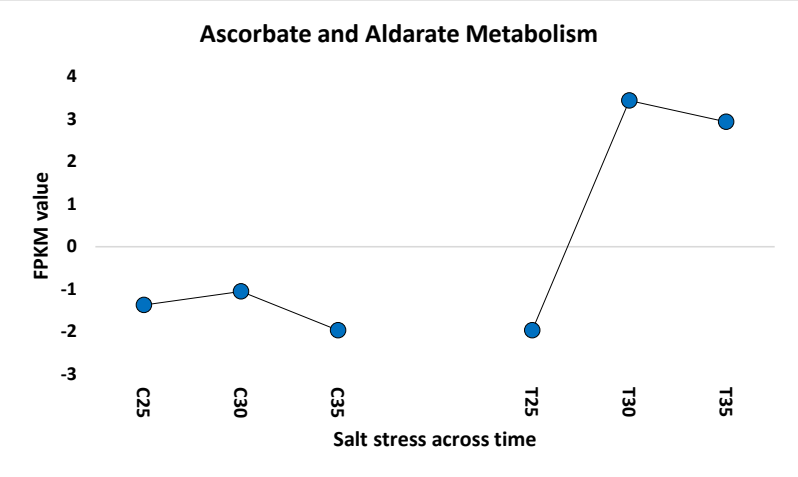

Figure 11. *The performance of ascorbate peroxidase (APX) gene in the "Ascorbate and aldarate metabolism" pathway in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals and harvested at the second day (days 25, 30 and 35, respectively)*

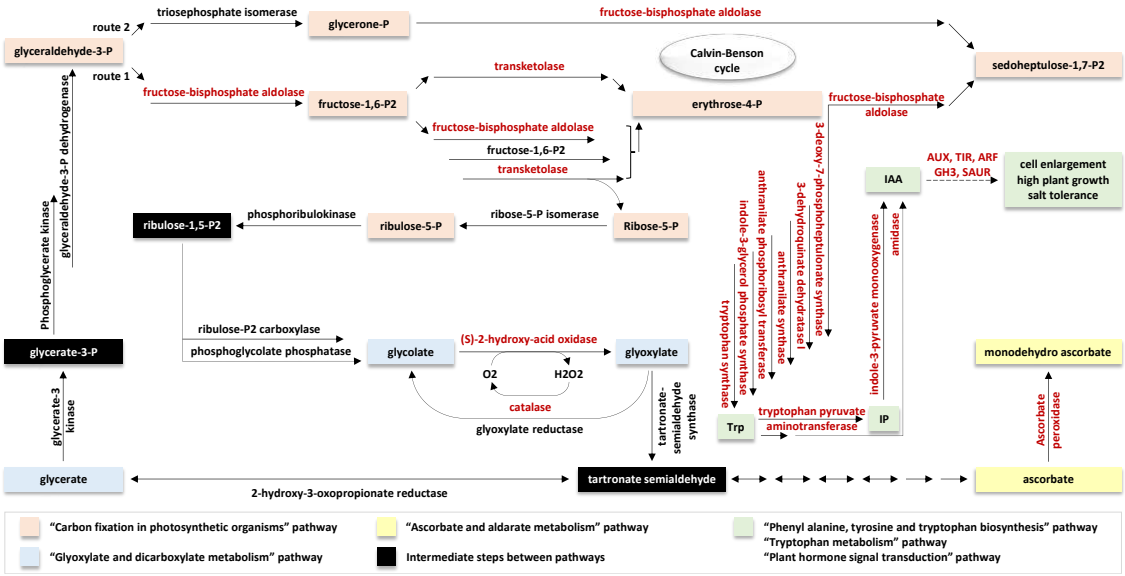

Figure 12. *A schematic representation of genetic regulatory network referring to the crosstalking pathways under salt stress in Jojoba plants. Enzymes in red refer to the enriched enzymes of six pathways under salt stress based on physiological (catalase and ascorbate peroxidase) and molecular (fructose-bisphosphate aldolase, transketolase and ascorbate peroxidase) analyses. Trp = tryptophan, IAA = indole acetic acid, IP = indole pyruvate*

Discussion

Chlorophyll is essential for photosynthesis as it allows plants to absorb energy from light (Carter, 1996; He et al., 2020). Such solar energy has a balancing network that can adapt over long time scale to provide certain metabolic demands due to the change in environmental conditions (Walker et al., 2020). There are many emerging research in plant photosynthesis indicating that stoichiometry of interaction of light reactions with

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carbon metabolism is mandatory in regulating photosynthetic rate under adverse conditions (Sharkey, 2020). Recent work of Zhou et al. (2020) successfully engineered the transition between photosystem I (PSI) and PSII as a smart and promising way to improve efficiency of natural photosynthetic process. There are two types of chlorophyll in the photosystems of green plants namely chlorophyll-a and chlorophyll-b (Carter, 1996; Speer, 1997). Of which, chlorophyll-b was proven to act solely in light harvesting (Kume et al., 2018). Chlorophyll absorbs light mostly in the blue portion of the electromagnetic spectrum as well as in the red portion (Muneer et al., 2014). These molecules are embedded in the thylakoid membranes of chloroplasts to induce photosystems (Carter, 1996). Photosynthesis converts light energy into chemical energy that can be used as a fuel for organisms' activities. This chemical energy is stored in carbohydrate molecules, e.g., sugars, which are synthesized from carbon dioxide and water (Green and Durnford, 1996). Carbon dioxide is converted into sugars in a process called carbon fixation (Whitmarsh and Govindjee, 1999).

Carbon fixation is the process by which carbon dioxide is converted to organic compounds in photosynthetic organisms. The organic compounds are then used to store energy and considered as building blocks for other important biomolecules (Geider et al., 2001). The results in *Figure 2a* indicate that amount of chlorophyll-a slightly reduced at 100 mM NaCl salt stress, while increased at 200 mM NaCl. On the other hands, the results for chlorophyll-b and carotenoids indicated increases at 100 mM NaCl salt stress, while significant decreases at 200 mM NaCl (*Fig. 2b, c*). These results indicate that salt-related genes for the production of chlorophyll-a required longer time (day 30) to respond to salt stress than the time required for genes controlling the production of either chlorophyll-b or carotenoids (day 25). No significant differences were found for the production of the three pigments when plantlets exposed to 50 mM NaCl for one day. This indicates that one-day exposure to salt stress is not enough time to induce salt-related genes for the production of the three pigments. Such a stepwise response of the genes for the production of the three pigments can be a mechanism of salt-stress tolerance in Jojoba.

Overall, salt-induced water stress results in the reduction of chloroplast stroma volume, thus, generation of reactive oxygen species (ROS) and inhibition of photosynthetic rate (Price and Hendry, 1991; Allen, 1995; Cha-Um and Kirdmanee, 2009). Pigment levels in Jojoba plant leaves reduced significantly at 50 and 100 mM NaCl in Chl_a, while Chl_b and charotinoids significantly reduced at the high salt concentration (200 mM NaCl). This indicates that stroma volume and photosynthetic rate were not affected at the low and intermediate levels of salt stress in Jojoba leaves. These results indicate that Jojoba tolerates moderate levels of salt stress.

Decrease in total chlorophyll content may also be observed under salinity stress due to ion accumulation and functional disorders observed during stoma opening and closure (Seemann and Critchley, 1985; Romero-Aranda, and Syvertsen, 1996; Molazem et al., 2010; Nawaz et al., 2010). Another reason for the decreased chlorophyll content under salt conditions is stated to be the result of rapid leaf maturing (Yeo et al., 1991). Decrease in chlorophyll content under salinity stress is observed more clearly in salt sensitive genotypes in comparison to cultivars with intermediate level of tolerance (such as Jojoba) (Krinsky, 1978; Seeman and Critchley, 1985; Sharkey et al., 1985; Maibangsa et al., 1999; Nafie and El-Khallal, 2000; Hayat et al., 2001; Sivakumar et al., 2002; Durai, 2006; Khan et al., 2009; Tiwari et al., 2010; Akça and Samsunlu, 2012; Ali et al., 2013; Sharkey et al., 2020).

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The results of the present study indicated that the two enzymes fructose-bisphosphate aldolase and transketolase were upregulated under salt stress referring to enhanced level of photosynthesis. The enzyme fructose-bisphosphate aldolase is among a subgroup of the larger lyase group. The aldolases can be divided into two classes depending on the type of enzyme catalysis. Type I enzymes is principally found in eukaryotes, while type II is found predominantly in bacteria and archaea (Dalby et al., 1999; Ziveri et al., 2017). *Figure 6* shows an increase in the enzyme at the three salt concentrations compared to the control. In plants, "carbon fixation in photosynthetic organisms" pathway plays a crucial role in initiating complex responses to stress conditions. Dixit et al. (2001) indicated that salt-tolerant plants have enhanced energy accumulation at high salt concentrations via enhanced photosynthesis. Overall, photosynthesis adds to the ability of the plant to resist reactive oxygen species (ROS) as functional activity of chlorophyll responds negatively to ROS content.

The enzyme transketolase participates in the Calvin cycle of photosynthesis (Sax et al., 2000). Transketolase is found in animal, plant and microorganisms, whereas in plants it contains one major isoform located in the chloroplast (Henderson and Toone, 1999; Henkes et al., 2001). Henkes et al. (2001) investigated the consequences of decreased transketolase activity for primary and secondary metabolism in tobacco by transferring a construct containing an antisense sequence via genetic transformation. The results showed a local loss of chlorophyll and carotenoids in the midrib when enzyme activity inhibited by > 50%, spreading onto minor veins and lamina in severely affected transformants. Consequently, decreased expression of transketolase led to a preferential decrease of sugars, whereas starch remained high until photosynthesis strongly inhibited. The decrease of chlorophyll is an indirect consequence of low transketolase activity, because it did not occur until expression strongly inhibited and many aspects of metabolism were altered. Our results showed increases in chlorophyll-b and carotenoids at 100 mM NaCl, while at 200 mM NaCl for chlorophyll-a. This is because production of transketolase increases in Jojoba plant under salt stress (*Fig. 7*) as a mechanism to maintain the level of chlorophyll-(a,b) and carotenoids in plant leaves.

During the conversion of glycolate to glyoxylate, H_2O_2 is supposed to be intensively produced due to the action of (S)-2-hydroxy-acid oxidase that is upregulated in Jojoba under salt stress (*Fig. 8*). The fact is that H_2O_2 level significantly reduced in Jojoba (*Fig. 3*) as it is converted to O_2 during the production of CAT. Accordingly, the latter significantly increased in favor of H_2O_2 reduction under salt stress in Jojoba (*Fig. 4*). APX significantly increased in both physiological and molecular analysis (*Figs. 5* and *12,* respectively). High expression of APX in Jojoba complements crosstalking of "ascorbate and aldarate metabolism" and "glyoxylate and dicarboxylate metabolism" pathways with tartronate semialdehyde stands as an intermediate step between the two pathways (*Fig. 12*).

H_2O_2 is the most important stable non-radical ROS (Ślesak et al., 2007; Sofo et al., 2015), without a net charge (Halliwell, 2006; Sofo et al., 2015). The amount of cellular H_2O_2, together with other ROS, is a good marker of the extent of oxidative stress. As a consequence, the balance of ascorbate peroxidase (APX), and catalase (CAT) activities, representing the main enzymatic H_2O_2 scavenging mechanism in plants, is crucial for the suppression of toxic H_2O_2 levels in a cell (Apel and Hirt, 2004; Ślesak et al., 2007; Kovalchuk, 2010; Sofo et al., 2015). CAT has the ability to scavenge ROS, which can lead to oxidative damage and damage transfer resulted from

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salt stress. If ROS accumulate in plants, light-harvesting efficiency will be affected, protein synthesis as well as signal transduction processes will slow down due to changes in osmotic pressure. Ascorbate peroxidase (APX), as well as other peroxidases, such as CAT and SOD, has the ability to scavenge ROS (Sofo et al., 2015). Activity of APX enzyme increased in Jojoba at 100 and 200 mM NaCl compared to the control (*Fig. 6*), which is supported by molecular analysis of the gene encoding the enzyme that showed an increase at the three salt concentrations (*Fig. 12*). APX is generated by tartronate semialdehyde in the "ascorbate and aldarate metabolis" pathway (*Fig. 12*).

H_2O_2 can also act as signal molecule in regulating plant growth, morphogenesis and development (Ślesak et al., 2007; Sofo et al., 2015), such as in auxin signaling and gravitropism of maize roots (Joo et al., 2001; Sofo et al., 2015), and in somatic embryogenesis stimulation of *Lycium barbarum* (Cui et al., 1999; Sofo et al., 2015). H_2O_2 has considered as an essential molecule of signal transduction in both abiotic and biotic stresses. Matsuda et al. (1994) demonstrated that application of H_2O_2 at low concentrations induces stress tolerance in plants due to the induction of the synthesis of certain substances similar to other normally synthesized during chilling stress (Sofo et al., 2015). The effects of NaCl on the balance of H_2O_2 level and CAT activity were previously studied in diverse groups of plants, such as unicellular alga, e.g., *Chlorella* sp., aquatic macrophyte, e.g., *Najas graminea*, and mangrove plant, e.g., *Suaeda maritime*. All of these organisms showed high tolerance to NaCl as CAT activity increased significantly in response to high NaCl treatment (Mallik et al., 2011; Sofo et al., 2015). Results of the latter studies, in addition of the present study, support the influence of H_2O_2 at low concentrations in inducing stress tolerance.

Pathways "phenylalanine, tyrosine and tryptophan biosynthesis" and "tryptophan metabolism" likely crosstalk towards production of tryptophan, then, production of the auxin indole acetic acid (or IAA). "tryptophan metabolism" pathway crosstalk with "plant hormone signal transduction" pathway in which tryptophan produced from "phenylalanine, tyrosine and tryptophan biosynthesis" pathway is metabolized towards production of IAA via two-step avenue (*Fig. 12*). Then, auxin provokes other downstream pathway namely "plant hormone signal transduction" to strengthen plant's ability to survive and maintain proper growth rates under stress conditions (*Fig. 12*). Towards the route downstream auxin production, a battery of metabolites are produced under stress condition, e.g., auxin/indole acetic acid (or AUX/IAA), auxin response factors (or ARFs), E3 ubiquitin ligase SCF^{TIR1} (or TIR1), Gretchen Hagen 3 (or GH3), AUX/LAX (or LAX) and small a*uxin*-up RNA (or SAUR). Actions of these auxin-responsive metabolites combine to enlarge cells and improve plant growth under salt stress. IAA signaling is controlled by complex ARFs and interacting repressors of AUX/IAA proteins (Gray et al., 2001). ARFs binds promoter elements of auxin-responsive genes *LAX* and *SAUR*, while AUX/IAA proteins bind ARFs to block their action (Ulmasov et al., 1997a, b). TIR1 enzyme is the main contributor to AUX/IAA protein degradation as it activates ARFs and derepresses downstream auxin responsive pathways, thus promotes plant growth and development (Gray et al., 2001). GH3 is a class of auxin-induced conjugating enzymes that secures homeostasis and regulation of active form of auxin in the cell as well as blocks action of excessively available auxin (Paponov et al., 2008). Activated forms of ARFs stimulate SAUR that acts as a regulator of cell elongation (Knauss et al., 2003) and a stimulator of shoot elongation (Paponov et al., 2008).

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Conclusion

In conclusion, we assume we have widened our knowledge of the physiological and molecular mechanisms underlying salt stress tolerance in Jojoba. This information can help using this plant as a source of biodiesel in salinized soil or areas of scarce water supply. The recovered information can also help in the development of new economically important crop plants with improved salt tolerance via metabolic engineering. The information can promote the possible cultivation of this plant as a source of biodiesel in moderately salinized soil or areas of water scarce.

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APPENDIX

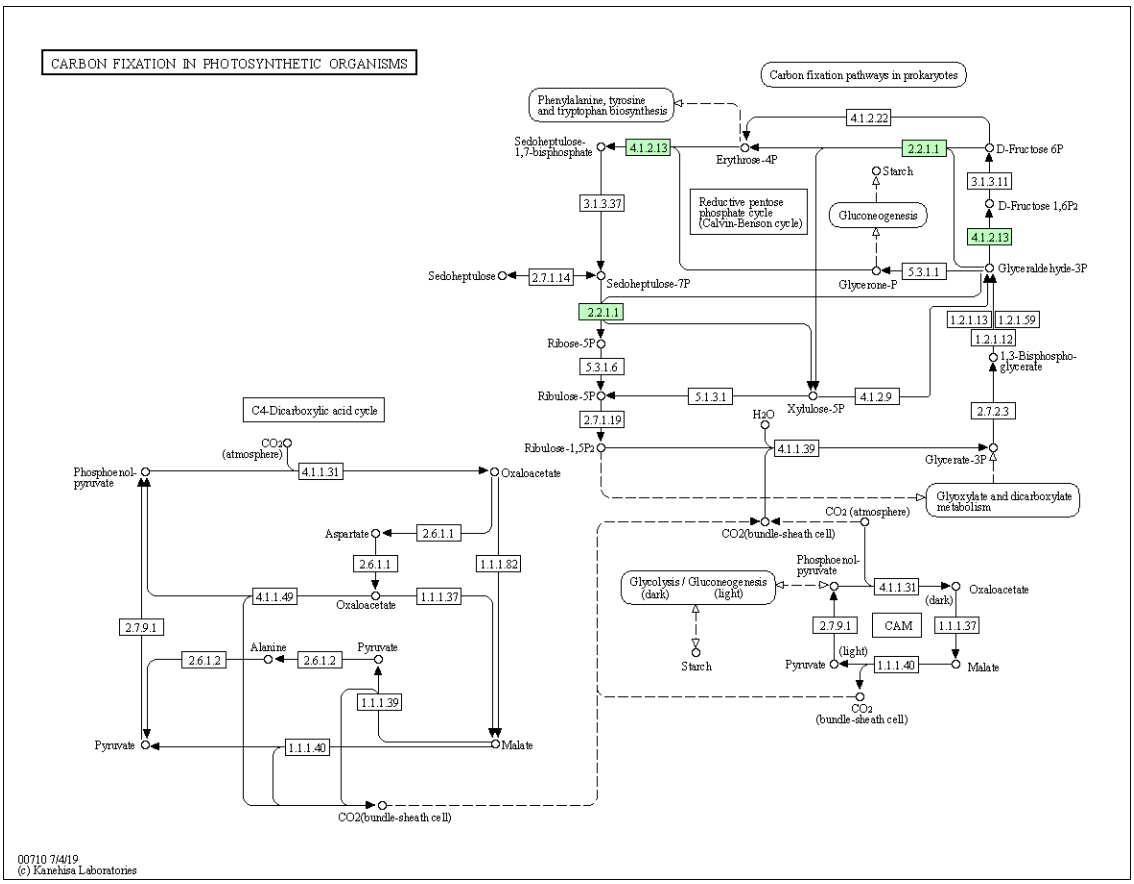

Figure A1. *Enriched metabolites in the "Carbon fixation in photosynthetic organisms" pathway due to salt stress (200 mM NaCl) in leaves of Jojoba plant. Colored boxes refer to enriched enzyme(s) under the stress*

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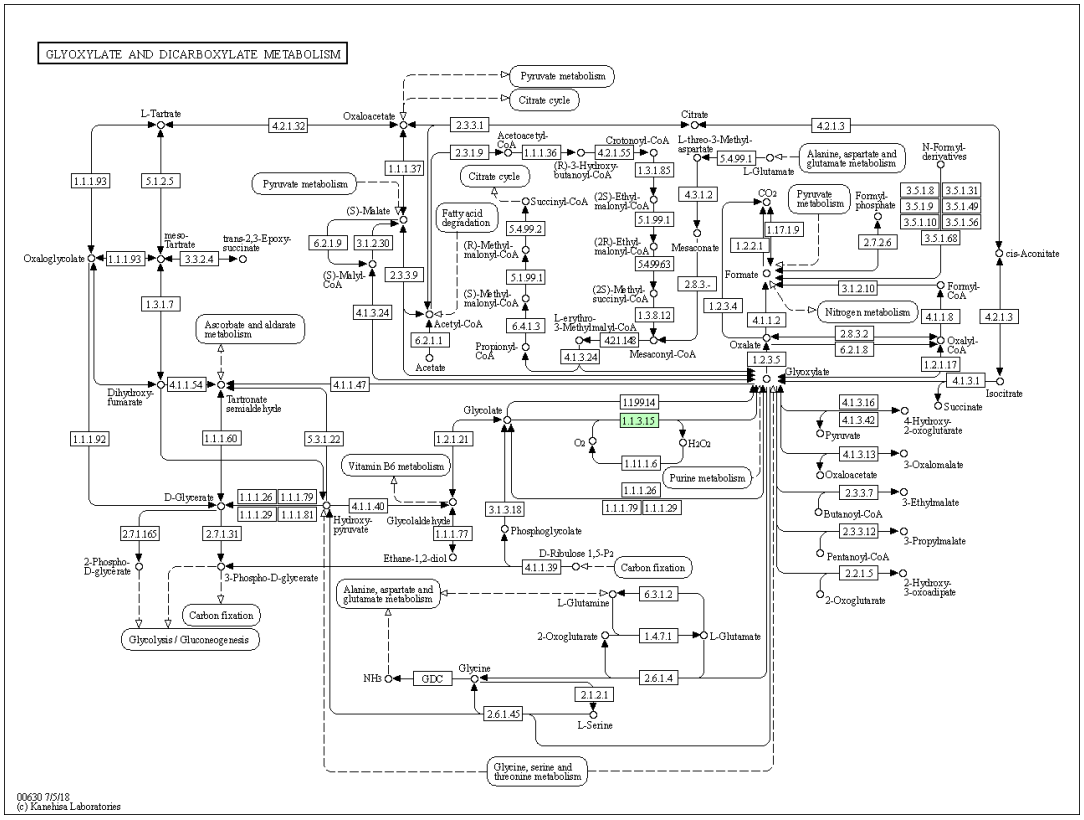

Figure A2. *Enriched metabolites in the "Glyoxylate and dicarboxylate metabolism" pathway due to salt stress (200 mM NaCl) in leaves of Jojoba plant. Colored boxes refer to enriched enzyme(s) under the stress*

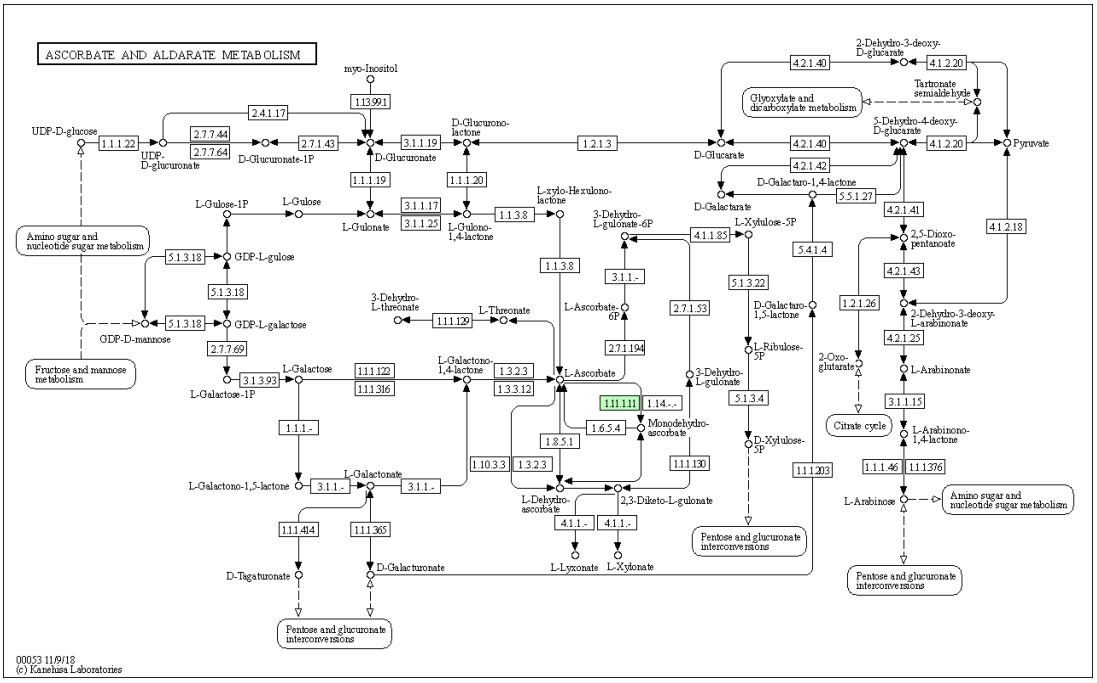

Figure A3. *Enriched metabolites in the "Ascorbate and aldarate metabolism" pathway due to salt stress (200 mM NaCl) in leaves of Jojoba plant. Colored boxes refer to enriched enzyme(s) under the stress*

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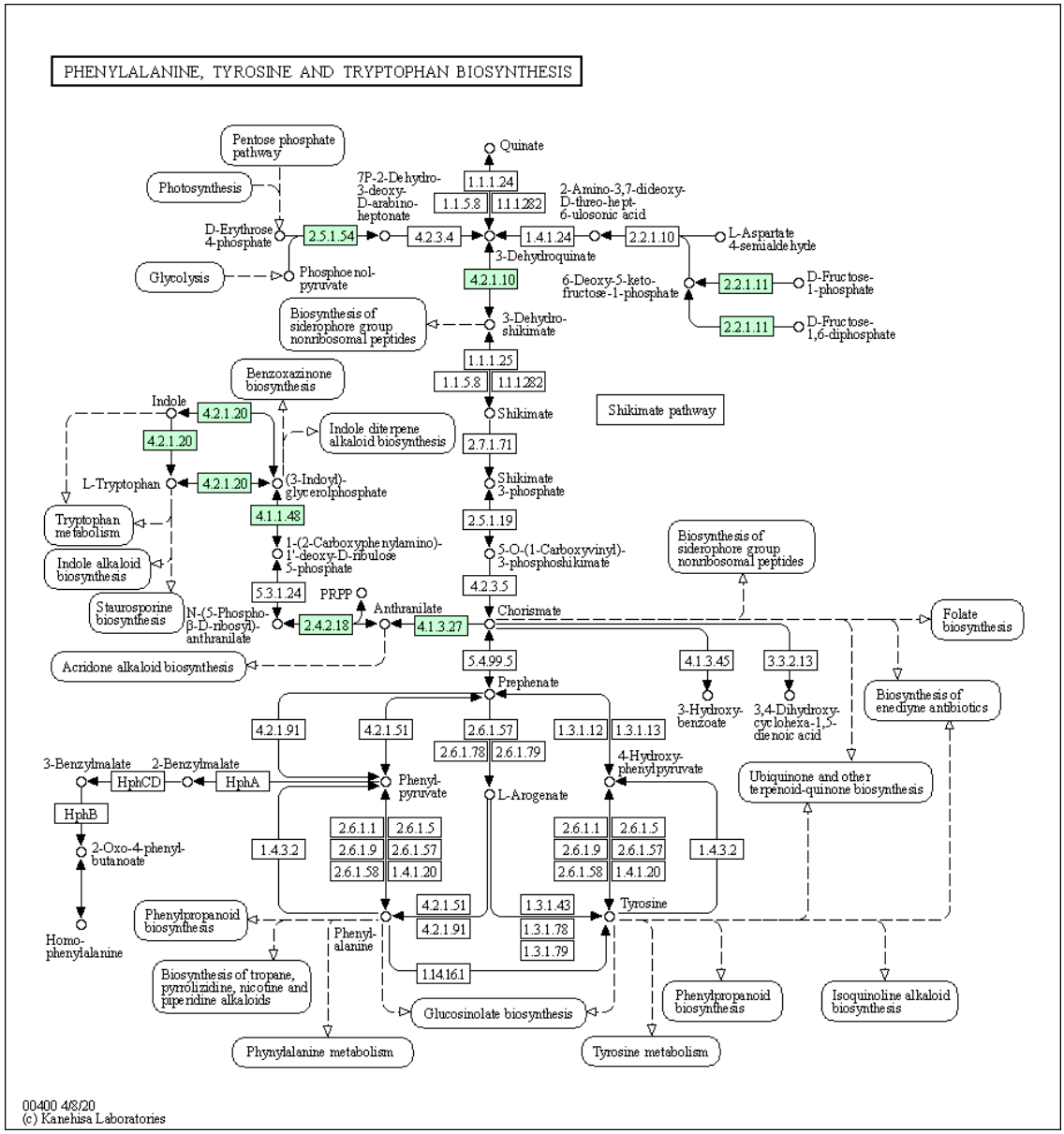

Figure A4. *Enriched metabolites in the "Phenylalanine, tyrosine and tryptophan biosynthesis" pathway due to salt stress (200 mM NaCl) in leaves of Jojoba plant. Colored boxes refer to enriched enzyme(s) under the stress*

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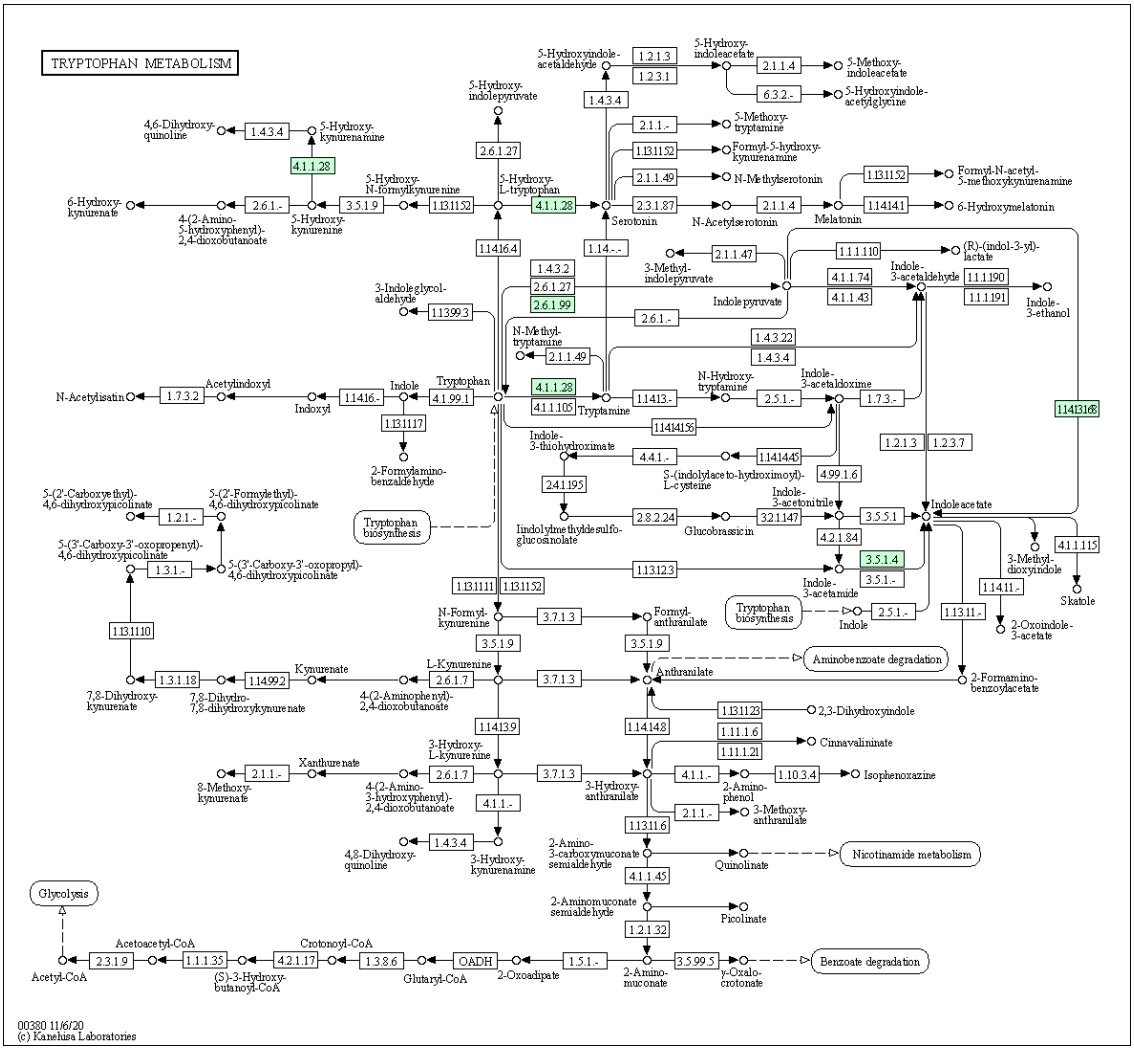

Figure A5. *Metabolites in the "Tryptophan metabolism" pathway that can be enriched due to salt stress (200 mM NaCl) in leaves of Jojoba plant. Colored boxes refer to enriched enzyme(s) under the stress*

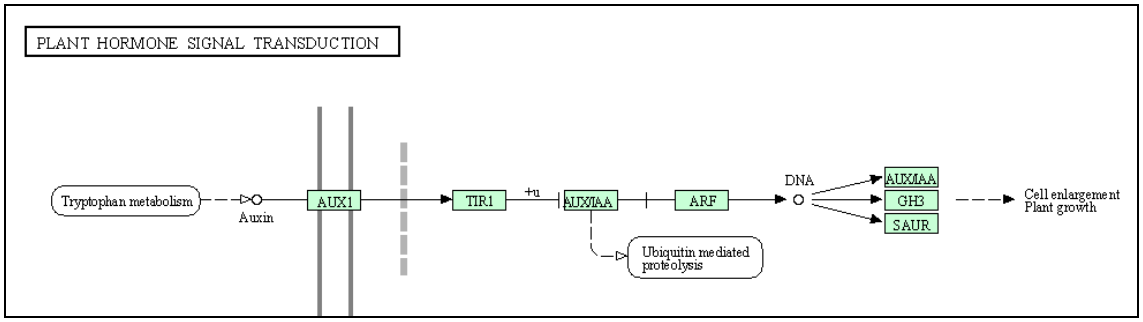

Figure A6. *Metabolites in the "Plant hormone signal transduction" pathway that can be enriched due to salt stress (200 mM NaCl) in leaves of Jojoba plant. Colored boxes refer to enriched enzyme(s) under the stress*

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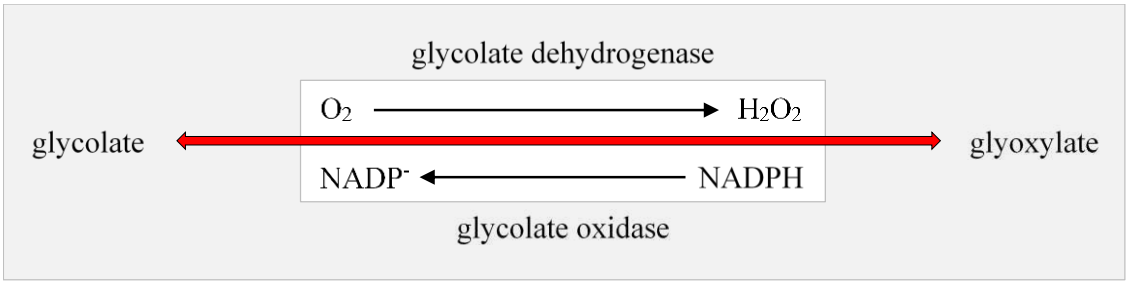

Figure A7. *Glycolate oxidation can be catalyzed either by a glycolate dehydrogenase (top) or a glycolate oxidase (bottom) (Kern et al., 2020)*

Table A1. *Mean values of chlorophyll-a, chlorophyll-b and carotenoids (mg/g FW) in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals (days 25, 30 and 35, respectively). Means were calculated for three replicates. Statistical analysis was done with P value of < 0.05. NS = non-significant, Sig. = significant*

	Sample	N	Mean	SD	t	P-value	Sig.
Chlorophyll-a	C25	3	2.56	0.17	1.123	0.324	NS
	T25	3	2.42	0.12			
	C30	3	1.95	0.21	1.092	0.336	NS
	T30	3	1.70	0.35			
	C35	3	1.36	0.26	0.873	0.432	NS
	T35	3	1.71	0.64			
Chlorophyll-b	C25	3	3.14	0.31	0.412	0.701	NS
	T25	3	3.04	0.05			
	C30	3	5.05	0.68	-1.800	0.146	NS
	T30	3	5.85	0.35			
	C35	3	5.48	0.21	2.814	0.048	Sig.
	T35	3	4.76	0.39			
Carotenoids	C25	3	8.67	0.82	0.658	0.546	NS
	T25	3	7.96	0.48			
	C30	3	12.92	0.49	4.211	0.014	Sig.
	T30	3	14.78	0.59			
	C35	3	13.57	1.67	3.410	0.027	Sig.
	T35	3	10.18	0.39			

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Table A2. *Mean values of hydrogen peroxide (μmol^{-1} cm^{-1}), catalase (CAT) activity (U/g FW) and ascorbate peroxidase (APX) activity (U/g FW) in leaves of non-salinized (C) and salinized (T) Jojoba plants at three NaCl concentrations (50, 100, and 200 mM) increased incrementally with five-day intervals (days 25, 30 and 35, respectively). Means were calculated for three replicates. Statistical analysis was done with P value of < 0.05. NS = non-significant, Sig. = significant*

	Sample/Concentration	N	Mean	SD	t	P-value	Sig.
H_2O_2 Conc.	C25	3	13.12	0.56	8.845	0.001	Sig.
	T25	3	9.32	0.49			
	C30	3	11.71	0.35	5.426	0.006	Sig.
	T30	3	8.99	0.80			
	C35	3	12.34	0.31	-6.280	0.003	Sig.
	T35	3	15.22	0.73			
CAT Enzyme activity	C25	3	13.11	2.78	-8.580	0.001	Sig.
	T25	3	27.16	0.56			
	C30	3	41.88	1.46	-23.538	0.000	Sig.
	T30	3	168.53	9.20			
	C35	3	35.35	6.34	-2.340	0.079	NS
	T35	3	45.93	4.59			
APX Enzyme activity	C25	3	4.58	0.95	-0.247	0.817	NS
	T25	3	5.98	9.80			
	C30	3	7.06	1.81	-3.622	0.022	Sig.
	T30	3	11.23	0.83			
	C35	3	14.84	2.34	-0.507	0.639	NS
	T200	3	15.63	1.41			

ELECTRONIC APPENDIX

This paper has an electronic appendix.

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SOIL BACTERIAL DIVERSITY AND COMPOSITION OF DIFFERENT FOREST TYPES IN GREATER XING'AN MOUNTAINS, CHINA

SONG, D. D. – REN, L. – LI, X. – MA, D. L.* – ZANG, S. Y.

College of Geographical Sciences, Harbin Normal University, Harbin 150025, PR China

**Corresponding author*
e-mail: madalong728@163.com

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Abstract. Soil microorganisms are an important component of forest ecosystems and have an important role in maintaining ecosystem stability. High-throughput sequencing technology was used to analyze the microbial composition and diversity of three typical forest, including *Larix gmelinii* forest (LG), *Pinus sylvestris* forest (PS), and *Betula platyphylla* forest (BP) in the Greater Xing'an Mountains, China. The results showed that there were significant differences in soil physical and chemical properties (pH, total organic carbon, total nitrogen and total phosphorus) among the three forest types. Soil bacterial alpha diversity (Shannon and Simpson) was not significantly different between forest types, but principal co-ordinates analysis (PCoA) results showed that soil bacterial beta diversity varied significantly (PERMANOVA: $R^2 = 0.5014$, $P < 0.01$). In the three forest types of soil, the composition of the dominant bacterial phyla was basically the same. The relative abundance of *Acidobacteria* in BP soil was 34.64%, *Proteobacteria* was 27.00%, *Chloroflexi* was 10.32%; the relative abundance of *Proteobacteria* in LG soil dominant bacteria was 30.62%, *Acidobacteria* was 18.31%, *Actinobacteria* was 16.01%; the relative abundance of *Proteobacteria* in PS soil was 25.54%, *Acidobacteria* was 24.59%, *Actinobacteria* was 16.46%. Redundancy analysis (RDA) results showed that pH, TP, TN and TOC were the main environmental factors affecting the soil bacterial community structure. The findings of this study are intended to provide a new perspective on microbial ecology for a deeper understanding of the biogeochemical cycle in forest soils in the Greater Xing'an Mountains, China.

Keywords: *forest types, permafrost, high-throughput sequencing, soil bacterial community, diversity, soil properties*

Introduction

Microorganisms are the most active part of the forest ecosystem and the key driving force for the formation and transformation of soil materials (Curlevski et al., 2010). Throughout the formation and development of soil, it plays an irreplaceable role in the soil carbon and nitrogen cycle (Demeter et al., 2018), nutrient conversion (Wakelin et al., 2016), plant productivity (Friggens et al., 2019), maintaining soil structure (Banerjee et al., 2018). It is one of the indispensable indicators for evaluating soil quality (Ryan et al., 2012 and Tu et al., 2015). Soil microbial diversity refers to the types of all microorganisms contained in the soil ecosystem, the genes they possess, and the degree of diversity in the interaction between these microorganisms and the surrounding environment (Tardy et al., 2015). Forest ecosystems play an important role in regulating the global material balance. Exploring the differentiation patterns of soil microbial communities under different forest vegetations is an important part of understanding forest ecosystem functions.

Greater Xing'an Mountains is located in the high latitude permafrost region in the northern hemisphere. It is one of the regions most affected by climate change and

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human activities in our country and in the world. Greater Xing'an Mountains is also the only cold temperate coniferous forest area in China, which has an irreplaceable role and position in maintaining the ecological balance of northeastern, China (Luo et al., 2018). In recent years, due to the effects of human interference and the degradation of permafrost in this area, forest productivity has decreased, vegetation reverse succession has accelerated, and the tree line of plantation trees has risen (Li et al., 2018). Nowadays, this local forest vegetation composition has been transferred from original conifer type (*Larix gmelinii*) into broad-leaved types (*Betula platyphylla*, *Populus davidiana* and *Quercus mongolica*). These changes will have a huge impact on the regional ecosystem and break the existing soil microbial composition structure. Moreover, changes in soil microbial community and function also impact aboveground plant composition and diversity (Siles and Margesin, 2016). Therefore, soil microorganisms are of great significance to the forest ecosystem and have critical roles in vegetation renewal and successional processes. This study of how vegetation impacts soil microbes is an increasingly popular topic for ecologists, especially research regarding the functional mechanisms of ecosystems. Therefore, clarifying the characteristics and evolution rules of forest soil microbial communities in the Greater Xing'an Mountains is the key grasping the biogeochemical cycle process in the cold region, and the correspondence is of great significance to global change.

The different types of litter and root exudates undertaken by soil under different forest vegetations will inevitably have a significant impact on microbial diversity and community structure (Ren et al., 2017). In order to further clarify the coupling relationship and the mechanism between vegetation and microbes, this paper using different forest types of soil in Greater Xing'an Mountains microorganisms are the research objects. We hypothesis: 1) how the soil bacterial community structure and diversity varied from long-term forest succession process? 2) Soil physico-chemistry properties were key factors affecting the bacterial community composition. Illumina Miseq high-throughput sequencing technology was used to sequence and analyze the 16S rRNA genes of bacteria in soil samples. It aims to provide a perspective of microbial ecology for a deeper understanding of the microbial structure and function of the forest soils in the Greater Xing'an Mountains.

Materials and methods

Study area

The research area is located in Mohe Forest Ecosystem Research Station in Mohe County, Heilongjiang Province, China. The climate belongs to the continental monsoon climate in the cold temperate zone. The summer is mild and short, and the winter is long and cold. The annual average temperature is -4.9 °C, the extreme minimum temperature is -49.5 °C, the frost-free period is 85–105 d, and the annual precipitation is 350–500 mm, mostly concentrated in summer, which accounted for 60–70% of annual total precipitation. The typical zonal vegetation is dominated by *Larix gmelinii*, *Pinus sylvestris* var. *mongolica*, *Betula platyphlla*, and *Populus davidiana.* The soil is dark brown forest soil (Dong et al., 2019). Due to human interference such as logging or fire, a large number of *Larix gmelinii* forests have been destroyed and large areas of secondary *Betula platyphylla* forests have been derived.

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Soil sample collection

Three typical 100 m × 100 m fixed experimental plots were selected in typical locations with similar site conditions (*Table 1*), including the main forest types *Larix gmelinii* forest (LG), *Pinus sylvestris* forest (PS), and *Betula platyphylla* forest (BP), and 3 quadrats of 20 m × 20 m each in different forest plots (*Fig. 1*). At the beginning of May 2019, the active soil layer was in a rapid melting stage, and the temperature gradually increased. The upper soil layer was in a melting state (average depth of about 23 cm), while the lower part was still frozen, respectively. Collect 5 samples of the melting active layer (0–20 cm) in the sample area according to the "S" route. After removing debris, tree roots and other debris, seal them in a sterilized ziplock bag and bring them back to the laboratory. The soil sample was evenly mixed and divided into 2 parts. One part was air-dried through a 2 mm sieve for the determination of soil physical and chemical properties. The other part was stored in a refrigerator at -80 °C and used for determination of soil microorganism sequence within one week.

Table 1. *Basic overview of three forest type plots*

Forest types	Latitude (North)	Longitude (East)	Altitude (m)	Slope (°)	Aspect	Canopy density	Active soil layer (m)
Larix gmelinii forest	53°28′03.2″	122°20′38.6″	320	9	Northeast	0.8	0.8-1.5
Pinus sylvestris forest	53°28′03.3″	122°21′07.6″	305	10	Northeast	0.7	1.1-1.8
Betula platyphylla forest	53°26′32.4″	122°12′18.0″	336	12	Northeast	0.8	1.5-2.5

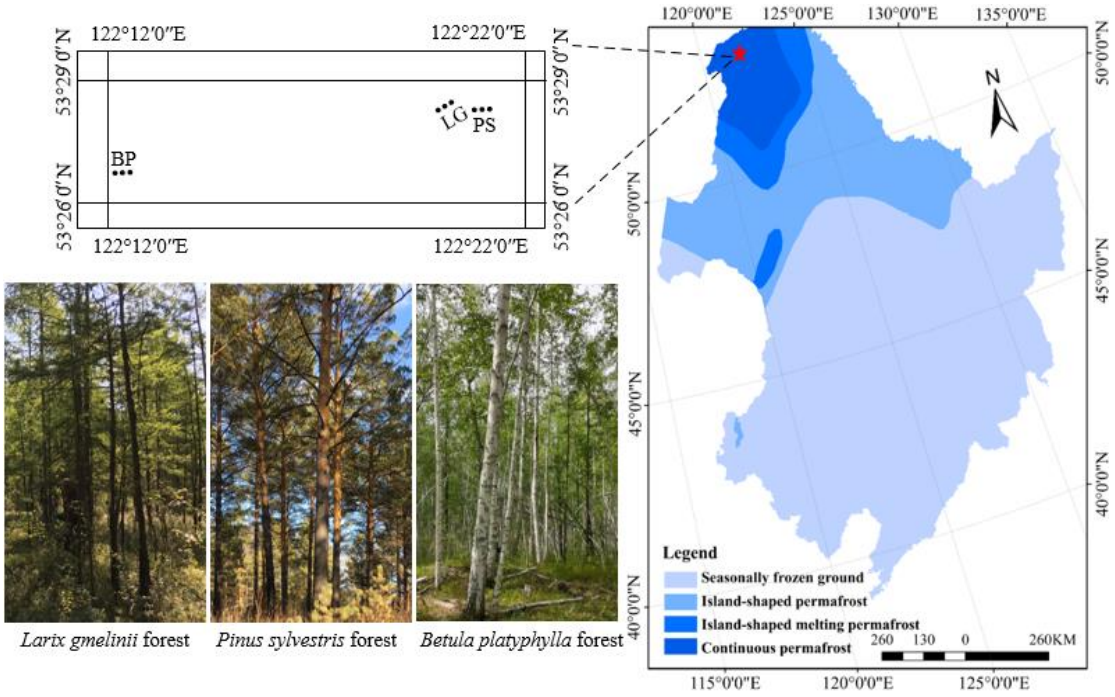

Figure 1. *Map of the study site*

Determination of physical and chemical properties of soil samples

Determination of soil moisture content: put the fresh soil sample back into the aluminum box and weigh it on the balance, accurate to 0.01 g, tilt the box lid on the

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aluminum basin, and put it into the preheated to 10 ± 2 °C. Bake in a constant temperature drying oven for 8 h, and take out the weighing calculation; the soil pH is measured with an acid meter (PHSJ-3F, INESA Scientific Instrument Co., Ltd, China) with a water-soil ratio of 2.5:1; the TOC and TN were measured by an elemental analyzer (VarioEL III, Elementar Analysensysteme GmbH, Germany). Total phosphorus: The $NaHCO_3$ extraction and $HClO_4$-H_2SO_4 digestion approaches were implemented to identify the total phosphorus.

DNA extraction and high-throughput sequencing

Using the Power Soil DNA extraction kit (MoBio Laboratories, USA), according to the instructions, weigh 0.5 g of fresh soil and extract genomic DNA from all soil samples (n = 9). The extracted genomic DNA was detected by 1% agarose gel electrophoresis. Universal primers 338F (5'-ACTCCTACGGGAGGCAG CA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used for bacterial 16S rRNA genes. The amplified product was detected by 2% agarose gel electrophoresis, and recovered from the gel using the AxyPrep DNA gel extraction kit (Axygen Biosciences, USA), washed with Tris-HCl, and verified by 2% agarose gel electrophoresis. Use the QuantiFluor™-ST fluorometer to quantify the polymerase chain reaction (PCR) products, and adjust the samples as necessary for sequencing. Sequencing was performed by Shanghai Majorbio Bioengineer Co., Ltd. (Shanghai, China) using the Illumina MiSeq platform.

16S rRNA gene sequence analysis

The original fastq sequence file is quality filtered by Trimmomatic, and then stitched by FLASH, which requires to meet the following standards: (i) 2 nucleotide mismatches are allowed, and delete the fragments containing ambiguous bases; (ii) sequences with an overlap length exceeding 10 bp are merged according to their overlapping sequences; (iii) a sequence less than 50 bp and an average quality score of less than 20. Using UPARSE, the operational taxonomic unit (OTU) is classified with 97% similarity, and UCHIME is used to identify and remove chimeric sequences. The classification of all 16S rRNA gene sequences was analyzed by the ribosomal database project (RDP) classification algorithm against the Silva (SSU123) 16S rRNA database.

Statistics analysis

Use Mothur software to calculate community diversity parameters (Simpson, Shannon-Wiener index) and community richness parameters (Chao, ACE index) that belong to alpha diversity analysis. Principal co-ordinates analysis (PCoA) was performed by using the R software to visually analyze based on vegan package, and calculate the redundancy analysis (RDA) based on the OTU level. One-way analysis of variance (ANOVA) was used to analyze the effects of different forest types on soil physico-chemistrical properties and microbial diversity, and Duncan test was used to test the significance of each indicator between different forest types ($\alpha = 0.05$). All sample results are mean ± standard deviation (SD). The result of $P < 0.05$ between groups was proved to be statistically significant.

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Results

Changes in soil physical and chemical properties under different forest types

The soil physical and chemical properties of different forest types in Greater Xing'an Mountains were obviously different (*Table 2*). The soil pH range was 4.49~5.67, indicating that the soils of the three forest types were acidic, and the lowest pH value of the BP was 4.49, which was significantly lower than that of the other two forest types ($P < 0.05$). The soil water content (SWC) in three different forest types differed significantly, the SWC of PS (36.36%) was highest and SWC of LG (27.95%) was lowest. Total organic carbon (TOC) in three different forest types differed significantly, which the TOC of PS (57.86 g/kg) was highest and the TOC of BP (34.93 g/kg) was lowest. Total nitrogen (TN) in three different forest types differed significantly, which the TN of LG (5.37 g/kg) was highest and the TN of BP (2.26 g/kg) was lowest. Total phosphorus (TP) in three different forest types differed significantly, which the TP of LG was highest (0.59 g/kg) and the TP of PS was lowest (0.45 g/kg).

Table 2. *Soil physical and chemical properties in different forest types*

Soil physical and chemical properties	*Larix gmelinii* forest (LG)	*Pinus sylvestris* forest (PS)	*Betula platyphylla* forest (BP)
pH	5.67 ± 0.31 a	5.48 ± 0.24 a	4.49 ± 0.16 b
Soil Water Content (%)	27.95 ± 2.48 c	36.36 ± 2.05 a	32.71 ± 1.53 b
Total Organic Carbon (g/kg)	49.27 ± 3.73 b	57.86 ± 2.42 a	34.93 ± 1.17 c
Total Nitrogen (g/kg)	5.37 ± 0.23 a	4.45 ± 0.34 b	2.26 ± 0.19 c
Total Phosphorus (g/kg)	0.59 ± 0.05 a	0.45 ± 0.02 c	0.51 ± 0.09 b

The values in the table are mean ± standard deviation; different letters indicate significant differences at the 0.05 level

Soil bacterial OTU of different forest types

The Venn result showed in *Figure 2*. There were 5080 soil bacterial OTUs in three forest types. Among them, there were 876 (34.91%) soil bacterial OTU sequences shared in *Larix gmelinii* forest (LG), *Pinus sylvestris* forest (PS) and *Betula platyphylla* forest (BP). The lowest number of BP soil bacteria-specific OTUs was 114, accounting for 4.54% of the total, the highest LG soil bacteria-specific OTUs was 498, accounting for 19.85% of the total, and the PS soil bacteria-specific OTUs was 202, accounting for 8.05% of the total. The results of the data show that the number of bacterial OTUs was higher in the LG and PS soils, and the number of unique OTUs in the LG soil was the largest.

Changes of soil microbial alpha and beta diversity in different forest types

From *Table 3*, we can infer that the Shannon and Simpson indices did not differ significantly ($P > 0.05$), but the ACE and Chao1 indices differed significantly ($P < 0.05$). The Shannon index ranged from 5.51 (BP) to 5.70 (PS), and the Simpson index ranged from 0.009 (PS) to 0.013 (LG). The ACE index ranged from 1128.84 (BP) to 1576.15 (PS), and the Chao1 index ranged from 1128.84 (BP) to 1573.92 (PS), PS was significantly higher than BP and LG ($P < 0.05$).

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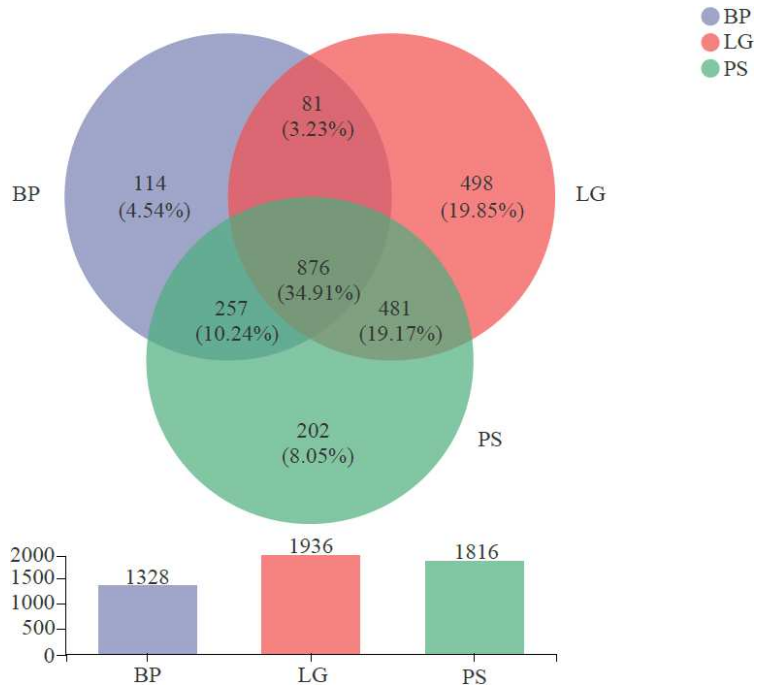

Figure 2. *Venn diagram of bacterial community structure based on operational taxonomic unit (OTU) level of different forest types*

Table 3. *Soil bacterial alpha diversity in different forest types*

Forest type	Shannon	Simpson	ACE	Chao1
BP	5.51 ± 0.23 a	0.010 ± 0.002 a	1128.84 ± 41.84 b	1128.84 ± 60.69 b
LG	5.58 ± 0.46 a	0.013 ± 0.009 a	1279.97 ± 69.53 b	1284.38 ± 89.31 b
PS	5.70 ± 0.33 a	0.009 ± 0.004 a	1576.15 ± 63.08 a	1573.92 ± 54.77 a

Larix gmelinii forest (LG), *Pinus sylvestris* forest (PS), and *Betula platyphylla* forest (BP). The values in the table are mean ± standard deviation; different letters indicate significant differences at the 0.05 level

The beta diversity of bacterial communities in different forest types was measured by PCoA of Bray-Crutis distance. The variance contribution rate of principal component 1 (PC1) was 41.89%, and the contribution rate of principal component 2 (PC2) was 23.96%, a total of 65.85% for the whole soil bacterial community composition (*Fig. 3*). The differences in bacterial community structure between different forest types are significant (PERMANOVA: $R^2 = 0.5014$, $P < 0.01$), the differences within the samples are not significant, and the differences are mainly from different samples. Moreover, the bacterial community structures of PS and BP were similar but different with LG.

Analysis of soil bacterial community structure composition in different forest types

From the perspective of the overall bacterial community structure, all OTUs belong to 58 bacterial phyla. If the sequence cannot be classified to a known phylum level, the phyla were uniformly classified as "others". From the relative abundance on all phylum level of the three forest types, the predominant phyla in all samples were *Proteobacteria* (23.44%), *Acidobacteria* (22.35%), *Chloroflexi* (20.84%), and *Actinobacteria* (14.13%) (*Fig. 4a*).

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The soil bacterial composition of *Betula platyphylla* forest on phylum level showed in *Figure 4b*. The relative abundance of *Acidobacteria* in BP soil dominant bacteria was 34.64%, *Proteobacteria* was 27.00%, *Chloroflexi* was 10.32%, *Actinobacteria* was 10.10%. The soil bacterial composition of *Larix gmelinii* forest on phylum level showed in *Figure 4c*. The relative abundance of *Proteobacteria* in LG soil was 30.62%, *Acidobacteria* was 18.31%, *Actinobacteria* was 16.01%, *Chloroflexi* was 14.58%. The soil bacterial composition of *Pinus sylvestris* forest on phylum level showed in *Figure 4d*. The relative abundance of *Proteobacteria* in PS soil was 25.54%, *Acidobacteria* was 24.59%, *Actinobacteria* was 16.46%, *Chloroflexi* was 13.67%.

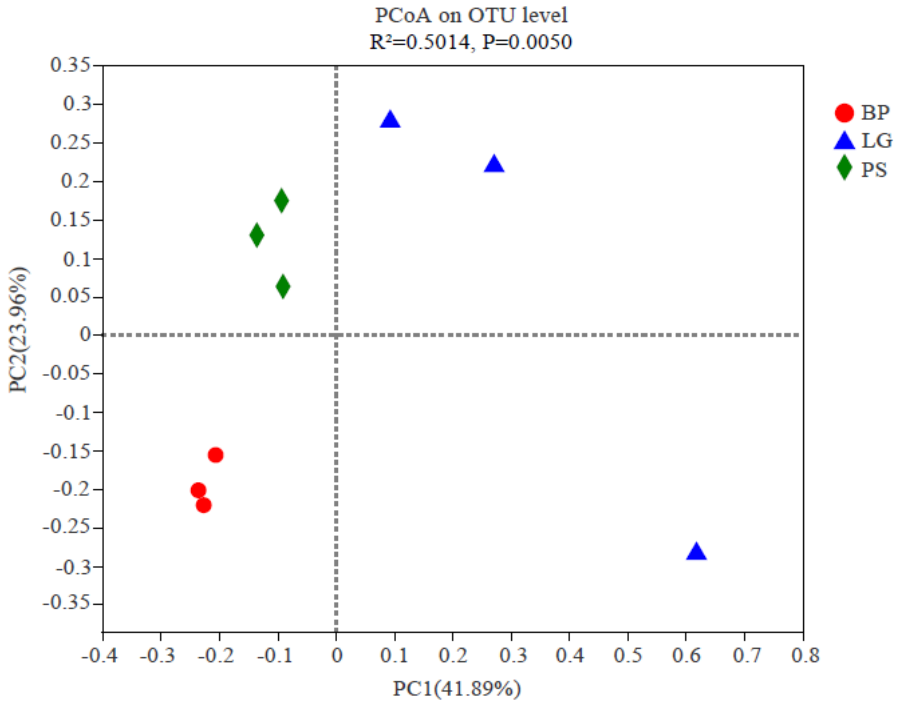
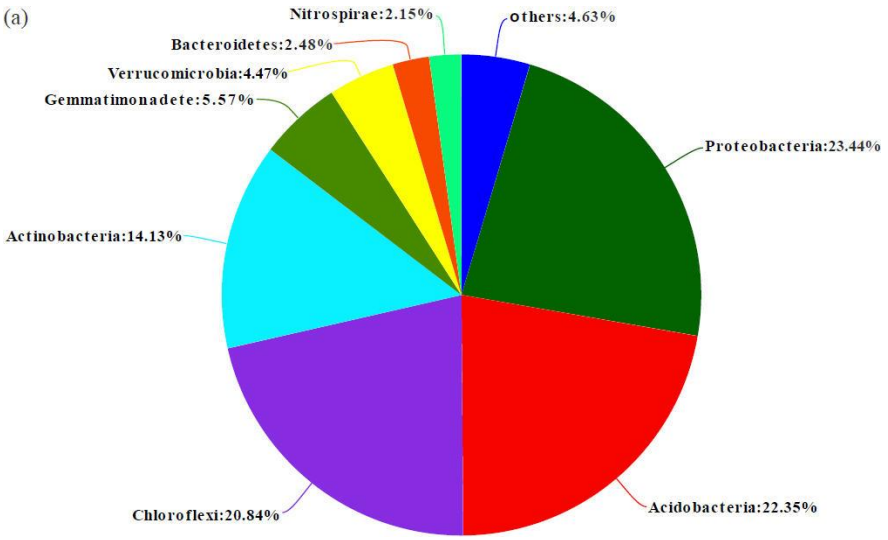


Figure 3. *Principal co-ordinates analysis (PCoA) of soil bacterial communities in different forest types*



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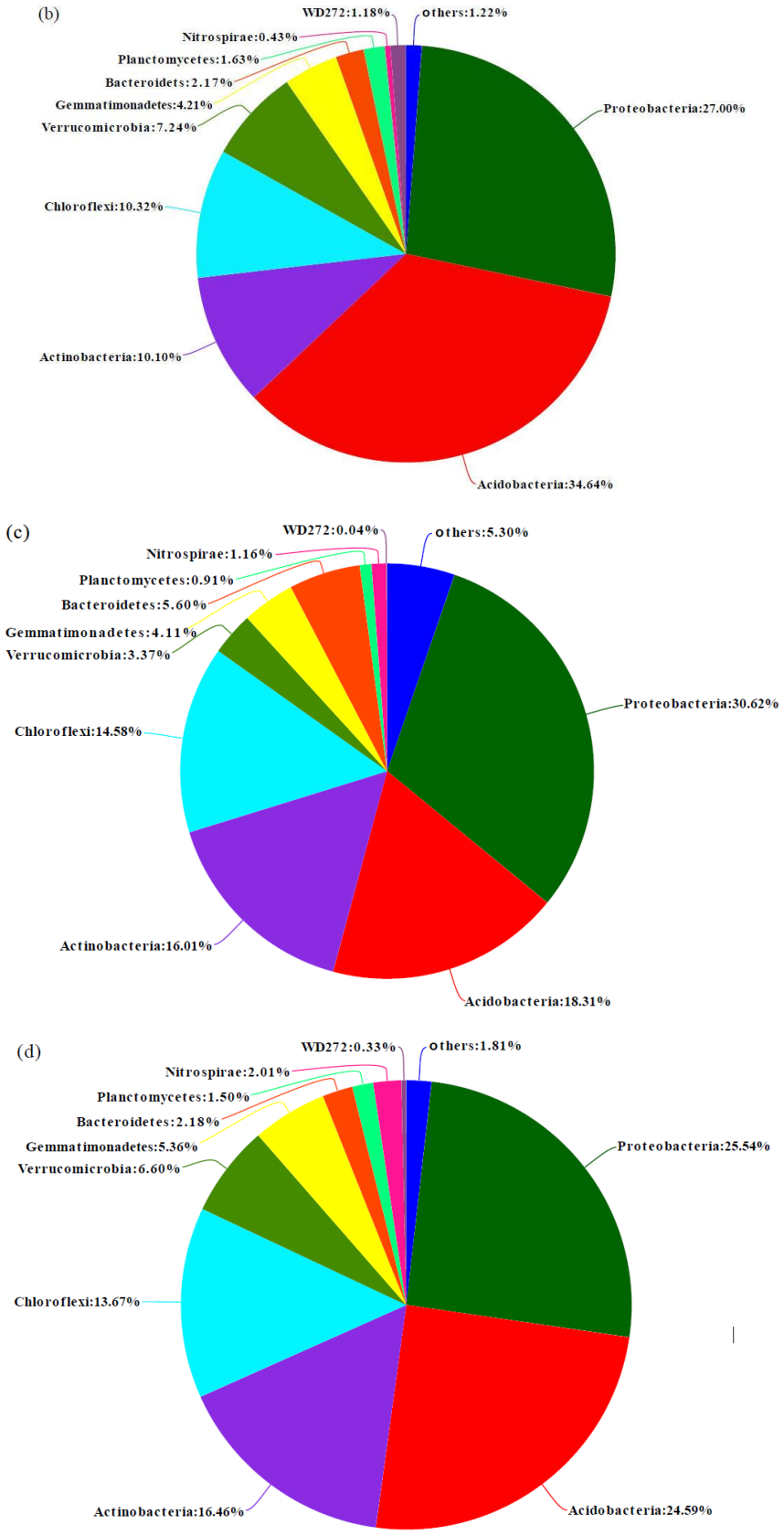

Figure 4. *The composition of bacterial community structure at the level of phylum in different forest types. (a) All, (b) Betula platyphylla forest (BP), (c) Larix gmelinii forest (LG), (d) Pinus sylvestris forest (PS)*

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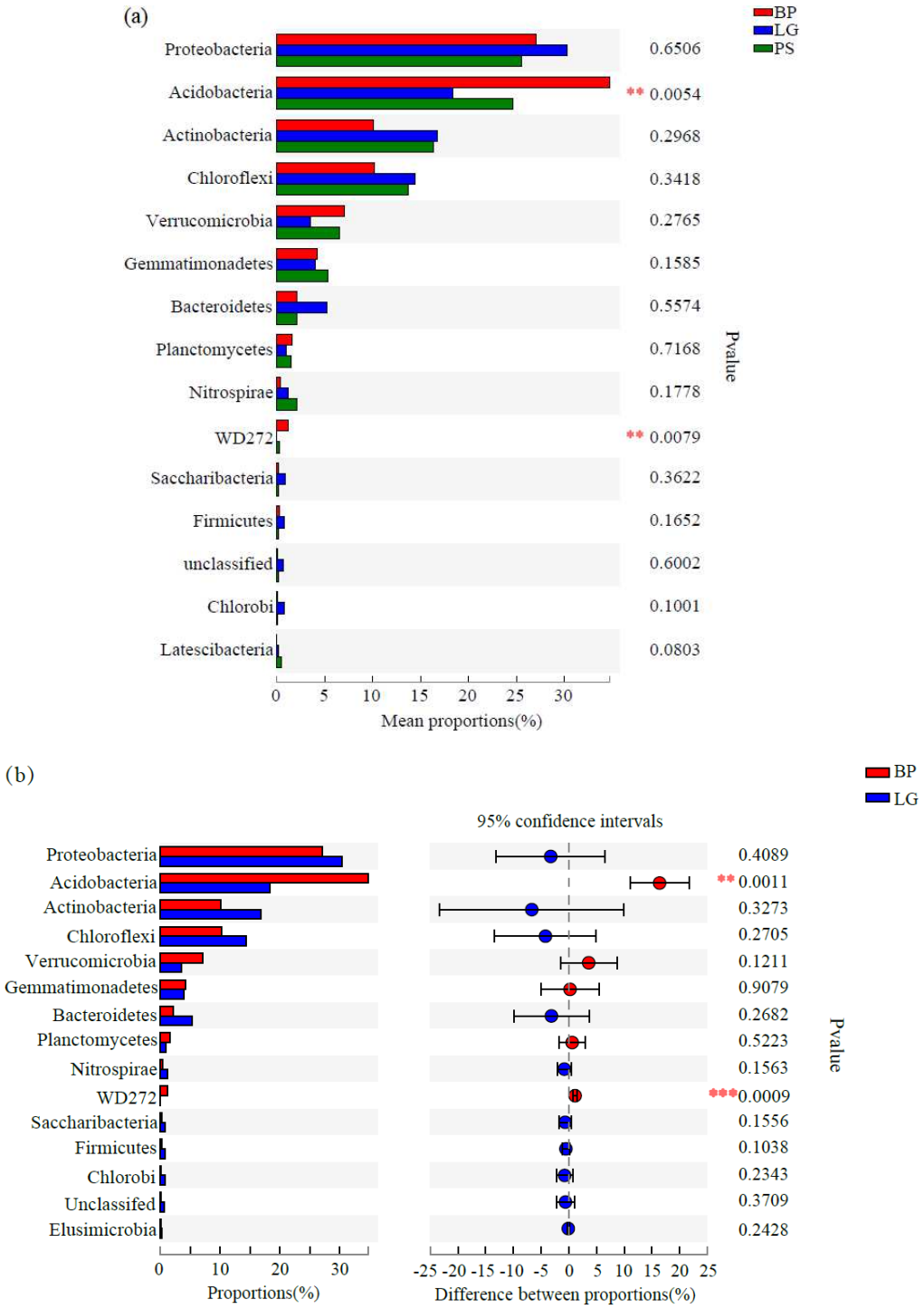
The two-sample T-test method was used to analyze the differences on the phylum level. There were two phyla significant differences in the three forests, *Acidobacteria* ($P < 0.01$) and *WD272* ($P < 0.01$, *Fig. 5a*). There were two phyla significant differences between BP and LG, mainly *Acidobacteria* ($P < 0.01$) and *WD272* ($P < 0.001$, *Fig. 5b*). There were four significant differences between BP and PS, mainly *Acidobacteria* ($P < 0.05$), *Gemmatimonadetes* ($P < 0.05$), *WD272* ($P < 0.01$), *Latescibacteria* ($P < 0.05$, *Fig. 5c*). There were two significant differences between LG and PS, mainly *Acidobacteria* ($P < 0.05$) and *Firmicutes* ($P < 0.05$, *Fig. 5d*).



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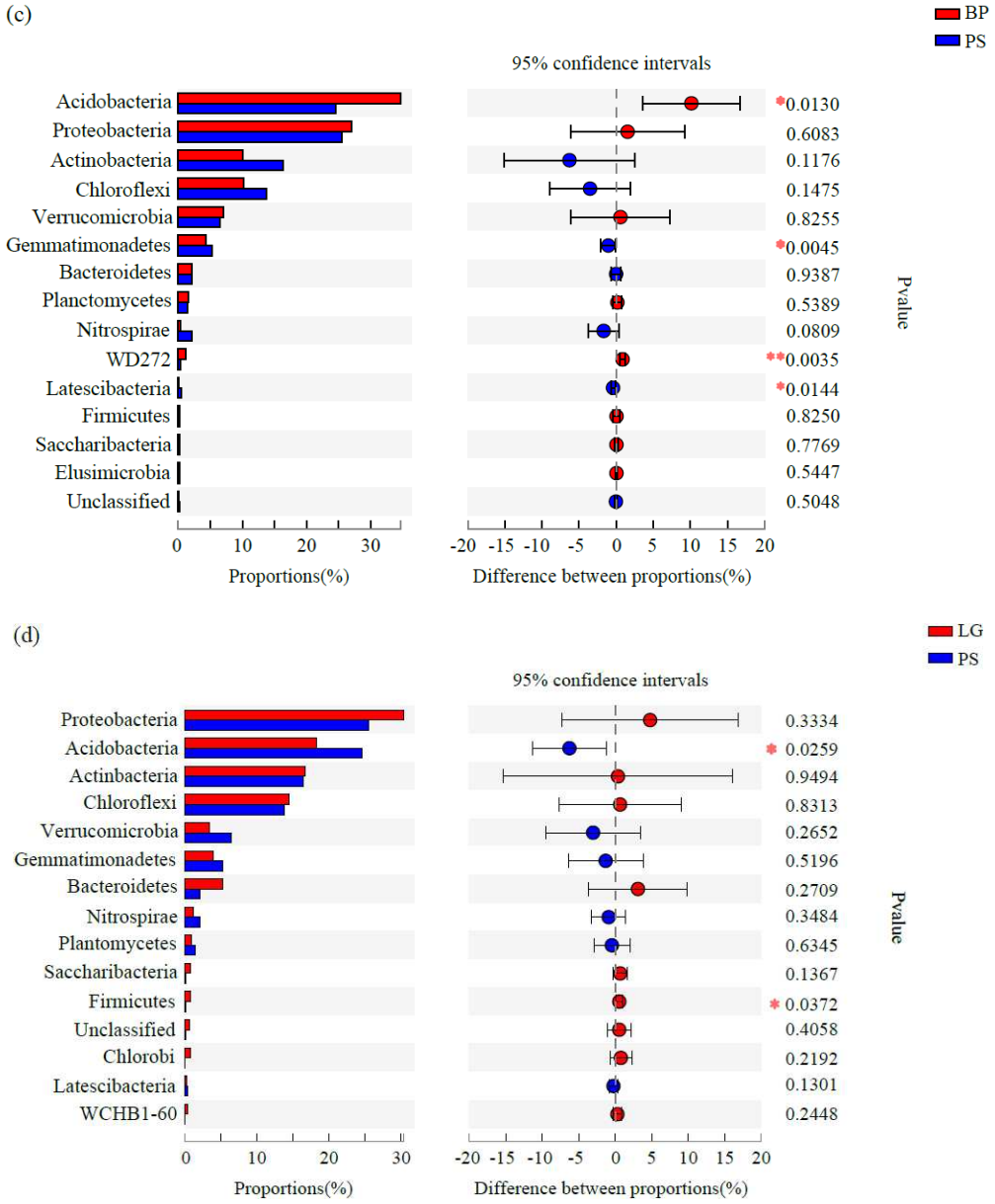

Figure 5. *Differences in the abundance of bacterial phylum in different forest types. * $0.01 < P \leq 0.05$, ** $0.001 < P \leq 0.01$ and *** $P \leq 0.001$*

Redundancy analysis of soil bacterial communities and physicochemical properties of different forest types

The physicochemical properties of the soil were analyzed redundancy analysis (RDA) with the bacterial community composition at the OTU levels. The analysis results showed in *Figure 6*. The first-order axis explained 49.31% and the second-order axis explained 18.48% of all information. The longer rays of pH indicate that it had a greater influence on the bacterial community composition, while the shorter rays of TN value indicate that it had less influence on the bacterial community composition. The bacterial community structures of LG were obvious positive affected by pH and TN, while the bacterial community structures of BP were obvious positive affected by TOC. The bacterial community structures of PS were obvious positive affected by TP.

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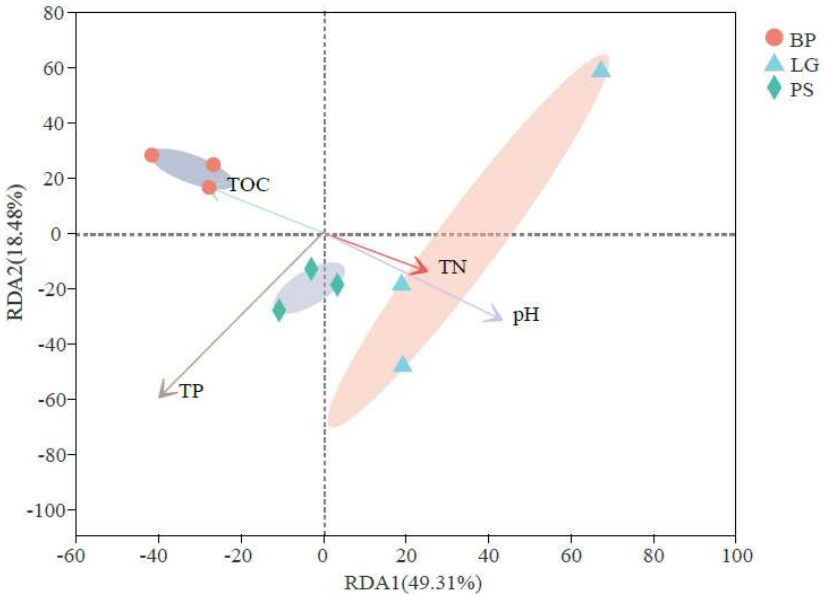

Figure 6. *Redundancy analysis (RDA) of soil bacterial community structure and soil physical and chemical properties*

Discussion

Soil physico-chemistry properties of the different forest types

In this study, we can observe that soil physico-chemistry properties varied significantly among different forest types (*Table 2*). According to our results, *Betula platyphylla* forest has reduced the soil total organic carbon and total nitrogen, which was consistent with the study of Yu et al. (2020). This influence may be attributed to long-term forest types change from original *Larix gmelinii* forest into broadleaf forest succession stage, which led to the soil nutrition lost when the dominant vegetation destroyed. In the cold regions, litter decomposes slowly because of the long winter and low temperature each year, and when the forest vegetation destroys, the soil nutrient, e.g. TOC and TN will lose quickly and cannot recover for a short time (Ramirez et al., 2010). Therefore, in our study, TOC, TN in LG were higher than those in BP. So the forest types would seem to play an important role in regulating soil physico-chemistry properties inside the same cold climate and, in particular, the difference between broadleaf forest and coniferous forest should be noted. Despite the numerous recalcitrant substances were found in the litter from *Larix gmelinii* forest, such as lignin, resin, tannin, and wax, the dense coniferous litter covering the soil surface impeded air circulation, and reduced the loss of soil nutrients (Thomson et al., 2015). On the contrary, *Betula platyphylla* forest is often a component of temperate deciduous broadleaf forests; it produces relatively large amount litter, and its nutrient contents in the soil are relatively high (Deng et al., 2019). However, in the cold temperature zone, we found that the litter cannot decompose soon because of the cold temperature and long winter as well as the soil microbial activity. So this is the reason why our result is not consistent with Deng et al. (2019) study. Therefore, in the high latitude permafrost region, the soil nutrition cannot be accumulated soon by litter and once the dominant forest suffered destroyed and led to soil nutrition lost, it would not get recovery by a short time.

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Changes of soil bacterial diversity response to different forest types

We found that different forest types had distinct soil bacterial abundant diversity (ACE and Chao1) (*Table 3*). But we do not find obvious different in soil bacterial Shannon and Simpson diversity. There were some studies proved that the crucial role of soil physico-chemistry properties is in altering the soil bacterial diversity during forest succession (Thomson et al., 2015). Soil pH, especially in terms of C, N, and P availability, are paramount factors (Tan et al., 2013) and significantly influence bacterial diversity (Zhong et al., 2010). It is reported that bacterial diversity would be decreased when soil pH was below 6.5, and soil bacterial diversity would be increased when soil pH close to neutral (Bergkemper et al., 2016). In our study, however, we found that a relatively small pH range (4.49 to 5.67), making it different to find correlation with bacterial diversity. But previous studies have indicated that bacterial abundant diversity is largely affected by TOC and TN (Siles and Margesin, 2016). Therefore, in our study, TOC and TN concentrations were significantly correlated with bacterial abundant diversity (ACE and Chao1), which was consistent with the observations obtained by Lin et al. (2014).

Changes of soil bacterial composition in different forest types

Similar to the diversity of bacterial communities, the abundance of the main bacterial phylum is affected by different vegetation types. Different forest types contain different bacterial structures. In this study, *Proteobacteria* and *Acidobacteria* were the most abundant phyla in the soil, which was consistent with the research of Sun et al. (2014), which found that the main bacterial types in the forests of Northeast China were *Proteobacteria* and *Acidobacteria*. We also found that *Acidobacteria*, *WD272*, *Gemmatimonadetes*, *Latescibacteria*, *Firmicutes* varied greatly between different forest types (*Fig. 5*).

In our study, the relative abundance of *Acidobacteria* among the three forest types was highest, and in BP was higher than that in LG and PS. *Acidobacteria* is a dominant phylum in broad leaf forests because it plays a crucial role in litter deposition. In this study, litters in conifer forests are difficult to decompose, but in the broad leaf forests, the litters are easy to decompose. Therefore, this difference of litter composition may be the reason why the abundance of *Acidobacteria* was higher in BP forests. Similar researches were found by Kopecky et al. (2011). The relative abundance of *Actinobacteria* in coniferous forests was significantly higher than that in broad-leaved forests may be due to differences in soil organic carbon, total nitrogen, and total phosphorus in different forest types (*Table 2*). *Actinobacteria* is usually positive to soil nutrition. We found that the TOC, TN and TP in conifer forest soil were significantly higher than those in broad leaf forest. This may result the abundance of *Actinobacteria* was higher in coniferous forest types. This has been consistent with study of Guo et al. (2018), which found that with different stages of forest succession, the *Actinobacteria* abundance and soil organic matter accumulation showed a significantly positive correlation. In addition, *Firmicutes* were significantly higher in LG than in PS and BP, which also shows that *Firmicutes* is more adaptable to environments in poorer habitat conditions (Hartmann et al., 2014). The physical-chemical composition of broad-leaved forest and coniferous forest vegetation, the competition among plant species and the change of plant diversity will cause the change of plant litter, organic components and soil bacterial composition.

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Effects of soil environmental factors on different forest types bacterial community

In terrestrial ecosystems, soil physical-chemical properties are important factors affecting soil microbial communities (Fierer and Jackson, 2006), and are inversely related to the soil bacterial structure composition (Zhou et al., 2017). In our study, we found that there is a clear correlation between bacterial structure composition and pH, TOC, TN and TP, most likely due to the significant difference in soil physical-chemical properties in this study (*Fig. 6*). The litters of three forest types were different, and the soil physico-chemistry properties were mainly input by litters. Therefore, we can infer that the soil available nutrition of coniferous forest and broad-leaved forest was obviously changed by aboveground litter, and finally affected by soil bacterial diversity. Similar study has proved this phenomenon (Lin et al., 2014).

PCoA results showed that soil bacterial communities of different forest types showed obvious differentiation (*Fig. 3*), moreover, the bacterial communities of BP and PS were similar and different with those of LG forest, which seemed more various between three replications. Soil bacterial community was controlled by soil physico-chemistrical properties and vegetation. In this study, we selected three forest types, the original *Larix gmelinii* forest, and the successional forest, including *Betula platyphylla* forest and *Pinus sylvestris* forest. The vegetation composition of original *Larix gmelinii* forest was simplified and the herb was less. However, vegetation compositions of the successional stages were complicated and the herbs also were abundant and similar. Therefore, the bacterial community composition of PS and BP were similar and differed from LG forest. In addition to soil environmental factors, litter decomposition rate, water and heat conditions in active layer, root biomass and exudates may also be important factors driving soil microbial community construction in high latitude cold region forest ecosystem.

Conclusions

Our results indicate that the soil physical and chemical characteristics and bacterial community composition of different forest types in the Greater Xing'an Mountains were significantly different. Coniferous forests have higher total organic carbon and total nitrogen than broad-leaved forests. In this study, it was found that soil pH, TOC, TN and TP may be the limiting factors of bacterial community structure and composition, and will significantly affect the diversity of bacteria. The results of this study are of great significance for us to understand the impact of forest type changes on soil bacterial community structure. Therefore, to study the coupling relationship between vegetation, microbes and soil environment can provide a deeper understanding for forest ecosystem processes. Further multi-dimensional comprehensive experiments will be carried out in the future to clarify the response mechanism of soil microbial community structure and function to environmental heterogeneity under the background of climate change.

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PRINCIPLE COMPONENT ANALYSIS (PCA) OF BEAN GENOTYPES (*Phaseolus vulgaris* L.) CONCERNING AGRONOMIC, MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

GIRGEL, U.

Kahramanmaras Sutcu Imam University, Goksun Vocational School, Goksun, Kahramanmaras, Turkey
e-mail: umitgirgel@ksu.edu.tr; phone: +90-344-300-1536

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Abstract. This study was established in Bayburt University with 3 replications according to the Randomized Complete Block Design (RCBD) pattern in Bayburt University Organic Agriculture Research and Application Treatment Area in order to determine the agro-morphological, biochemical and quality characteristics of 5 local bean (*Phaseolus vulgaris* L.) genotypes (Aydintepe, Mollakoy, Konursu, Yukarikirzi, Suludere) and 2 registered bean varieties (Ala Ciftci, Mispir). During the research, Principle Component Analysis (PCA) was performed on dry bean genotypes. The first principal component had 37.899% of the total variation (PC1). The second principle component (PC2) explained 19.975% of the total variation. The third principle component had 15.906 of the total variation (PC3). The cumulative ratio of the three primary components in total variation was 73.780%. In the first principle component, Carotenoid (0.905), Chlorophyll-B (0.798), Pod Number per Plant (0.745), Pod Length (0.701), Chlorophyll-C (0.684), Chlorophyll-A (0.608), Branch Number (0.563), First Pod Height (0.491) and Thousand Seed Weight (0.314) had the highest coefficients. The genotypes used in the study differ greatly from each other in terms of agronomic, morphological and biochemical characteristics. This is important for breeders trying to create variability, and it may be recommended to include these genotypes as genitors in breeding studies.
Keywords: *local genotypes, variety, Phaseolus vulgaris L., Principle Component Analysis (PCA)*

Introduction

Bean (*Phaseolus vulgaris* L.) is an edible legume plant belonging to The Leguminosae family. It has been reported by researchers that in the family, beans are the commonly produced species in the world (Singh et al., 2007). Leguminosae family is the second commonly important family in agriculture after Gramineae family. Although grains are a very important source of energy, legumes are an important source of protein for humanity (Singh, 2005, 2007). This family meets 33 percent of human protein needs (Graham and Vance, 2003). On the other hand, *Phaseolus* species are important worldwide for human and animal consumption (Graham and Ranalli, 1997; Logozzo et al., 2007; Aquino-Bolanos et al., 2016). Beans are consumed as canned and frozen as well as fresh and dried beans (Paredes et al., 2009).

Lewis et al. (2005), reported that legumes include agroforestry species, oilseed crops, major grain legumes, ornamental crops, forage crops etc.

Legumes are economically important because it is used in world trade as linoleum, chemicals, lubrication, paints, ethanol coatings, pharmaceutical products, soap, resins, cosmetics, plastic coatings etc. (Singh et al., 2007).

Beans are grown on five continents and these continents are Asia, North and South America, East Africa and West-Southeast Europe (Siemonsma and Na Lapang, 1992). According to 2018 data in the world, 33 million hectares of dry beans were harvested and 28.9 million tons were produced. In the same year 225 thousand tons of dry bean

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production was performed in 89 thousand hectares of land in Turkey. Yield for dry bean in the world was 874 kg/ha and in Turkey was 2531 kg/ha (FAO, 2019).

Central America (Mesoamerica) and South America (Andea) regions are the bean gene pools (Gepts, 1998; Checa et al., 2006; Angioi et al., 2010; Bitocchi et al., 2012; Cortes, 2013). The Central America (Mesoamerica) gene pool extends from Mexico to Colombia, and the South America (Andea) gene pool extends from Southern Peru to Northwest Argentina (De la Funte, 2012). Dry beans (*Phaseolus vulgaris* L., 2n = 2x = 22) are a type of self-pollinated product (Yeken et al., 2018).

After the beans were cultured, many genotypes that differ in morpho-agronomic characteristics were developed and this diversity is used in breeding and expansion of the gene pool (Sinkovic et al., 2019).

Principal Component Analysis (PCA) is a multivariate statistical technique that aims to reduce the dimensionality of high dimensional data sets (Wiley, 1981). It does so by computing much smaller variables (Principle Components) that represent the original data set. Each new variable is a linear combination of the original variables. The first principle component is the linear combination of original variables that explains the maximum amount of variance. The second principal component is perpendicular to the first principal component and describes the maximum amount of remaining variance in the data. All essential components are perpendicular to each other, so there is no unnecessary information (Dona et al., 2009).

Difficulty may be encountered in interpreting and summarizing analysis results with too many variables. In such cases, principal component analysis (PCA), one of the multivariate statistical methods, is widely used (Sangun, 2007). In this way, principal component analysis has been used in many studies (Rencher, 2002; Marcus, 2004; Pierce et al., 2006; Shittu et al., 2007; Widodo et al., 2007; Madakbas and Ergin, 2011; Rencher and Christensen, 2012; Canci et al., 2019). On the other hand, Principal Component Analysis (PCA), which is used to eliminate the dependency structure between variables or for dimension reduction, is used as an analysis used alone, as well as a data preparation technique for other analyzes (Sharma, 1996). On the other hand, PCA analysis in dry bean was used to calculate the Euclidean distances between cultivars (Adams, 1977).

This research was carried out in order to obtain information that could be the basis for future cultivar development studies in the bean plant. For this purpose, important Agronomic, morphological and biochemical characteristics of bean genotypes and standard varieties collected from different locations were examined and the PCA analysis results of these characteristics were presented.

Materials and Methods

Site Description

In this study was established in Bayburt University Aydıntepe Vocational School Research Area (40°24’05.7” N, 40°08’31.3” E) in Turkey. In the research, Aydintepe, Ala Ciftci, Mollakoy, Konursu, Mispir, Yukarikirzi, Suludere bean (*Phaseolus vulgaris* L.) genotypes were used. Two of them (Ala Ciftci, Mispir) were registered bean variety and the others are local bean genotypes.

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Experiment

The experiment was laid out in a randomized complete block design (RCBD) with three replications with 3 replications. 4 rows of planting were made in each plot, and the seeds were planted by hand at a depth of 5-6 cm in rows opened with a marker and 50 cm between inter-row spacing and 10 cm intra-row spacing was used (The plot size is 5.0 m x 0.5 m x 4 row = 10 m^2). According to Sehirali (1988), the water requirement of the bean plant depending on the climatic conditions was provided by the sprinkler system. Weeds were destroyed manually according to the situation in the environment. On the other hand, 6.0 kg P_2O_5 and 2.5 kg N_2 per decare fertilizer was used at the time of planting. Growing rules for bean plants were applied equally to all plots (Meral et al., 1998; Bozoglu et al., 2002; Karadavut et al., 2011; Sozen et al., 2012; Sozen and Karadavut, 2016; Girgel and Cokkizgin, 2019).

Measurements

The following features measured according to Hardwick et al. (1978), IBPGR (1982), Berrocal-Ibarra et al. (2002), Karadavut et al. (2011) Asemanrafat and Honar (2017), Boydston et al. (2018), Saleh et al. (2018); the sample was taken on ten plants and its average was determined plant height, stem diameter, branch number, first pod height, pod length, pod width, pod number per plant, seed number per pod. It was decided to reach 50% of the plot for the following features, number of days to emergence, number of days to flowering, number of days to physiological maturity. Thousand seed weight was calculated according to this: 100 seeds were counted 4 times; the average was taken and multiplied by 10 (Girgel and Cokkizgin, 2019). The seed yield value was found by converting into kg/ha with proportion after the plots were harvested (Hardwick et al., 1978; Meral et al., 1998; Karadavut et al., 2011; Sozen and Karadavut, 2016; Girgel and Cokkizgin, 2019).

Chlorophyll-A, Chlorophyll-B, Chlorophyll-C, carotenoid, proline, malondialdehyde, total phenolic compounds parameters determined according to Chandler and Dodds (1983), Lichtenthaler (1987), and Kabbadj et al. (2017).

Statistical Analyses

The effect levels of the characters determining Principle Component Analysis (PCA) and Correlation Coefficient Analysis. PCA and Correlation coefficients were calculated using the xlstat statistical analysis program, which is a program that uses the Microsoft Excel infrastructure (XLSTAT, 2020).

Results and Discussions

Summary Statistics

According to results; number of days to emergence, number of days to flowering, plant height, stem diameter, branch number, first pod height, pod length, pod width, pod number per plant, seed number per pod, thousand seed weight, seed yield, number of days to physiological maturity, chlorophyll-a, chlorophyll-b, chlorophyll-b, carotenoid, proline, malondialdehyde, total phenolic compounds varied between 11.667-20.000 day, 58.000-69.333 day, 39.473-42.967 cm, 3.603-4.837 mm, 2.667-4.000 number, 15.433-18.700 cm, 9.000-11.587 cm, 1.320-1.810 cm, 2.333-4.000 number, 3.333-5.333 number, 341.667-450.333 g, 877.33-1546.67 kg/ha, 115.667-127.000 day, 210.947-282.526 µg/g, 82.023-128.235 µg/g, 126.287-159.569 µg/g, 0.696-1.578 µg/g, 1.120-1.926 µmol/gr, 1.577-2.796

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nmol/g, 19.210-23.434 mmol GA/g, respectively (*Table 1*). These results were found to be lower than what they obtained in the Bilashini Devi et al. (2018) study. This situation is the result of plant genetics, climate and environmental factors. Our findings are in agreement with other studies (Kamaluddin and Ahmed, 2011; Madakbas and Ergin, 2011; Gopinath et al., 2014; Hosseinpour et al., 2014; Aydogan, 2017; Kadioglu et al., 2020).

Table 1. *Summary Statistics for Bean Genotypes*

Variable	Minimum	Maximum	Mean	Std. deviation
NDE	11.667	20.000	16.095	2.904
NDUF	58.000	69.333	63.095	4.086
PH	39.473	42.967	41.209	1.265
SD	3.603	4.837	4.103	0.500
BN	2.667	4.000	3.114	0.418
FPH	15.433	18.700	16.650	1.161
PL	9.000	11.587	10.190	0.858
PW	1.320	1.810	1.507	0.168
PNPP	2.333	4.000	3.305	0.589
SNPP	3.333	5.333	4.219	0.710
TSW	341.667	450.333	401.667	46.187
SY	877.33	1546.67	123.976	25.813
NDPM	115.667	127.000	123.810	4.264
CHL-A	210.947	282.526	255.054	22.870
CHL-B	82.023	128.235	102.275	16.229
CHL-C	126.287	159.569	144.333	12.493
CARO	0.696	1.578	1.094	0.269
PROL	1.120	1.926	1.531	0.254
MDA	1.577	2.796	2.036	0.437
TPC	19.210	23.434	21.855	1.437

NDE:Number of days to emergence (day), NDUF:Number of days to flowering (day), PH: Plant height (cm), SD:Stem diameter (mm), BN:Branch number (number), FPH:First pod height (cm), PL:Pod lenght (cm), PW:Pod width (cm), PNPP:Pod number per plant (number), SNPP:Seed number per pod (number), TSW:Thousand seed weight (g), SY:Seed yield (kg/ha), NDPM:Number of days to physiological maturity (day), CHL-A:Chlorophyll-A (µg/g), CHL-B:Chlorophyll-B (µg/g), CHL-C:Chlorophyll-B (µg/g), CARO:Carotenoid (µg/g), PROL:Proline (µmol/gr), MDA: Malondialdehyde (nmol/g), TPC:Total phenolic compounds (mmol GA/g)

Correlation Coefficient Analysis

According to the correlation coefficient analysis (Pearson, 1900), positive-significant relationships were found between number of days to flowering and Malondialdehyde (r=0.854), stem diameter and branch number (r=0.757), first pod height and carotenoid (r=0.772), pod length and pod number per plant (r=0.870), seed number per pod and thousand seed weight (r=0.765), Chlorophyll-A and Chlorophyll-B (r=0.866), Chlorophyll-A and Chlorophyll-C (r=0.909), Chlorophyll-A and Carotenoid (r=0.861), Chlorophyll-B and Chlorophyll-C (r=0.843), Chlorophyll-B and Carotenoid (r=0.884), Chlorophyll-C and Carotenoid (r=0.814) (*Table 2*). Especially chlorophyll A, chlorophyll B, and chlorophyll C were found to be positively correlated as they are properties associated with photosynthesis. In terms of agronomical characters' correlation coefficients were similar to Tofiq et al. (2016) results. On the other hand, similar views were reported regarding the correlation coefficients between yield components (Aydogan, 2017). This situation reveals that there is a close relationship between biochemical characters especially between Chlorophyll elements with Carotenoid.

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Table 2. *Correlation matrix (Pearson (n))*

Variables	NDE	NDUF	PH	SD	BN	FPH	PL	PW	PNPP	SNPP	TSW	SY	NDPM	CHL-A	CHL-B	CHL-C	CARO	PROL	MDA
NDUF	-0.135																		
PH	0.329	-0.604																	
SD	-0.342	0.006	-0.110																
BN	-0.291	0.118	0.162	**0.757**															
FPH	0.067	0.303	0.430	0.201	0.734														
PL	0.237	0.265	-0.138	-0.405	-0.497	-0.298													
PW	0.229	0.208	-0.294	0.305	0.267	0.013	0.456												
PNPP	0.279	-0.145	-0.057	-0.379	-0.699	-0.650	**0.870**	0.303											
SNPP	0.374	-0.655	0.566	0.327	0.461	0.300	-0.556	0.126	-0.378										
TSW	0.737	-0.233	0.403	-0.057	0.211	0.423	-0.412	0.074	-0.391	**0.765**									
SY	0.433	-0.248	0.365	0.562	0.256	0.118	-0.141	0.108	0.023	0.460	0.347								
NDPM	0.575	-0.389	0.736	-0.250	0.083	0.352	0.332	0.336	0.273	0.489	0.448	0.179							
CHL-A	-0.146	0.302	0.168	0.294	0.469	0.633	-0.654	-0.554	**-0.788**	0.078	0.238	0.260	-0.338						
CHL-B	0.119	-0.007	0.370	0.521	0.563	0.583	-0.679	-0.340	-0.678	0.453	0.472	0.667	-0.095	**0.866**					
CHL-C	-0.091	0.160	0.020	0.351	0.455	0.469	**-0.857**	-0.484	**-0.881**	0.283	0.418	0.243	-0.441	**0.909**	**0.843**				
CARO	0.015	-0.081	0.548	0.297	0.623	**0.772**	-0.748	-0.452	**-0.823**	0.514	0.518	0.334	0.083	**0.861**	**0.884**	**0.814**			
PROL	-0.495	-0.717	0.065	0.078	-0.212	-0.619	-0.219	-0.310	0.169	0.117	-0.404	-0.149	-0.202	-0.346	-0.282	-0.215	-0.231		
MDA	0.010	**0.854**	-0.572	-0.105	-0.239	-0.059	0.639	0.264	0.356	**-0.813**	-0.436	-0.085	-0.300	-0.014	-0.224	-0.210	-0.429	-0.574	
TPC	0.484	0.217	-0.194	-0.730	**-0.881**	-0.437	0.466	-0.260	0.518	-0.510	-0.012	-0.112	-0.112	-0.153	-0.286	-0.194	-0.403	-0.234	0.487

Values in bold are different from 0 with a significance level alpha=0.05.

NDE:Number of days to emergence (day), NDUF:Number of days to flowering (day), PH: Plant height (cm), SD:Stem diameter (mm), BN:Branch number (number), FPH:First pod height (cm), PL:Pod lenght (cm), PW:Pod width (cm), PNPP:Pod number per plant (number), SNPP:Seed number per pod (number), TSW:Thousand seed weight (g), SY:Seed yield (kg/ha), NDPM:Number of days to physiological maturity (day), CHL-A:Chlorophyll-A (µg/g), CHL-B:Chlorophyll-B (µg/g), CHL-C:Chlorophyll-B (µg/g), CARO:Carotenoid (µg/g), PROL:Proline (µmol/gr), MDA: Malondialdehyde (nmol/g), TPC:Total phenolic compounds (mmol GA/g)

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Principal Component Analysis

Principal component analysis (PCA), which is a size reduction method using the data set of the studied agricultural characteristics, applied. All of the total variation has been derived from 6 principal component axis and Eigenvalues, Variability values (%) and Cumulative values (%) showed that *Table 3*. The first principal component had 37.899% of the total variation (PC1). The second principle component (PC2) explained 19.975% of the total variation. The third principle component had 15.906 of the total variation (PC3). The cumulative ratio of the three primary components in total variation was 73.780%. The rest of principle components (PC4=12.710%, PC5=7.689% and PC6=5.821%) had 26.22% of the total variation. As a result of the PCA analysis, 6 principle component axes were obtained and these axes represented all of the total variation. The 6 principle components explained 100% of the total variation. Madakbas and Ergin (2011) also reported that all variations were explained with the first 6 principle components in their work.

Table 3. *Eigenvalues, Variability and Cumulative Values*

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	7.580	3.995	3.181	2.542	1.538	1.164
Variability %	37.899	19.975	15.906	12.710	7.689	5.821
Cumulative %	37.899	57.874	73.780	86.489	94.179	100.000

Scree Plot (Graphical representation of Eigenvalues) was given in *Fig. 1*. Eigenvalues were 7.580 for PC1 and 3.995 (PC2), 3.181 (PC3), 2.542 (PC4), 1.538 (PC5) and 1.164 (PC6), respectively. If the eigenvalues are above 1, it indicates that the evaluated principal component weight values are reliable (Mohammadi and Prasanna, 2003). On the other, Iezzoni and Pritts (1991) reported that if the eingenvalue value is greater than 1 (PCs with eigenvalue >1.0), it is more informative than the original variable.

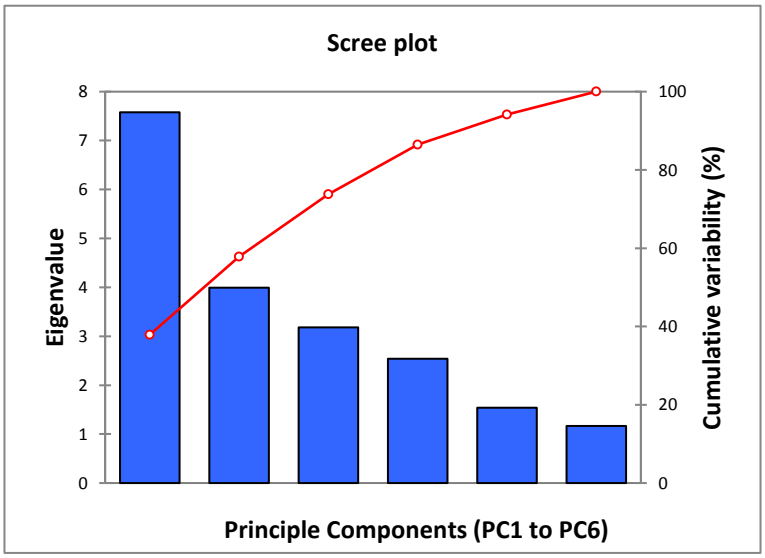

Figure 1. *Graphical representation of Eigenvalues*

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When this biplot is examined (*Fig. 2*), there is a positive relationship between the narrow angle features, for example NDPM with NDE or CARO with CHL-B etc. Right-angle features are not related to each other, for example CARO with NDPM. Wide-angle features have negative relationships with each other for example CARO with PNPP etc. The biplot technique enables the determination of the relationships between the variables as well as the detailed description of a multivariate data set (Yan and Rajcan, 2002).

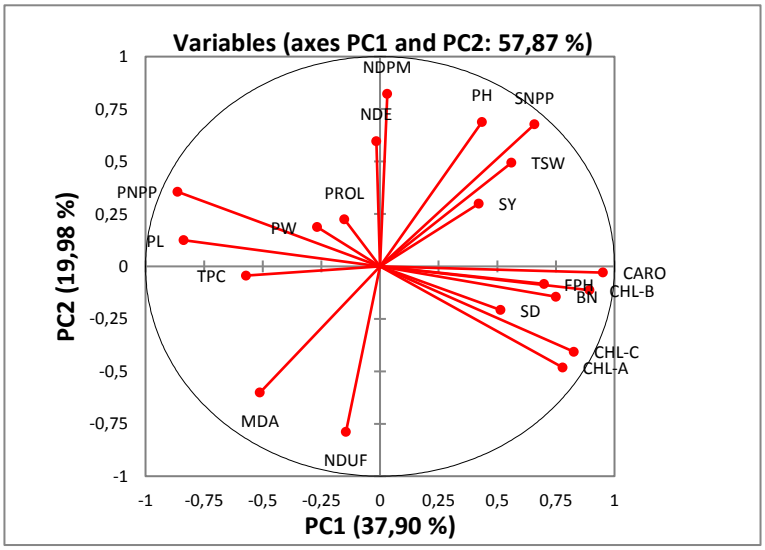

Figure 2. *Principal Component Analysis (PCA) biplot showing the distribution of agronomic, morphological and biochemical characteristics in the first principle component and the second principle component. NDE:Number of days to emergence (day), NDUF:Number of days to flowering (day), PH: Plant height (cm), SD:Stem diameter (mm), BN:Branch number (number), FPH:First pod height (cm), PL:Pod lenght (cm), PW:Pod width (cm), PNPP:Pod number per plant (number), SNPP:Seed number per pod (number), TSW:Thousand seed weight (g), SY:Seed yield (kg/ha), NDPM:Number of days to physiological maturity (day), CHL-A:Chlorophyll-A (µg/g), CHL-B:Chlorophyll-B (µg/g), CHL-C:Chlorophyll-B (µg/g), CARO:Carotenoid (µg/g), PROL:Proline (µmol/gr), MDA: Malondialdehyde (nmol/g), TPC:Total phenolic compounds (mmol GA/g)*

In the first principle component, CARO (0.905), CHL-B (0.798), PNPP (0.745), PL (0.701), CHL-C (0.684), CHL-A (0.608), BN (0.563), FPH (0.491) and TSW (0.314) had the highest coefficients, respectively (*Table 4*). In the second principle component, NDPM (0.677), NDUF (0.623), PH (0.474), SNPP (0.458), MDA (0.362) had the highest coefficients, respectively. For the third principle component PROL (0.903), NDE (0.484) had the highest coefficients, respectively. On the other hand, in the fourth principle component PW (0.701), TPC (0.456), SD (0.418) had the highest coefficients. In the fifth essential component SY (0.539) had the highest coefficients. Yeken et al. (2019) reported that the principle component values obtained from botanical features at these levels.

When the biplot graph of the genotypes used in the study is examined (*Fig. 3*), it is seen that the genotypes are quite different from each other and they are distributed in the graph. It is possible to say that only Ala Ciftci and Aydintepe genotypes can be similar. This situation can be fully explained by the reflection of genetic factors on the studied parameters (agronomic, morphological and biochemical characteristics). Similar results gained by Madakbas et al. (2006).

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Table 4. *Principle component analysis results of the studied agronomic, morphological and biochemical characteristics*

	PC1	PC2	PC3	PC4	PC5	PC6
NDE	0.000	0.356	**0.484**	0.050	0.071	0.039
NDUF	0.021	**0.623**	0.312	0.027	0.013	0.004
PH	0.189	**0.474**	0.004	0.036	0.060	0.238
SD	0.265	0.043	0.054	**0.418**	0.205	0.014
BN	**0.563**	0.021	0.000	0.391	0.024	0.001
FPH	**0.491**	0.007	0.221	0.039	0.220	0.023
PL	**0.701**	0.016	0.152	0.046	0.004	0.082
PW	0.072	0.035	0.095	**0.701**	0.011	0.086
PNPP	**0.745**	0.126	0.007	0.002	0.049	0.071
SNPP	0.434	**0.458**	0.009	0.020	0.010	0.069
TSW	**0.314**	0.244	0.191	0.030	0.003	0.218
SY	0.177	0.089	0.057	0.019	**0.539**	0.118
NDPM	0.001	**0.677**	0.133	0.038	0.120	0.030
CHL-A	**0.608**	0.232	0.031	0.091	0.000	0.038
CHL-B	**0.798**	0.013	0.034	0.017	0.096	0.042
CHL-C	**0.684**	0.166	0.001	0.097	0.023	0.029
CARO	**0.905**	0.001	0.011	0.038	0.022	0.024
PROL	0.023	0.050	**0.903**	0.010	0.007	0.006
MDA	0.262	**0.362**	0.311	0.016	0.019	0.031
TPC	0.327	0.002	0.172	**0.456**	0.042	0.002

Values in bold correspond for each variable to the factor for which the squared cosine is the largest

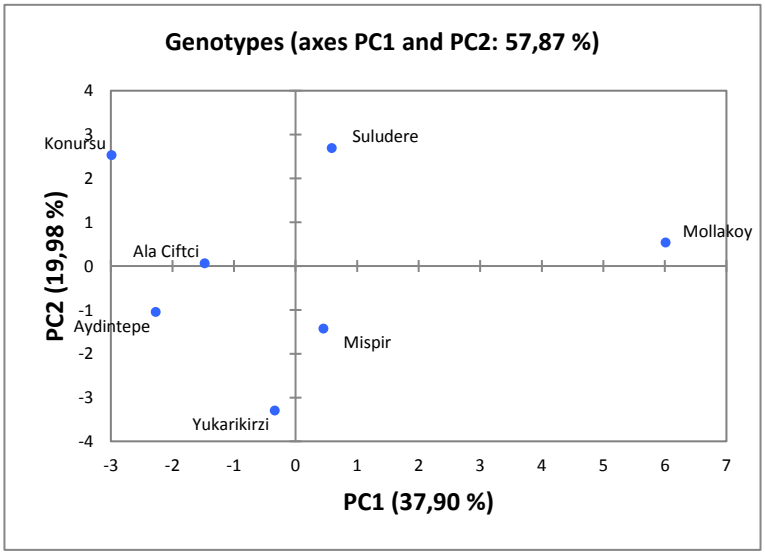

Figure 3. *Principal Component Analysis (PCA) biplot showing the distribution of bean genotypes in the first principle component and the second principle component*

The graphic in which the biplots of bean genotypes were combined with the examined agronomic, morphological, biochemical characteristics was given in *Fig. 4*. In this graph, as stated above, while narrow angle features have a positive relationship, wide angles have a negative relationship, while there is no correlation between right angles. On the

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other hand, similarly the Euclidean distances between cultivars were used to calculate in dry bean (Adams, 1977).

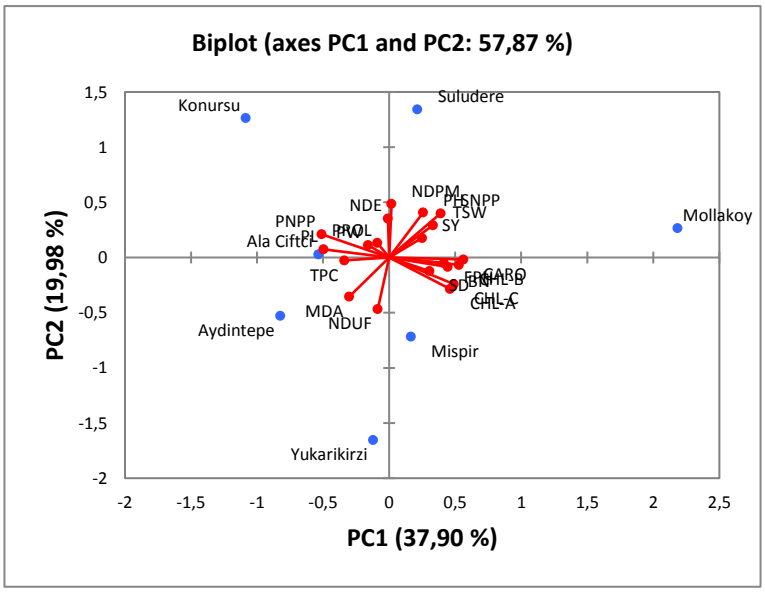

Figure 4. *Principal Component Analysis (PCA) biplot of the agronomic, morphological and biochemical characteristics of local bean genotypes for the first two principle components*

In terms of the genotypes PCA analysis results was given in *Table 5*. According to it; Mollakoy (0.921) and Konursu (0.411) have the highest coefficient value in the first Principle Component (PC1). In the second Principle Component (PC2), Yukarikirzi and Suludere had the highest coefficient values (0.651 and 0.438 respectively). Ala Ciftci (0.599) and Aydintepe (0.543) genotypes are the genotypes with the highest coefficient in the third Principle Component (PC3). And finally, the Mispir registered bean variety in the sixth Principle Component (PC6) had a high coefficient value (0.393). It stated that the populations differ more from each other. It is stated that Turkey is considered rich in beans biodiversity (Canci et al., 2019). Ashgari and Vojdani (1997), as a result of their studies, it has been determined that climate diversity and genetic difference were closely related.

Table 5. *Principle component analysis results of the bean genotypes*

	PC1	PC2	PC3	PC4	PC5	PC6
Aydintepe	0.267	0.058	**0.543**	0.027	0.046	0.059
Ala Ciftci	0.138	0.000	**0.599**	0.011	0.217	0.036
Mollakoy	**0.921**	0.007	0.000	0.067	0.004	0.000
Konursu	**0.411**	0.295	0.005	0.205	0.025	0.059
Mispir	0.019	0.189	0.048	0.305	0.045	**0.393**
Yukarikirzi	0.007	**0.651**	0.087	0.007	0.238	0.010
Suludere	0.021	**0.438**	0.017	0.400	0.079	0.044

Values in bold correspond for each variable to the factor for which the squared cosine is the largest

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Conclusions

It can be said that the genotypes used in the study differ greatly from each other. This is important for breeders trying to create variability, and it may be recommended to include these genotypes as genitors in breeding studies.

The important features in the first principle component, Pod number per plant, Pod length, Branch number, First pod height and Thousand seed weight, should be taken into account in the agronomic, morphological breeding studies. In the future, Carotenoid, Chlorophyll-B, Chlorophyll-C, Chlorophyll-A, properties could be taken into consideration in biochemical breeding studies.

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VARIATION IN AVIAN DIVERSITY IN RELATION TO PLANT SPECIES IN URBAN PARKS OF AYDIN, TURKEY

ASIK, Y.[1*] – KARA, B.[2]

[1]*Department of Landscape Architecture, Burdur Mehmet Akif Ersoy University, 15030 Burdur, Republic of Turkey*

[2]*Department of Landscape Architecture, Aydin Adnan Menderes University, 09970 Aydin, Republic of Turkey*

[*]*Corresponding author*
e-mail: yasik@mehmetakif.edu.tr; phone: +90-248-213-2782

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Abstract. Among wildlife species, birds are important indicators of biodiversity and habitat quality in urban ecosystems. Parks, which are among the important components of urban ecosystems, are home to many bird species with their diversity of plant species. Due to this aspect, parks make significant contributions to increase bird diversity. The bird species were observed with the naked eye and using an Olympus 10x50 DPS I brand binocular. Bird observations were performed on sunny days without rain and excessive wind in the mornings (07.00-09.00 a.m./2 h after sunrise) and evenings. Sixteen bird species observed in urban parks of Aydin comprised 11 native, 11 resident and 9 insectivorous species. The Eurasian Collared-Dove (*Streptopelia decaocto* Frivaldszky), Eurasian Jackdaw (*Corvus monedula* L.), Hooded Crow (*Corvus cornix* L.), Great Tit (*Parus major* L.), and House Sparrow (*Passer domesticus* L.) were the most frequently observed bird species in all parks. The Australian Pine (*Casuarina equisetifolia* L.) attracted the highest number of bird species with 12 species, followed by the Turkish Pine (*Pinus brutia* Ten.) with 11 species. A significant variance was found between plant species and avian diversity.
Keywords: *biodiversity, bird richness, habitat, landscape ecology, species distribution*

Introduction

Many cities in the world are home to many wildlife species since they have unlimited opportunities that meet vital needs such as food, water, shelter, and nesting (Pacheco and Vasconcelos, 2007; Baldock et al., 2015; Goertzen and Suhling, 2015; Kowarik and von der Lippe, 2018). Among these wildlife species, birds take an important place since they are common and easily observable (Suarez-Rubio et al., 2016; Bradbury, 2019; Moss and Martin, 2019) Cities are considered to be ecosystems with the potential to support various bird communities (Shochat et al., 2010). Of all bird species in the world, 20% live in cities (Aronson et al., 2014). Urban bird communities are divided into five groups based on their relationships with the urban ecosystem: urban avoiders, urban exploiters, urban adapters, residents, and migrants (Blair, 1996).

Parks have a high diversity of vegetation since they are usually the most heterogeneous green areas in the urban ecosystem (Gilbert, 1989; Hadidian et al., 1997; Rottenborn, 1999).

Because of the high diversity of vegetation, parks are among the important habitats for birds in cities (Jokimäki, 1999). Parks make significant contributions to the conservation of bird diversity and richness (Cornelis and Hermy, 2004; Khera et al., 2009; Carvajal-Castro et al., 2019).

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Birds are important indicators of habitat quality (Fontana et al., 2011a). The bird life in parks also increases the life quality of users. Park visitors are in search of a wildlife atmosphere, which differs from their work environments (Baines, 2000). Urban birds and their diversity make up a significant factor regarding how people can experience urban nature. Since communicating with birds means communicating with nature, it improves the physical, mental, and emotional health of urban dwellers (Moss and Martin, 2019).

All birds have different habitat requirements. However, they need food which they can feed on and feed their offspring with, and trees as well as shrubs where they can shelter and build a nest (Bradbury, 2019).

Bird communities in cities largely vary depending on the type and structure of vegetation (Sewell and Catterall, 1998; Fernández-Juricic and Jokimäki, 2001; White et al., 2005; Villegas and Garitano-Zavala, 2010). It was found that vegetation was positively associated with bird species richness. The more species of trees and shrubs there are that produce seeds and fruits and bloom at different times, the more bird species parks attract (Bauer, 2012; Bradbury, 2019). Trees are considered to be one of the most important plant components that increase bird species richness and diversity in parks since they provide opportunities for nutrition, shelter, and nesting (Fontana et al., 2011b; Aronson et al., 2014; Beninde et al., 2015). The shrub vegetation in parks is considered an important microhabitat since it decreases the problems to be caused by people by reducing their visibility, gives birds a chance to escape, and reduces the risk of hunting (Martín and López, 1995; Kramer and Bonenfant, 1997; Yang et al., 2015).

The most important problem related to wildlife in parks is that most plant species are exotic (Taylor, 2015). Most of the exotic species are less important for native bird species since they have a low food supply. Most of the plants in wildlife-friendly parks comprise native species (Bradbury, 2019).

The most important way for parks to increase the value of bird habitat is that plants are in layers in the form of trees, shrubs, and groundcovers as in nature (Bauer, 2012). This diversity of vegetation layers increases nutritional, sheltering, and nesting opportunities for different bird species (Marzluff and Ewing, 2001; Tews et al., 2004).

Information on the patterns of urban bird populations and communities appeared in the 1970s (Emlen, 1974). Most of the studies on urban birds addressed the key issues of abundance and distribution (Shochat et al., 2010). A lot of studies were conducted on birds within the context of urban ecology (Lepczyk and Warren, 2012; Shwartz et al., 2013; Gil and Brumm, 2014). In very few studies, the importance of vegetation structure for bird communities was examined (Keller et al., 2003; Macgregor-Fors and Schondube, 2011). However, variations in the distribution of bird species in cities depending on the vegetation profile remains unclear. There are no studies examining the relationship between bird diversity and plant species in the parks in Turkey, despite its potential significance. The study hypothesis is that in urban parks of Aydin, depending on the vegetation profile, the diversity of birds would change.

In the study, 1-the vegetation structure in the parks, which made up the study area, was revealed and 2- to what extent this vegetation affected the variation in bird species was investigated.

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Materials and Methods

Study area

The study area was the Pinarbasi Recreation Area, Aytepe Recreation Area, Nevzat BICER Park, and Ismet SEZGIN Park in Aydın city (*Figure 1*). While the population of Aydin city in 2019 was 293,816 (TUIK, 2020), its altitude is 59 m, and its surface area is 631 km^2. The Mediterranean climate prevails in the city. The mean annual temperature is 17.8 °C, and the mean annual amount of precipitation is 646 mm. The Pinarbasi Recreation Area is 32,195.63 m^2, the Aytepe Recreation Area is 15,828.97 m^2, Nevzat BICER Park is 14,663.16 m^2, and Ismet SEZGIN Park is 8,043.85 m^2. The Pinarbasi Recreation Area and Aytepe Recreation Area are on the urban fringe, and Nevzat BICER Park and Ismet SEZGIN Park are in the city center.

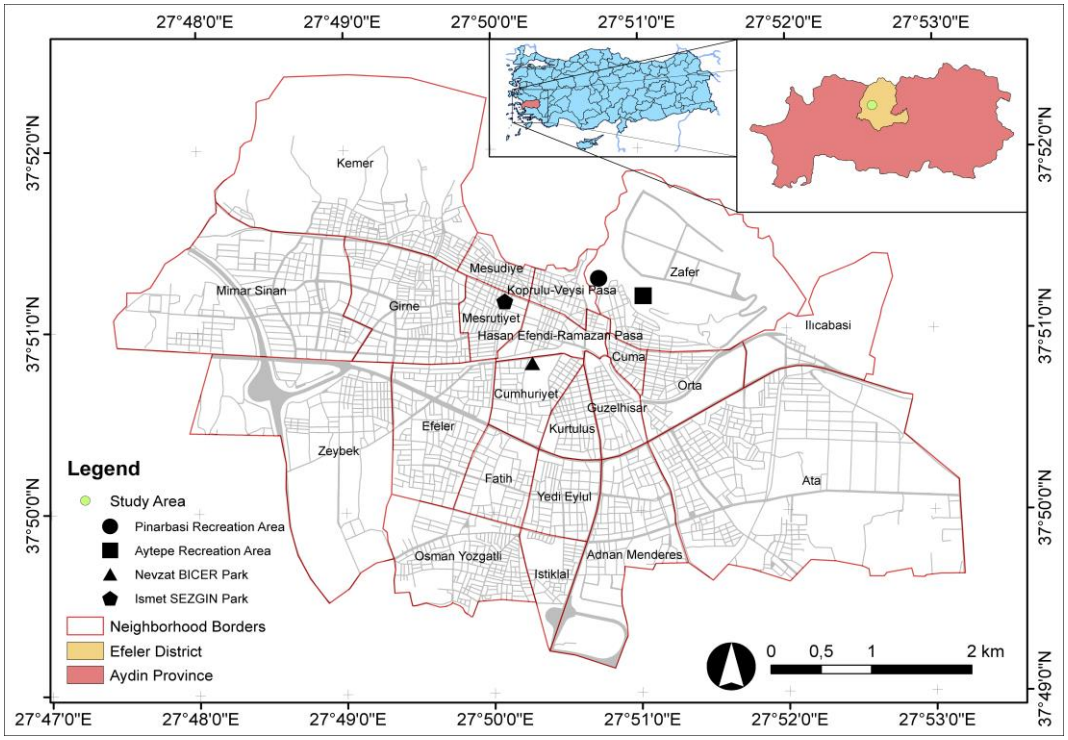

Figure 1. *Study area*

Observation tools

The bird species were observed with the naked eye and using an Olympus 10x50 DPS I brand binocular.

Bird data collection

Bird observations were performed on sunny days without rain and excessive wind, in the mornings (07.00-09.00 a.m./2 h after sunrise) and evenings (17.00-19.00 p.m./ 2 h before dark) (Meles and Bogale, 2018) during which bird activity was maximum, in the non-breeding season of 2019 between 16-26 September (Threlfall et al., 2016; Kale et al., 2018; Carvajal-Castro et al., 2019; Mao et al., 2019; Vaccaro et al., 2019). The non-breeding season is defined as the period during which wintering birds are likely to be

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present and the resident bird species are unlikely to breed (Braden et al., 2007). Bird data were collected by using a standard five-minute point-count method (Heezik et al., 2010; Yang et al., 2015; van Camacho-Cervantes et al., 2018; Wolff et al., 2018; Filloy et al., 2019; Vaccaro et al., 2019). All birds heard or seen (McCurdy, 2016; Threlfall et al., 2016; Callaghan et al., 2018; Filloy et al., 2019; Vaccaro et al., 2019) within a radius of 25 m from each observation point (Shanahan et al., 2011; Yang et al., 2015) were observed. The observations were performed by walking on the line between observation points (Chong et al., 2014; Yang et al., 2015; Kale et al., 2018). The observations were performed at 67 points, including 33 points in the Pinarbasi Recreation Area, 19 points in the Aytepe Recreation Area, 9 points in Nevzat BICER Park, and 6 points in Ismet SEZGIN Park (*Figure 2*). In four parks which made up the study area, 32 hours of observations were performed in 16 different time periods in 8 days. The observations were performed by two observers trained on visual and auditory bird identification (Verner and Milne, 1989).

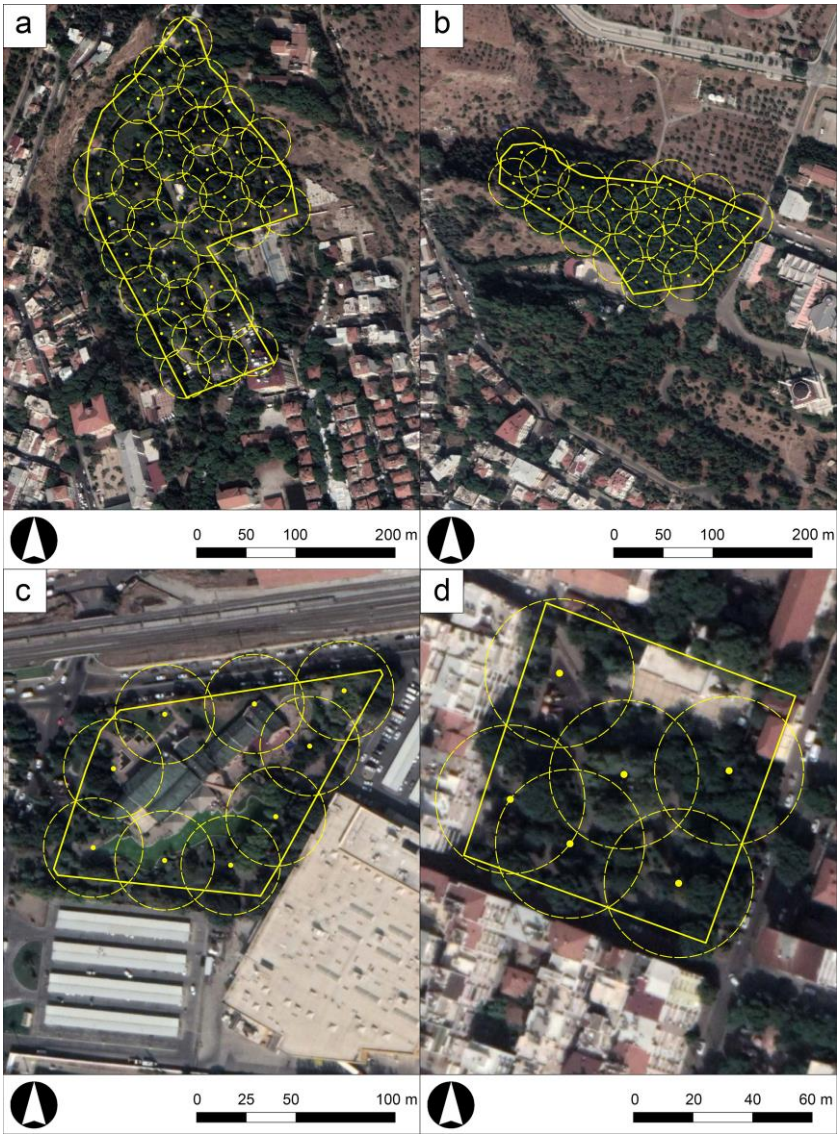

Figure 2. *Observation points (a) Pinarbasi Recreation Area, (b) Aytepe Recreation Area, (c) Nevzat BICER Park, (d) Ismet SEZGIN Park*

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Data analysis

The feature data used in bird identification were collected from published sources (Heinzel et al., 1995) and online bird databases (Bird Life International, 2011; eBird, 2012; Rodewald, 2015; The Cornell Lab, 2017). The published sources (Karamanoglu, 1976; Mamıkoglu, 2010; Akkemik, 2018) were used in the identification of plants. The observation points were created on a Quickbird satellite image using ArcMap 10.7 software. The birds were classified according to their scientific names and taxonomic structure (Kirwan et al., 2008; AOU, 2009; Gill and Donsker, 2020; TRAKUS, 2020), native and exotic status, residency status, urban associations (Howell and Webb, 1995), and guilds.

Statistical analysis

The statistical software package SPSS 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used to analyze statistically the study data. First, the data's normality assumption was tested. It was determined that the data not met the normality assumption. Therefore, it was applied non-parametric tests to the data. Using the Mann-Whitney U test, the connection between binary independent categorical variables was analyzed and the connection between triple independent categorical variables was analyzed using the Kruskal Wallis H test. Statistical analysis included only plants on which birds were observed and the birds on which they were observed, not all plant species and bird species. Plant heights for statistical analysis were classified as short, medium, high for <10 m, 10-20 m and >20 m, respectively. No individual measurements have been taken because all the plant species in the study area are adults. The average plant height was calculated, taking advantage of published sources used in data analysis section.

Results

Plant species and bird species in the study area

While the highest number of native tree species (15 species) was present in the Pinarbasi Recreation Area, the lowest number of native tree species (7 species) was present in the Aytepe Recreation Area and Nevzat BICER Park. While the highest number of exotic tree species (24 species) was present in Nevzat BICER Park, the lowest number of exotic tree species (1 species) was present in the Aytepe Recreation Area. The highest number of native shrub species (6 species) was present in the Pinarbasi Recreation Area and Nevzat BICER Park. However, the lowest number of native shrub species (2 species) was present in the Aytepe Recreation Area. The highest number of exotic shrub species (21 species) was present in Ismet SEZGIN Park, and the lowest number of exotic shrub species (4 species) was present in the Aytepe Recreation Area (*Table 1*).

In the observations performed in four parks in Aydin city, 16 bird species from 9 families were observed. While the highest number of bird species (14 species) was observed in the Pinarbasi Recreation Area, the lowest number of bird species (8 species) was observed in Nevzat BICER and Ismet SEZGİN Parks. The observed birds comprised 11 native, 5 exotic, 11 resident, 5 migrant, 14 urban adapter (5 exotic, 9 native) and 2 urban exploiter (native species) species. While 9 of the observed bird

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species were insectivorous species, 6 and 1 of them were omnivorous and granivorous species, respectively (*Table 2*).

Table 1. *Native and exotic plant species in the study area*

Plant Species	Species and Frequency	Native (N) or Exotic (E)	Urban Parks			
			Pinarbasi Recreation Area	Aytepe Recreation Area	Nevzat BICER Park	Ismet SEZGIN Park
Tree	Species	N	15	7	7	11
		E	21	1	24	13
	Frequency	N	246	616	40	92
		E	269	3	199	51
Shrub	Species	N	6	2	6	4
		E	11	4	15	21
	Frequency	N	452	61	32	64
		E	202	1078	385	724

Table 2. *Bird species observed in the study area*

Latin Name	Common Name	Family	Status*	Urban Parks**			
				PRA	ARA	NBP	ISP
Streptopelia decaocto Frivaldszky	Eurasian Collared-Dove	*Columbidae*	N-R-UA-G	●	●	●	●
Corvus monedula L.	Eurasian Jackdaw	*Corvidae*	N-R-UA-O	●	●	●	●
Corvus cornix L.	Hooded Crow	*Corvidae*	N-R-UE-O	●	●	●	●
Garrulus glandarius L.	Eurasian Jay	*Corvidae*	N-R-UA-O	●	●		
Pica pica L.	Eurasian Magpie	*Corvidae*	N-R-UA-O	●	●		●
Fringilla coelebs L.	Common Chaffinch	*Fringillidae*	N-R-UA-O	●	●		
Motacilla alba L.	White Wagtail	*Motacillidae*	E-M-UA-I	●		●	
Motacilla cinerea Tunstall	Grey Wagtail	*Motacillidae*	E-M-UA-I			●	
Motacilla flava L.	Western Yellow Wagtail	*Motacillidae*	E-M-UA-I	●			
Ficedula parva Bechstein	Red-breasted Flycatcher	*Muscicapidae*	E-M-UA-I		●		●
Muscicapa striata Pallas	Spotted Flycatcher	*Muscicapidae*	E-M-UA-I	●	●	●	
Cyanistes caeruleus L.	Eurasian Blue Tit	*Paridae*	N-R-UA-I	●	●		
Parus major L.	Great Tit	*Paridae*	N-R-UA-I	●	●	●	●
Passer domesticus L.	House Sparrow	*Passeridae*	N-R-UE-O	●	●	●	●
Dendrocopos syriacus (Hemprich & Ehrenberg)	Syrian Woodpecker	*Picidae*	N-R-UA-I	●	●		●
Sitta europaea L.	Eurasian Nuthatch	*Sittidae*	N-R-UA-I	●			
Number of Bird Species Observed:				**14**	**12**	**8**	**8**

Note: *Status: Native or Exotic: N (Native), E (Exotic); Residency Status: R (Resident), M (Migrant); Urban Association: UA (Urban Adapter), UE (Urban Exploiter); Guild: G (Granivore), I (Insectivorous), O (Omnivorous); **Urban Parks: PRA (Pınarbasi Recreation Area), ARA (Aytepe Recreation Area), NBP (Nevzat BICER Park), ISP (Ismet SEZGIN Park)

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The Eurasian Collared-Dove (*Streptopelia decaocto* Frivaldszky), Eurasian Jackdaw (*Corvus monedula* L.), Hooded Crow (*Corvus cornix* L.), Great Tit (*Parus major* L.), and House Sparrow (*Passer domesticus* L.) that were observed in all parks were also the most frequently observed bird species in the parks. In the morning observations, the most frequently observed bird species were the Eurasian Jackdaw in the Pinarbasi Recreation Area, the Eurasian Jay (*Garrulus glandarius* L.) and Great Tit in the Aytepe Recreation Area, and the House Sparrow in Nevzat BICER and Ismet SEZGIN parks. In the evening observations, the most frequently observed bird species were the Eurasian Jackdaw and Eurasian Blue Tit (*Cyanistes caeruleus* L.) in the Pinarbasi Recreation Area, the Great Tit in the Aytepe Recreation Area, the House Sparrow in Nevzat BICER park, and the Eurasian Collared-Dove in Ismet SEZGIN Park (*Table 2*).

Variation in bird species in relation to plant species in the study area

In the Pinarbasi Recreation Area, the highest number of bird species was observed in the Australian Pine (*Casuarina equisetifolia* L.) (12 species), Turkish Pine (*Pinus brutia* Ten.) (9 species), and Oriental Plane Tree (*Platanus orientalis* L.) (8 species). The lowest number of bird species (1 species) was observed in the Japanese Spindle Tree (*Euonymus japonicus* Thunb.), Common Privet (*Ligustrum vulgare* L.), Oriental Sweetgum (*Liquidambar orientalis* Mill.), Japanese Pittosporum [*Pittosporum tobira* (Thunb.) W. T. Aiton], and Black Locust (*Robinia pseudoacacia* L.). The highest number of bird species observed in the Australian Pine were the Hooded Crow, Eurasian Jay, Eurasian Blue Tit, and Eurasian Nuthatch (*Sitta europaea* L.) (*Table 3*).

In the Pinarbasi Recreation Area, among the most frequently observed species in the parks examined, the great tit was observed in 12 tree species and 2 shrub species, the House Sparrow was observed in 10 tree species and 2 shrub species, the Eurasian Jackdaw was observed in 7 tree species, the Hooded Crow was observed in 5 tree species, the Eurasian Collared-Dove was observed in 3 tree species (*Table 3*). While the Western Yellow Wagtail (*Motacilla flava* L.), one species observed only in the Pinarbasi Recreation Area, was not observed in any tree, it was observed only on the ground.

In the Australian Pine, Turkish Pine, and Oriental Plane Tree that attracted the highest number of bird species in the Pinarbasi Recreation Area, most of the bird species were mostly observed in the morning observations. Other tree species on which birds were mostly observed in the morning observations were the Boxelder (*Acer negundo* L.), Italian Cypress [*Cupressus sempervirens* 'Horizontalis' (Mill.) Loudon], Stone Pine (*Pinus pinea* L.), Atlas Cedar [*Cedrus atlantica* (Endl.) Lindl.], and White Mulberry (*Morus alba* L.). In the Australian Pine and Turkish Pine, most of the bird species were also observed in the evening observations. Other species on which the bird species were mostly observed in the evening observations were the Boxelder, White Mulberry, Stone Pine, and Oriental Plane Tree (*Table 3*).

In the Aytepe Recreation Area, the highest number of bird species (11 species) and the lowest number of bird species (2 species) were observed in the Turkish Pine and Olive (*Olea europaea* L.), respectively. The Eurasian Collared-Dove, Eurasian Blue Tit, and Great Tit were most frequently observed in the Turkish Pine (*Table 4*).

In the Aytepe Recreation Area, among the most frequently observed species in the parks examined, the Eurasian Collared-Dove, Great Tit, and House Sparrow were most frequently observed in the Turkish Pine, and the Eurasian Jackdaw and Hooded Crow

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were most frequently observed in the Italian Cypress (Horizontal form) and Turkish Pine (*Table 4*).

Table 3. *Plants on which birds were observed in the Pinarbasi Recreation Area and their time of observation*

Plant Species		**Bird Species***											
Latin Name	**Common Name**	***Sd***	***Cm***	***Cc***	***Gg***	***Pp***	***Fc***	***Ms***	***Cy***	***Pm***	***Pd***	***Ds***	***Se***
Acer negundo L.	Boxelder		M			M	M		ME	M2E	M		
Ailanthus altissima (Mill.) Swingle	Tree of Heaven							E	M	2M			
Casuarina equisetifolia L.	Australian Pine	M	2ME	2M2E	2M2E	2ME	M	E	2M2E	ME	ME	E	2M2E
Catalpa bignonioides Walt.	Southern Catalpa									M	M	M	
Cedrus atlantica (Endl.) Lindl.	Atlas Cedar		M	M	M					M			
Cedrus libani A.Rich.	Cedar of Lebanon			M				ME			M		
Cupressus sempervirens 'Horizontalis' (Mill.) Loudon	Italian Cypress		M		M	ME		E		2M	M		
Eucalyptus camaldulensis Dehnh.	River Red Gum								M	M2E	M		
Euonymus japonicus Thunb.	Japanese Spindle Tree									M			
Ligustrum ovalifolium Hassk.	California Privet					E					2M		
Ligustrum vulgare L.	Common Privet									M			
Liquidambar orientalis Mill.	Oriental Sweetgum										M		
Magnolia grandiflora L.	Southern Magnolia					M					2M		
Morus alba L.	White Mulberry				2ME		M			M2E			
Paulownia tomentosa (Thunb.) Sieb. & Zucc. ex Steud.	Princess Tree		M			M							
Pinus brutia Ten.	Turkish Pine	2M2E	2ME	M2E	2ME			E	M2E	M2E	M		E
Pinus pinea L.	Stone Pine				M			2E	M	2M	M		E
Pittosporum tobira (Thunb.) W.T.Aiton	Japanese Pittosporum										2M		
Platanus orientalis L.	Oriental Plane Tree	ME	2M	2M	2M	2M			M	2ME		E	
Populus nigra L.	Black Poplar				M	M			E				
Robinia pseudoacacia L.	Black Locust									M			

Note: Observation Time: M (Morning), E (Evening); *Bird Species: *Sd* (*Streptopelia decaocto* Frivaldszky), *Cm* (*Corvus monedula* L.), *Cc* (*Corvus cornix* L.), *Gg* (*Garrulus glandarius* L.), *Pp* (*Pica pica* L.), *Fc* (*Fringilla coelebs* L.), *Ms* (*Muscicapa striata* Pallas), *Cy* (*Cyanistes caeruleus* L.), *Pm* (*Parus major* L.), *Pd* (*Passer domesticus* L.), *Ds* (*Dendrocopos syriacus* (Hemprich & Ehrenberg)), *Se* (*Sitta europaea* L.)

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Table 4. *Plants on which birds were observed in the Aytepe Recreation Area and their time of observation*

Plant Species		Bird Species*										
Latin Name	**Common Name**	***Sd***	***Cm***	***Cc***	***Gg***	***Pp***	***Fc***	***Ms***	***Cy***	***Pm***	***Pd***	***Ds***
Cupressus sempervirens 'Horizontalis' (Mill.) Loudon	Italian Cypress	M	M E	M 2E		2M			M	2M		
Olea europaea L.	Olive									2M	M	
Pinus brutia Ten.	Turkish Pine	2M 2E	2M	2M E	2M E	2M	2M E	2M E	2M 2E	2M 2E	2M E	M

Note: Observation Time: M (Morning), E (Evening); *Bird Species: *Sd* (*Streptopelia decaocto* Frivaldszky), *Cm* (*Corvus monedula* L.), *Cc* (*Corvus cornix* L.), *Gg* (*Garrulus glandarius* L.), *Pp* (*Pica pica* L.), *Fc* (*Fringilla coelebs* L.), *Ms* (*Muscicapa striata* Pallas), *Cy* (*Cyanistes caeruleus* L.), *Pm* (*Parus major* L.), *Pd* (*Passer domesticus* L.), *Ds* (*Dendrocopos syriacus* (Hemprich & Ehrenberg))

In the Aytepe Recreation Area, in the Turkish Pine that attracted the highest number of bird species, all bird species were observed in the morning observations. Another tree species on which birds were mostly observed in the morning observations was the Italian Cypress (Horizontal form). Most of the bird species in the Turkish Pine were also observed in the evening observations. Another species on which bird species were mostly observed in the evening observations was the Italian Cypress (Horizontal form) (*Table 4*).

In Nevzat BICER park, the highest number of bird species was observed in the Silky Oak (*Grevillea robusta* A. Cunn. ex R. Br.) (4 species). The lowest number of bird species was observed in (1 species each) 9 tree species, including the Tree of Heaven [*Ailanthus altissima* (Mill.) Swingle] and Silk Tree (*Albizia julibrissin* Durazz.), and in 2 shrub species comprising the California Privet (*Ligustrum ovalifolium* Hassk.) and Four-Stamen Tamarisk (*Tamarix tetrandra* Pall. ex M. Bieb.) (*Table 5*).

In Nevzat BICER park, among the most frequently observed species in the parks examined, the House Sparrow was observed in 15 tree species and 2 shrub species, the Eurasian Collared-Dove and Eurasian Jackdaw were observed in 9 tree species, and the Hooded Crow and Great Tit were observed in 1 tree species (*Table 5*). The Grey Wagtail (*Motacilla cinerea* Tunstall), which was observed only in Nevzat BICER park, was not observed in any tree; it was observed only on the ground.

In the Silky Oak that attracted the highest number of bird species in Nevzat BICER park, most of the bird species were observed in the morning observations. Other tree species on which the birds were mostly observed in the morning observations were the Boxelder, White Mulberry, and Japanese Pagoda Tree (*Sophora japonica* L.). The species on which bird species were mostly observed in the evening observations were the Boxelder and Russian Olive (*Elaeagnus angustifolia* L.). Other species on which bird species were mostly observed in the evening observations were the White Mulberry and Weeping Willow (*Salix babylonica* L.) (*Table 5*).

In Ismet SEZGIN park, while the highest number of bird species (6 species) was observed in the Australian Pine, the lowest number of bird species (1 species) was observed in the California Privet, Common Privet, White Mulberry, and Turkish Pine. The Eurasian Jackdaw was most frequently observed in the Australian Pine (*Table 6*).

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Table 5. *Plants on which the birds were observed in Nevzat BICER Park and their time of observation*

Plant Species		Bird Species*					
Latin Name	**Common Name**	***Sd***	***Cm***	***Cc***	***Ms***	***Pm***	***Pd***
Acer negundo L.	Boxelder	M	2M 2E				2M 2E
Acer saccharinum L.	Silver Maple	E	M				2E
Ailanthus altissima (Mill.) Swingle	Tree of Heaven						M
Albizia julibrissin Durazz.	Silk Tree						M
Carpinus betulus L.	Common Hornbeam						2M
Cedrus atlantica (Endl.) Lindl. & Gordon	Atlas Cedar		M				
Cupressocyparis leylandii A.B.Jacks. & Dallim	Leyland Cypress	E					ME
Elaeagnus angustifolia L.	Russian Olive	M E	2E		E		
Grevillea robusta A.Cunn. ex R.Br.	Silky Oak	M	M	M			E
Jacaranda mimosifolia D. Don.	Jacaranda	M E			M		M E
Ligustrum ovalifolium Hassk.	California Privet						M E
Magnolia grandiflora L.	Southern Magnolia						M
Morus alba L.	White Mulberry	2M E	M				M 2E
Platanus occidentalis L.	American Sycamore					E	
Prunus cerasifera 'Atropurpurea'	Purple-Leaf Cherry Plum				M E		M
Prunus domestica L	Plum				M		
Prunus persica (L.) Batsch	Peach	M	M				
Robinia pseudoacacia L.	Black Locust				E		2M
Salix babylonica L.	Weeping Willow				ME		2E
Sophora japonica L.	Japanese Pagoda Tree	2M	M				M
Tamarix tetrandra Pall. ex M.Bieb.	Four-Stamen Tamarisk						M
Tilia cordata Mill.	Small-Leaved Lim						2M
Washingtonia robusta H. Wendl.	Mexican Fan Palm		M				

Note: Observation Time: M (Morning), E (Evening); *Bird Species: *Sd* (*Streptopelia decaocto* Frivaldszky), *Cm* (*Corvus monedula* L.), *Cc* (*Corvus cornix* L.), *Ms* (*Muscicapa striata* Pallas), *Pm* (*Parus major* L.), *Pd* (*Passer domesticus* L.)

In Ismet SEZGIN Park, among the most frequently observed species in the parks examined, the House Sparrow was observed in 8 tree species and 2 shrub species, the Eurasian Collared-Dove and Eurasian Jackdaw were observed in 8 tree species, and the Hooded Crow and Great Tit were observed in 2 tree species (*Table 6*).

In the Australian Pine that attracted the highest number of bird species in Ismet SEZGIN Park, most of the bird species were observed in the morning observations. Other tree species on which the birds were mostly observed in the morning observations were the Italian Cypress [*Cupressus sempervirens* 'Pyramidalis' (O. Targ. Tozz.) Nyman], Stone Pine, Kurrajong (*Brachychiton populneus* Schott.), Oriental Plane Tree, and Small-Leaved Lime (*Tilia cordata* Mill.). The species on which bird species were mostly observed in the evening observations were the Stone Pine and Italian Cypress (Pyramidal form). Another species on which bird species were mostly observed in the evening observations was the Oriental Plane Tree (*Table 6*).

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Table 6. *Plants on which the birds were observed in Ismet SEZGIN Park and their time of observation*

Plant Species		Bird Species*							
Latin Name	**Common Name**	***Sd***	***Cm***	***Cc***	***Pp***	***Fp***	***Pm***	***Pd***	***Ds***
Brachychiton populneus Schott.	Kurrajong	M	2M				M	M	
Casuarina equisetifolia L.	Australian Pine	M E	2M E		M	M		M	M
Cupressus sempervirens 'Pyramidalis' (O.Targ.Tozz.) Nyman	Italian Cypress	2M E	M 2E	M				2M 2E	
Eucalyptus camaldulensis Dehnh.	River Red Gum	M E						M E	
Gleditsia triacanthos L.	Honey Locust	M	M				M		
Ligustrum ovalifolium Hassk.	California Privet							2M	
Ligustrum vulgare L.	Common Privet							E	
Morus alba L.	White Mulberry							2M E	
Pinus brutia Ten.	Turkish Pine		M						
Pinus pinea L.	Stone Pine	2M 2E	2M 2E			E		2M E	
Platanus orientalis L.	Oriental Plane Tree	M	2M E					2M 2E	
Tilia cordata Mill.	Small-Leaved Lim	M	2M	M				M E	

Note: Obsevation Time: M (Morning), E (Evening); *Bird Species: *Sd* (*Streptopelia decaocto* Frivaldszky), *Cm* (*Corvus monedula* L.), *Cc* (*Corvus cornix* L.), *Pp* (*Pica pica* L.), *Fp* (*Ficedula parva* Bechstein), *Pm* (*Parus major* L.), *Pd* (*Passer domesticus* L.), *Ds* (*Dendrocopos syriacus* (Hemprich & Ehrenberg))

The Australian Pine attracted the highest number of bird species with 12 species, followed by the Turkish Pine with 11 species. Other plants that attracted the highest number of bird species were the Oriental Plane Tree with 8 species, and the Boxelder, Italian Cypress (Horizontal form), and Stone Pine with 6 species. Four of the six plant species that attracted the highest number of bird species were native species.

Native, resident, insectivorous and omnivorous birds show significant variance ($p<0.05$), depending on the location of the park, according to the results of the Mann Whitney U test. Depending on the location of the park, it has been determined that urban adapter and urban exploiter birds do not show significant variance. Native and urban adapter birds show significant variance between shrubs and trees; between broad-leaved and coniferous plants, native, urban adapter, urban exploiter, insectivorous and omnivorous birds show significant variance ($p<0.05$). Depending on the park location, plant species and the type of plant leaf, avian diversity shows a significant variance (*Table 7*). As there is no significant variance between native and exotic tree species and bird species and avian diversity, this is not included in the table.

The results of the Kruskal Wallis test are shown in *Table 8*, which shows bird species and avian diversity depending on the height of the plant. A statistically significant variance was observed between plant heights for native birds, omnivorous birds and levels of avian diversity, based on the test results ($p<0.05$). The level of attraction of native birds is much higher for high-sized plants than for short-sized plants; the level of attraction of native birds is significantly higher for medium-sized plants than short-sized

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plants. In attracting omnivorous birds from higher plants to shorter plants, there is a significant variance. More omnivorous birds than short plants are attracted by higher plants. There is a significant variance in avian diversity between the likelihood of finding more birds in high and medium-sized plants than in short-sized plants (*Table 8*).

Table 7. *Calculation results of Mann-Whitney U test on bird species and avian diversity related to park location, plant species and leaf type*

Bird Species	**Park Location**	**Mean Rank**	**U**	***p***
Native	Urban Center	25.09	524	0.025*
	Urban Fringe	34.78		
Resident	Urban Center	7.5	144	0.002*
	Urban Fringe	17.86		
Urban Adapter	Urban Center	20.46	337	0.096
	Urban Fringe	26.82		
Urban Exploiter	Urban Center	21.61	277	0.121
	Urban Fringe	25.29		
Insectivorous	Urban Center	10.71	177.5	0.008*
	Urban Fringe	19.34		
Omnivorous	Urban Center	21.77	471.5	0.002*
	Urban Fringe	34.08		
Avian Diversity	Urban Center	25.74	569	0.018*
	Urban Fringe	36.21		
Bird Species	**Plant Species**	**Mean Rank**	**U**	***p***
Native	Shrub	13.36	284.5	0.006*
	Tree	31.19		
Resident	Shrub	9.88	70.5	0.203
	Tree	15.82		
Urban Adapter	Shrub	8.5	109.5	0.041*
	Tree	24.55		
Urban Exploiter	Shrub	20	115	0.611
	Tree	23.38		
Insectivorous	Shrub	10.5	40	0.430
	Tree	16.38		
Omnivorous	Shrub	17.40	163	0.168
	Tree	27.47		
Avian Diversity	Shrub	12.64	303.5	0.003*
	Tree	32.34		
Bird Species	**Leaf Type**	**Mean Rank**	**U**	***p***
Native	Broad-leaved	25.53	133.5	0.003*
	Coniferous	40.73		
Resident	Broad-leaved	13.88	60.5	0.257
	Coniferous	17.94		
Urban Adapter	Broad-leaved	20.33	110	0.008*
	Coniferous	31.54		
Urban Exploiter	Broad-leaved	20.7	134.5	0.002*
	Coniferous	28.65		
Insectivorous	Broad-leaved	13.33	49	0.017*
	Coniferous	21.6		
Omnivorous	Broad-leaved	22.8	125.5	0.002*
	Coniferous	36.54		
Avian Diversity	Broad-leaved	25.72	122.5	0.000*
	Coniferous	43.75		

Note: * the significance level (p) <0.05

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Table 8. *Calculation results of Kruskal-Wallis test on bird species and avian diversity related to plant size*

Bird Species	Plant Size		Test Statistic	Std. Error	Std. Test Statistic	*p*	**Adjusted *p*
Native	Short	High	-16.393	5.250	-3.123	0.002	0.005*
	Short	Medium	-17.787	6.568	-2.708	0.007	0.020*
	High	Medium	1.394	5.581	0.250	0.803	1
Omnivorous	Short	High	-16.344	4.944	-3.306	0.001	0.003*
	Short	Medium	-8.500	5.981	-1.591	0.155	0.466
	Medium	High	-7.844	4.944	-1.586	0.113	0.338
Avian Diversity	Short	High	-17.080	5.292	-3.228	0.001	0.004*
	Short	Medium	-18.468	6.714	-2.750	0.006	0.018*
	High	Medium	1.388	5.781	0.240	0.810	1

Note: * the significance level (p) <0.05 and **significance values have been adjusted by the bonferroni correction for multiple tests

Discussion

Many birds are usually observed in urban environments. However, very few of them are native species (McCurdy, 2016). However, native species made up most bird species (11 species/68.75%) observed in the four urban parks examined in Aydin city.

The species common in urban landscapes are exotic exploiters or general native species tolerant to various urban conditions (White et al., 2005; Antos, 2006; McKinney, 2006). Resident species dominated the bird community in the city center and made up over 90% of the species observed. However, their number decreased as the building density decreased (McCurdy, 2016). All bird species observed in this study comprised urban adapter (5 exotic and 9 native species/87.5%) and urban exploiter (2 native species/12.5%) species. Resident species (11 species) made up most of the bird species observed (68.75%). However, they increased (towards the urban fringe) as the building density decreased.

The ratio of granivores in the bird community reaches a maximum in the city center (23%), and the ratio of the species in this group decreased towards natural regions and reached 8% (McCurdy, 2016). In this study, only one granivore species (Eurasian Collared-Dove) was identified. The number of insectivorous species with the highest ratio (56.25%) in the bird population increased towards natural areas.

In the studies investigating bird diversity in the USA (Tucson), Canada (Quebec), Germany (Leipzig), Scotland (St. Andrews), and Israel (Tel-Aviv), it was determined that the synanthropic species, including urban adapter and urban exploiter species such as the House Sparrow, Rock Dove (*Columba livia* Gmelin), Common Starling (*Sturnus vulgaris* L.), Eurasian Magpie (*Pica pica* L.), Eurasian Blackbird (*Turdus merula* L.), Hooded Crow, Eurasian Jackdaw, European Robin (*Erithacus rubecula* L.), and Great Tit, were the common bird species in cities (Shwartz et al., 2008; Strohbach et al., 2009; Carbó-Ramírez and Zuria, 2011; Camacho-Cervantes et al., 2018; Hensley et al., 2019). In this study, the Eurasian Collared-Dove, Eurasian Jackdaw, Hooded Crow, Great Tit, and House Sparrow were most frequently observed in the parks, and similar results were obtained.

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Similarly to the results of previous studies (White et al., 2005; Shwartz et al., 2008; Yang et al., 2015), the results also revealed that the woody plant species richness had a positive effect on bird species richness. Specific habitat characteristics that have been effective in increasing bird species diversity in urban green areas are tall tree woodlands and hollow old trees (Fernández-Juricic, 2004; Sandström et al., 2006; Stagoll et al., 2012; Threlfall et al., 2016). During the winter months, insectivorous species are more frequently observed in wider green areas with more tree and shrub species and in taller trees (Carbó-Ramírez and Zuria, 2011). Insectivorous bird species such as the Spotted Flycatcher (*Muscicapa striata* Pallas), Eurasian Blue Tit, Great Tit, Syrian Woodpecker [*Dendrocopos syriacus* (Hemprich & Ehrenberg)], and Eurasian Nuthatch were more frequently observed in tall coniferous trees, such as Atlas Cedar, Cedar of Lebanon (*Cedrus libani* A. Rich.), Italian Cypress (Horizontal form), Turkish Pine, and Stone Pine, and in tall broad-leaved trees such as the Tree of Heaven, Boxelder, Australian Pine, Southern Catalpa (*Catalpa bignonioides* Walt.), River Red Gum (*Eucalyptus camaldulensis* Dehnh.), White Mulberry, Oriental Plane Tree, Black Poplar (*Populus nigra* L.), and Black Locust in the Pinarbasi Recreation Area and Aytepe Recreation Area with more tree and shrub species and the wider area. Older and taller trees are more likely to have hollows compared to smaller trees (Carlson et al., 1998; Manning et al., 2006; Lindenmayer et al., 2014), and Woodpeckers, hollow-nesting species and forest birds increase from the city center to the periphery (Sandström et al., 2006). Under this view, the Eurasian Blue Tit, Syrian Woodpecker, and Eurasian Nuthatch, which are hollow-nesting species, were observed in the old and tall coniferous and broad-leaved trees in the Pinarbasi Recreation Area and Aytepe Recreation Area near the urban fringe.

There is a relationship between bird species richness and vegetation structure. The features of the vegetation structure are important for birds in urban areas (Lancaster and Rees, 1979; Mills et al., 1989; Fernández-Juricic, 2004). That the Eurasian Jay and Common Chaffinch (*Fringilla coelebs* L.) were observed in coniferous and broad-leaved tree species comprising the Boxelder, Australian Pine, Atlas Cedar, Italian Cypress (Horizontal form), White Mulberry, Turkish Pine, Stone Pine, Oriental Plane Tree, and Black Poplar supports the view that these bird species are observed in all kinds of woodlands (Bloomsbury, 2019).

The bird species richness in the Pinarbasi Recreation Area with more deciduous trees was found to be higher than the bird species richness in the Aytepe Recreation Area with more coniferous trees. This result is compatible with the view that the bird species richness is higher in parks where there are more deciduous trees than coniferous trees (Thompson et al., 1993). But it has been observed that the richness of coniferous trees for bird species is higher than broad-leaved trees.

Well-protected understory may provide birds with abundant hunting products such as arthropods (Kirchner, 1977; Conner et al., 1986; Keller et al., 2003). The abundant food source enabled the observation of the Eurasian Magpie, Great Tit, and House Sparrow in the shrubs formed by the Japanese Spindle Tree, California Privet, Common Privet, Japanese Pittosporum, and Four-Stamen Tamarisk that made up the understory. While the House Sparrow was observed in four of five shrub species, the Great Tit, the only insectivorous species among these species, was observed in the Japanese Spindle Tree and Common Privet. So, doubling the vegetation layers significantly increases the number of insectivorous bird species in particular (Threlfall et al., 2016).

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There is a significant relationship between bird diversity and the amount of natural vegetation (Sandström et al., 2006; Threlfall et al., 2016; Muñoz-Pedreros et al., 2018). The diversity of bird species increases as the vegetation increases towards the urban fringe (Blair, 1999; Sandström et al., 2006). Since vegetation is usually exotic, there is an increase in the diversity of exotic bird species. However, sometimes, native vegetation allows for a higher proportion of native bird species (White et al., 2005; Chace and Walsh, 2006; Daniels and Kirkpatrick, 2006; Threlfall et al., 2016). Although the variance in this study is not statistically significant, when consideration is given to observed bird species and plant species, the number of native bird species was found to be higher in the Pinarbasi Recreation Area and Aytepe Recreation Area on the urban fringe where there were more native tree species, which was due to the fact that high diversity in natural vegetation provided more nesting space, shelter, and food for many bird species (Chong et al., 2014).

The Grey Wagtail and Western Yellow Wagtail have specific habitat requirements; such as being observed only in open vegetative areas (Yang et al., 2015). Likewise, the White Wagtail (*Motacilla alba* L.), Grey Wagtail, and Western Yellow Wagtail were observed in open areas by the pool in the Pinarbasi Recreation Area and Nevzat BICER park.

The Eurasian Jackdaw, Hooded Crow, and House Sparrow were three of the most frequently observed species in all parks since they are omnivorous species, are not selective in terms of food, and are highly adapted to urban conditions. The Great Tit and Eurasian Collared-Dove, which are the other species most frequently observed in the parks, are highly adapted to urban conditions.

While the trees on which the birds were observed in the morning provide food, the trees on which the birds were observed in the evening provide shelter. The reason the Australian Pine, Turkish Pine, and Oriental Plane Tree in the Pinarbasi Recreation Area attracted the highest number of bird species is that they are usually a food source for birds. In the Pinarbasi Recreation Area, the Australian Pine and Turkish Pine also attract most bird species at night and provide them with shelter.

The Turkish Pine, which attracted the highest number of bird species in the Aytepe Recreation Area, offers food for all bird species and shelter for most of them. The Italian Cypress (Horizontal form) is another species that is mostly a food source for most of the bird species.

The reason the Silky Oak in Nevzat BICER park attracted the highest number of bird species is that it is mostly a food source for birds. The Boxelder, White Mulberry, and Japanese Pagoda Tree, other tree species on which the birds were mostly observed in the morning observations, are also mostly food sources for birds. The Boxelder and Russian Olive, on which bird species were mostly observed in the evening, mostly serve as a shelter for birds. Since the Silver Maple (*Acer saccharinum* L.) has a dense texture, it is used for spending the night by the House Sparrows. The dense leaves of the tree allow the birds to hide from predators at night.

In Ismet SEZGIN Park, the Australian Pine attracts the highest number of bird species because it is a food source for birds. The Italian Cypress (Pyramidal form), Stone Pine, Kurrajong, Oriental Plane Tree, and Small-Leaved Lime, other tree species on which the birds are mostly observed in the morning, are also food sources for birds. The Stone Pine and Italian Cypress (Pyramidal form), on which bird species are mostly observed in the evening, also serve as a shelter.

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The birds were intensely observed in the trees in the parks examined in the study because trees are more sheltered from attacks by predators such as cats because of their height. Small shrubs are rarely used as they leave birds vulnerable to attack by cats living in parks.

Conclusions

Understanding the relationships between biodiversity and urban green areas is important for the management and especially conservation of urban green areas (Temple and Wiens, 1989; Ives et al., 2016; Ibáñez-Álamo et al., 2017). To understand the vegetation and to associate it with wildlife activity and pattern in a particular area are considered to be the best approaches for predicting the species that can use a metropolitan area and their capacity (Morrison et al., 1992; Clergeau et al., 1998).

The relationship between native bird species and native plants was found to be higher compared to exotic plants (Donnelly and Marzluff, 2004; Daniels and Kirkpatrick, 2006). The heterogeneity of native vegetation makes up the best starting point for the conservation of native bird species diversity in urban environments and the minimization of urban exploiter and exotic species (Chace and Walsh, 2006; Palmer et al., 2008; Shwartz et al., 2008).

It is necessary to give priority to natural or semi-natural vegetation for the management of urban areas that support bird wildlife, and the species that require high maintenance should be considered as a second option (Chong et al., 2014). Rather than exotic woody species, different native plant species should be mainly used in urban parks because of their low water requirements and their compatibility with the soil structure and climate of the region (Livingston et al., 2003; Chace and Walsh, 2006; Daniels and Kirkpatrick, 2006; Burghardt et al., 2009). Native trees, shrubs, and groundcovers are very important since they are sources of food and nectar and attract many insects that birds feed on. Birds are fed with the seeds of these native plants and the insects they attract. Therefore, native plants are important parts of a wildlife park focusing on bird habitat (Bauer, 2012).

The ratio of shrubs and trees of different sizes should be increased in urban parks of Aydin to increase bird diversity. Native plant species that attract native bird species should be used in the planting design studies of the parks to be built in Aydin and other cities in Turkey and the world (*Table 9*).

While the White Mulberry and Turkish Pine should be used if the Eurasian Jay is desired to be invited to parks, the Italian Cypress (Horizontal form), Turkish Pine, and Oriental Plane Tree should be used if the Eurasian Magpie is desired to be invited, the Turkish Pine should be used if the Common Chaffinch is desired, the Stone Pine should be used if the Red-Breasted Flycatcher (*Ficedula parva* Bechstein) is desired to be invited, the Cedar of Lebanon, Turkish Pine, and Stone Pine should be used if the Spotted Flycatcher is desired, the Turkish Pine should be used if the Eurasian Blue Tit is desired to be invited, the White Mulberry, Turkish Pine, and Oriental Plane Tree should be used if the great tit is desired to be invited, the Turkish Pine and Oriental Plane Tree should be used if the Syrian Woodpecker is desired, and the Turkish Pine and Stone Pine should be used if the Eurasian Nuthatch is desired to be invited.

The Turkish Pine, Oriental Plane Tree, Italian Cypress (Horizontal form), Stone Pine, and White Mulberry should be planted in parks as food sources for the Eurasian Jay,

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Great Tit, Eurasian Magpie, Eurasian Blue Tit and Common Chaffinch, Spotted Flycatcher and Syrian Woodpecker.

Table 9. *Native plant species that attract bird species in parks (developed from Bradbury, 2019)*

Plant Types	Family	Plants	Provides
Ground cover	*Fabaceae*	*Lupinus albus* L.	Insects
	Lamiaceae	*Lavandula stoechas* L.	Seeds
	Urticaceae	*Urtica dioica* L.	Insects, seeds
	Araliaceae	*Hedera helix* L.	Berries, insects, shelter
	Grossulariaceae	*Ribes orientale* Desf.	Berries, insects, shelter
	Santalaceae	*Viscum album* L.	Berries, insects, shelter
Shrub	*Adoxaceae*	*Sambucus nigra* L.	Berries, insects, shelter
		Viburnum lantana L.	Berries, insects
		Viburnum opulus L.	Berries, insects, shelter
	Aquifoliaceae	*Ilex aquifolium* L.	Berries, insects, shelter
	Betulaceae	*Corylus avellane* L.	Insects, seeds, shelter
	Ericaceae	*Arbutus unedo* L.	Fruit, insects, shelter
	Oleaceae	*Ligustrum vulgare* L.	Fruit, shelter, insects
	Rhamnaceae	*Rhamnus cathartica* L.	Fruit, insects, shelter
	Rosaceae	*Crataegus monogyna* Jacq.	Berries, insects, shelter
		Rosa canina L.	Fruit, insects, shelter
		Prunus avium L.	Fruit, shelter, insects
		Prunus laurocerasus L.	Berries, insects, shelter
		Pyracantha coccinea M. Roem.	Fruit, shelter
		Pyrus communis L.	Fruit, insects, shelter
		Sorbus acuparia L.	Berries, insects, shelter
		Sorbus torminalis (L.) Crantz.	Berries
Tree	*Styracaceae*	*Styrax officinalis* L.	Berries, insects, shelter
	Taxaceae	*Taxus baccata* L.	Fruit, insects, shelter
	Cornaceae	*Cornus mas* L.	Fruit, shelter, insects
	Cupressaceae	*Cupressus sempervirens* 'Horizontalis' (Mill.) Loudon	Fruit, insects, shelter
	Elaeagnaceae	*Elaeagnus angustifolia* L.	Fruit, insects, shelter
	Lauraceae	*Laurus nobilis* L.	Fruit, shelter, insects
	Moraceae	*Morus alba* L.	Fruit, shelter, insects
	Pinaceae	*Cedrus libani* A.Rich.	Insects, seeds, shelter
		Pinus brutia Ten.	Fruit, insects, shelter
		Pinus pinea L.	Fruit, insects, shelter
	Platanaceae	*Platanus orientalis* L.	Insects, seeds, shelter
	Salicaceae	*Populus alba* L.	Insects, seeds, shelter
		Populus nigra L.	Insects, seeds, shelter
		Salix alba L.	Insects, shelter

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The Italian Cypress (Horizontal form), Russian Olive, Turkish Pine, White Mulberry, Stone Pine, and Oriental Plane Tree should be used in the planting of parks since they provide shelter for the Great Tit, Spotted Flycatcher, Eurasian Jay, Common Chaffinch, Red-Breasted Flycatcher, Eurasian Blue Tit, Eurasian Nuthatch, and Syrian Woodpecker.

Rather than being an aesthetic element, parks should be a network of life where nature is imitated and a mini-ecosystem in which birds, bees, insects, and other creatures live. Thus, parks that support wildlife will also increase bird diversity (Bauer, 2012).

The planting design should be included in future studies if it is aimed to protect high natural biodiversity in urban parks.

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MINERAL-MICROBE INTERACTIONS: BACTERIALLY INDUCED HYDRATION OF BIOTITE

HU, R.[1] – LI, S.[1] – LI, F.[1*] – YANG, M.[1] – JIN, Z.[2] – LI, X.[1] – ZHANG, F.[2]

[1]*College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China*

[2]*State Key Laboratory of Loess and Quaternary Geology, Institute of Earth Environment, Chinese Academy of Sciences, Xi'an, Shaanxi 710075, China*

**Corresponding author*
e-mail: fchli@njau.edu.cn; phone: +86-25-8439-5014

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Abstract. The interaction between microbes and minerals occurring in the soil is an important geochemical process in the Earth's surface system. Investigating the weathering of potassium-bearing silicate minerals by microorganisms is of great importance in understanding the process of soil development and the provision of nutrient for plants. One of the major gaps in studies of weathering is the lack of sufficient evidence from X-ray diffraction to indicate of the formation of secondary minerals in the weathering of primary minerals. The present study aims to investigate whether or not secondary minerals formed during laboratory experiments can be detected by XRD (X-ray diffraction, XRD). For this reason, a series of culture experiments using biotite and *Paenibacillus mucilaginous* strain YM-1 were performed over a period of 450 days. The results demonstrate that preliminary treatment by sedimentation-centrifugation according to Stokes's law to enrich the fine particles with grain size less than 1 μm and subsequent detection by XRD are practicable methods for investigating the presence of secondary minerals. The XRD results of the fraction with grain size less than 1 μm indicated that a part of the biotite flakes altered into hydrobiotite on day 60 in experiments with strain YM-1, while hydrobiotite occurred on day 390 for experiments without bacteria. In summary, it is demonstrated that strain YM-1 of *Paenibacillus mucilaginous* evidently accelerated biotite weathering.
Keywords: *fraction of fine particles, bioweathering, Paenibacillus mucilaginous, sedimentation-centrifugation, enrichment method*

Introduction

The weathering of primary silicate minerals is of great importance for resolving many problems related to soil sciences and geochemistry. Firstly, clay minerals are the dominant weathering residues of primary silicates, and their formation and subsequent transformation helps bring to light the development of the soil (Kretzschmar et al., 1997). Secondly, cations released from minerals by weathering are an important source of nutrient elements for plants (Kretzschmar et al., 1997; Föllmi et al., 2009). Thirdly, mineral weathering on the continental surface controls the chemical composition of groundwater, rivers, lakes, and oceans over long timescales (Bonneville et al., 2011).

Biotite, as a representative of primary phyllosilicate minerals, exists extensively in different rocks, sediments and soils. In the past three decades, the weathering process (Banfield and Eggleton, 1988; Banfield and Murakami, 1998; Emberson et al., 2017) and weathering rate of biotite (Murphy et al., 1998; White, 2003) in the field and the effects of pH and temperature on dissolution kinetics in the laboratory (Kalinowski and Schweda, 1996; Murakami et al., 2003 ; Samson et al., 2005; Bray et al., 2015; Webb

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and Girty, 2016) have been investigated. The chemical weathering process and rate of biotite have been investigated also in the laboratory (Kalinowski and Schweda, 1996; Murakami et al., 2003; Bray et al., 2015; Tamrat et al., 2018), and several studies have suggested that microorganisms selectively colonize and weather minerals for nutritional purposes (Bennett et al., 2000; Rogers and Bennett, 2004). For example, *Paenibacillus mucilaginosus* and *P. edaphicus* selectively weather K-bearing silicate minerals (Ehrlich, 1996; Barker et al., 1998). The first direct indications that microorganisms enhance biotite weathering were found by Frankel (1977). Subsequently, a large number of studies have shown that microbes accelerate the elemental release from biotite (Baker et al., 1998; Balogh-Brunstad et al., 2008; Ward et al., 2013; Ahmed and Holmstrom, 2015). Many works have also suggested that microbial metabolism not only observably accelerates element release from primary silicates (Balogh-Brunstad et al., 2008; Zavarzina et al., 2016; Setiawati and Mutmainnah, 2016), but also effectively induces the formation of secondary minerals (Fortin and Beveridge, 1997; Murakami et al., 2003).

Several indications of biotite weathering into kaolinite (Ahn and Peacor, 1987), vermiculite (Banfield and Eggleton, 1988) and kaolinite + halloysite (Dong et al., 1998) have been found using transmission electron microscopy in the course of chemical or biological weathering experiments under laboratory conditions. Secondary minerals observed by TEM (transmission electron microscopy, TEM) are commonly seen as microscopic granules, but not only is it difficult to determine the quantities of secondary minerals in a sample, it is also difficult to ascertain whether or not they have been newly formed during weathering experiments. Although, fortunately, XRD technology allows secondary minerals to be detected, it is possible that they might only be detected in weathering residues if they are present in sufficient quantities.

Few previous studies have focused on the detection of secondary minerals by XRD. In the present work, a batch of weathering experiments was carried out over 450 days using biotite and *P. mucilaginous* strain YM-1 bacteria isolated from a soil sample. Grains smaller than 1 μm were examined by XRD with the aim of clarifying if the amount of secondary minerals formed during weathering of biotite is sufficient for detection by XRD. We hope to find a more efficient and precise method to get information about the formation of secondary minerals than the previous applied process.

Materials and methods

Characteristics of the biotite sample

The biotite sample used in this study was provided by Rentang Company Limited, Nanjing, China. The original biotite flakes have a maximum size of about 15 cm × 15 cm, and are black-brown in color. The flakes were cut into approximately 1 cm × 1 cm squares using a steel forfex, and ground in an agate mortar. Flakes > 100 mesh (0.15 mm) and < 65 mesh (0.25 mm) were obtained by a dry sieving. The chemical composition of the sample, determined by XRF (X-ray fluorescence spectrometer, XRF), was as follows (wt%): SiO_2 36.41, Al_2O_3 21.31, FeO + Fe_2O_3 24.43, MgO 5.43, CaO 0.05, K_2O 9.15, Na_2O 0.19, TiO_2 1.54 and MnO 0.55, total 99.06% including a 1.44% loss on ignition. In addition, the fluorine (F) content determined by an anion - selective electrode was 0.96 wt%.

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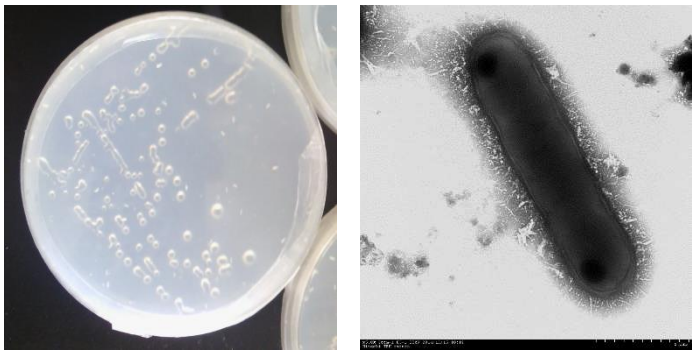
Isolation and identification of bacteria

The bacteria used in the culture experiments were obtained from local soil. A soil sample (alfisols, pH 6.40) was taken 7–10 cm beneath the ground surface from farmland at Qilinmen, Nanjing, Jiangsu Province, China (32°03′18″N, 118°55′21″E), a normal farmland with no special plants. In this study, a SB (Super Broth, SB) solid medium was used for bacterial selection, enrichment and purification. The biotite weathering tests were performed in a SB liquid medium. The composition of the solid medium was: 5 g sugar, 2 g Na_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCO_3$ and 18 g agar per liter of deionized water. The liquid medium was obtained from the solid medium using 0.5 g yeast-displacing agar. One strain, denoted YM-1, was isolated from the soil sample. The bacterial colonies were colorless and hemispherical with a smooth surface. The YM-1 bacterial cells are rod-shaped with diameters ranging from 0.8 to 1.3 μm and 2.5–5.5 μm long (*Fig. 1*). Gram staining showed that strain YM-1 is gram-negative. Strain YM-1 was identified as being closely related to *Paenibacillus mucilaginous* 480D^T (AF006077) (Shelobolina et al., 1997) with 99.72% similarity, based on comprehensive phylogenetic analyses of 16S rRNA gene sequences (*Fig. 2*). Login number on NCBI is JF499917 for strain YM-1.



a b

Figure 1. *The morphology of the YM-1 bacterial. (a) The morphology of the colony grown on the medium; (b) the morphology of the bacterial cell grown on the solid medium after four days*

Experimental methods

Several colonies of strain YM-1 were selected and dissolved in 300 ml of the liquid medium, and a seed culture was obtained after 48 h incubation at 30 °C and 180 rpm on a rotary shaker. Then 48 ml of the liquid medium (pH about 7.4) and 500 mg of the biotite powder were dispensed into 250-ml sterile conical flasks. All flasks were autoclaved at 121 °C for 20 min. After cooling to room temperature, a 2-ml aliquot of seed culture was added to the flasks. The bacterial density was found to be about 4.52 × 106 cfu/ml in the initial culture. The flasks were incubated at 28 °C ± 0.5 °C in still conditions, and periodically shaken by hand each day. Single tests were ceased every five days up to the 30th day, then every 30 days until the 450th day. To maintain bacterial activity, 2 ml of fresh seed culture was added to the remaining sample flasks every 30 days. All tests were carried out in triplicate. Simultaneously, a batch of CK (control check, CK) tests without bacteria was performed for comparison. When each test was complete, the liquid and solid constituents were separated using the sedimentation-centrifugation method in accordance with Stokes's law.

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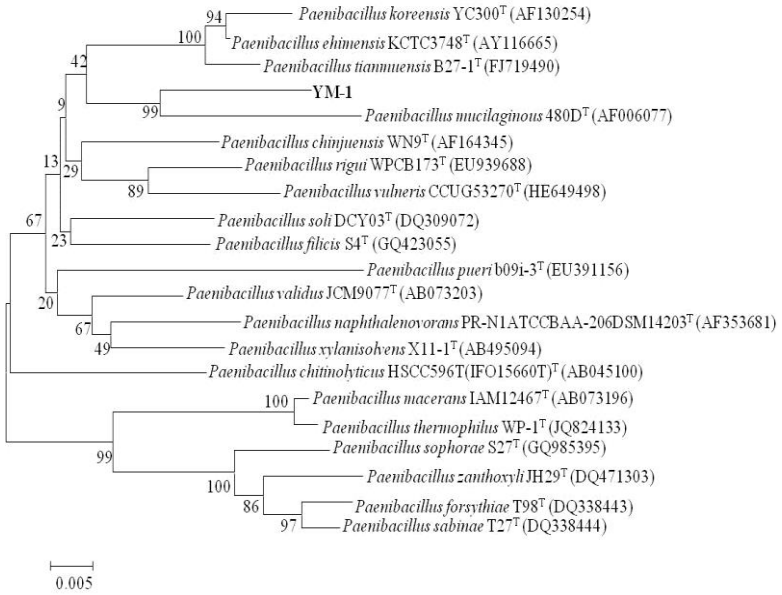

Figure 2. *Phylogenetic tree of strain YM-1 based on 16S rRNA gene sequence comparison within related genus*

The tests that were stopped prior to and including the 60th day were mainly used to determine bacterial density and pH; after 60 days the samples were tested by XRD to detect secondary mineral formation during the weathering process. To obtain the fine particles necessary to do this the < 1 μm fraction was separated from the solid constituent by sedimentation–centrifugation, then transferred to a glass slide and dried at room temperature for oriented particle specimens.

Observation and measurement

The plate count method was used for bacterial enumeration (Huang et al., 2013). The pH of the solution was measured with a pH meter (pHS-3C, KangYi Instrument co., China). XRD (Rigaku D/Max-B (III), Japan) with Cu-Kα radiation was used to identify the mineral species. Each sample was scanned continuously at 2° (2θ) min-1, from 3° to 60° (2θ) at 35 kV and 20 mA in 0.01° (2θ) steps. Qualitative identification of the mineral composition was performed using Jade 6.0 software. An TEM (H-7650, Hitachi, Japan) was used to observe the morphology of the bacteria. Several colonies suspended in double-distilled water were dropped onto a copper mesh. The dried sample was stained with phosphotungstic acid for 30 s and dried before TEM imaging. An EVO18-type scanning electron microscope (EVO18-type, Carl Zeiss, Germany) equipped with an energy dispersive detector was used for imaging the minerals and analyzing their chemical compositions. The dried samples were coated with 8 nm of gold before SEM (scanning electron microscope, SEM) imaging.

Results

Change of bacterial density and pH

The temporal curve in *Figure 3* shows that bacterial density increased with incubation time and peaked at 93.67×10^6 cfu/ml on day 25, then decreased gradually to

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17.24×10^6 cfu/ml on day 60. Conversely, the pH gradually decreased in the initial stage until day 30 (5.86), then began to rise slowly until day 60.

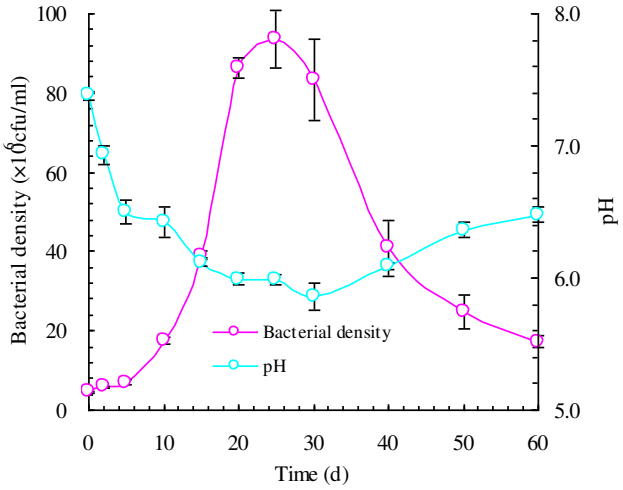

Figure 3. *Temporal changes of bacterial density and pH*

The occurrence of secondary minerals

The XRD results showed that the diffraction peak (d between 1.0999 and 1.4903 nm) appeared in the patterns of the samples on and after day 30 (*Fig. 4; Table 1*). This suggests that a part of the biotite flakes may have been altered to hydrobiotite. The abiotic experiments showed a diffraction peak of secondary minerals on and after day 390 (*Fig. 5; Table 1*). This indicates that secondary minerals also formed in abiotic conditions, but that their formation evidently occurred later than in the biotic experiments.

Table 1. *d value of diffraction peak of the hydrobiotite and percentage of vermiculite in the hydrobiotite*

Time (d)	Biotic experiments		Abiotic experiments	
	d value (nm)	Vermiculite (%)	d value (nm)	Vermiculite (%)
30	-	-	/	/
60	1.1085	29.8	/	/
90	1.1189	32.3	-	-
120	1.1692	44.0	-	-
150	1.1136	31.0	/	/
180	1.1143	31.2	/	/
210	1.1042	28.7	/	/
240	1.1129	30.9	-	-
270	1.0999	27.6	-	-
300	1.2543	61.7	/	/
330	1.3954	86.2	-	-
360	1.4085	88.2	-	-
390	1.4076	88.1	1.4506	94.5
420	1.4903	100.0	1.4698	97.2
450	1.4685	97.0	1.4602	95.8

"-" denotes absence of new diffraction peak; "/" denotes that the particle fraction with grain size of less than 1 μm was not obtained

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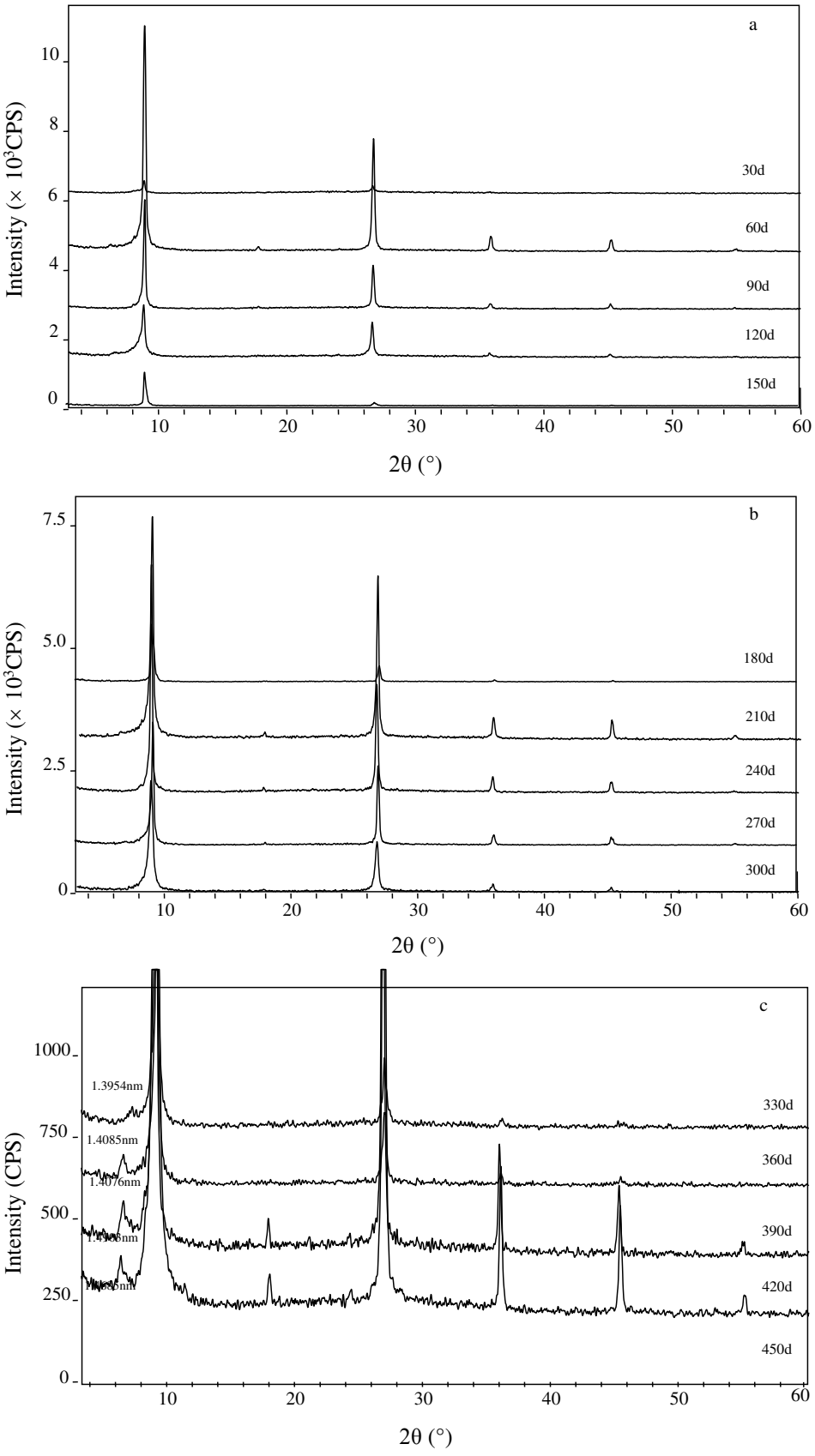

Figure 4. *Representative XRD patterns of the < 1 μm fraction in the biotic experiments*

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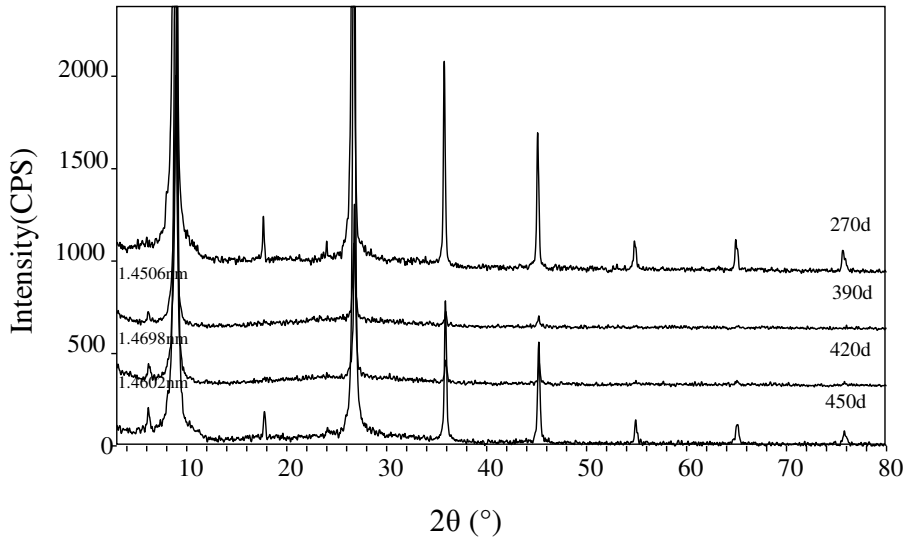

Figure 5. *Representative XRD patterns of the < 1 μm fraction in the abiotic experiments*

Bacterial cells (*Fig. 6a*) and mineral fragments (*Fig. 6b*) were observed by SEM. The EDS (energy dispersive detector, EDS) results indicated that these fine fragments mainly consisted of Si, Al, Fe, Mg and K, which when considered together with the XRD results, suggested that the hydrobiotite may be composed of biotite layers and vermiculite layers.

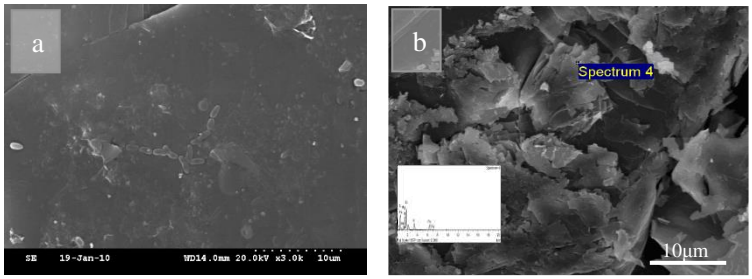

Figure 6. *SEM images and the EDS pattern of the solid particles after incubation 330 days. (a) Several bacterial cells arrayed as chain on the surface of biotite flakes; (b) several mineral particles consisted mainly of Si, Al, Fe, Mg and K*

Discussion

Content of vermiculite in the hydrobiotite

To quantitatively describe the vermiculite content in the hydrobiotite, we introduce *Equations 1* and *2,* which are applicable to mixed layer minerals (Lu, 1993):

$$1 / d_N = \alpha / d_B + \beta / d_V \quad \text{(Eq.1)}$$

$$\alpha + \beta = 100\% \quad \text{(Eq.2)}$$

where d_N is the d_{001} value of the mixed layer mineral; d_B and d_V are the d_{001} values of biotite (= 1.00 nm for samples in this study) and vermiculite (commonly = 1.49 nm) (Xu

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and Cao, 1993), respectively; and α, β are the percentages of vermiculite and biotite layers in the hydrobiotite flakes, respectively. The calculation results are listed in *Table 1*. The percentage of vermiculite layer gradually increased with incubation time from approximatively 30% on day 60 to about 100% on day 420 (*Fig. 7*)—that is, biotite changed into vermiculite stage by stage throughout the biotite-vermiculite interstratified mineral. This confirmed that hydrobiotite may be an intermediate product in the transformation process from biotite into vermiculite.

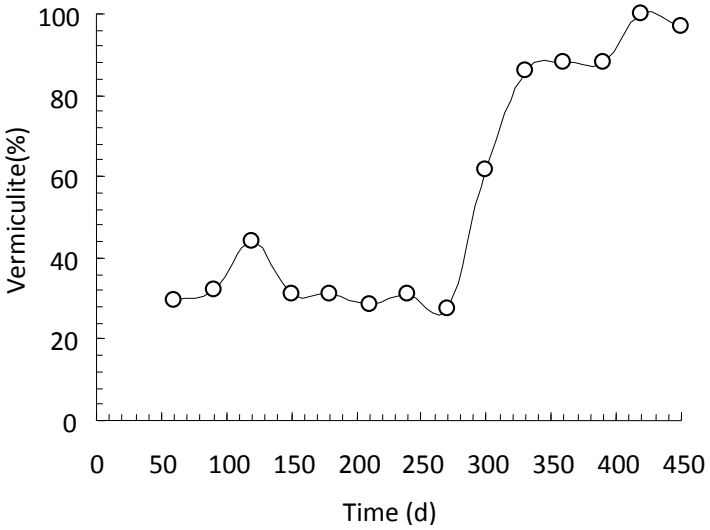

Figure 7. *Temporal change of vermiculite percentage in the hydrobiotite*

Hydration of biotite

Biotite sometimes weathers to hydrobiotite in which there is a regular alternation of 1.0 nm biotite and 1.4 nm vermiculite units yielding distinct X-ray reflections at 1.2 and 2.4 nm (Farmer and Wilson, 1970). However, complete regular interstratification has previously only been achieved by reversing the reaction in which vermiculite is reverted to a mica-like phase (Farmer and Wilson, 1970). This study implies that hydrobiotite with irregular interstratification is perhaps more common than regular interstratified hydrobiotite. For instance, vermiculite from Tseganbrak Xinjiang, the largest vermiculite deposit in China, consists mostly of irregular phlogopite and vermiculite layers (Xu and Cao, 1993). Different cations are present in the interlayer regions of biotite and vermiculite, with K^+ predominant in biotite, and Mg^{2+} in vermiculite. In this study, the formation of hydrobiotite would be explained if K^+ were replaced by a small quantity of hydrated Mg^{2+}, leading to modification of the layer structure and lowering the overall layer charge deficit. Here, the Mg^{2+} was derived from the original biotite and SB culture. Biotite and vermiculite are both phyllosilicates with a similar 2:1 structure type. Thus, the vermiculite might be formed in a layer-by-layer transformation (Murakami et al., 2003), causing the interlayer distance to increase from 1.00 nm to 1.49 nm structural expansion in the crystallographic c-axis direction. The neoformed vermiculite layers and relict biotite layers constitute a hydrobiotite with irregular interstratification. The increase of interlayer distance up to 1.49 nm on day 420 indicates that a part of the biotite flakes were almost completely transformed into vermiculite.

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Previous studies have indicated that weathering takes place mainly at the edges of the biotite grains and from the edges parallel to the crystal face (001) inward to a depth of a few tens to hundreds of micrometers (Murakami et al., 2003). Thus, the weathering rate at the edges is two orders of magnitude larger than at the basal surface (Turpault and Trotignon, 1994). The transformation of the fine biotite flakes into vermiculite is more rapid due to the larger specific surface area than is available in the thick flakes. In other words, an irregular interstratification structure consisting of biotite and vermiculite is likely to form when K^+ around the edges of the biotite flake is replaced by hydrated Mg^{2+}.

Conclusions

When it is produced in the laboratory, verification of the weak crystalline nature of a mineral comes from XRD analysis, Fourier-transform infrared analysis, thermogravimetric analysis, and/or from Raman spectroscopy. Such analyses are possible because the weak crystalline mineral is the only phase present. If the weak crystalline mineral is present in company with a fine crystalline mineral, however, its detection by XRD or any other analytical method becomes problematical, especially if the fine crystalline mineral constitutes only a small proportion of the total precipitate. Secondary clay minerals (e. g. vermiculite) are more weakly crystalline than a primary silicate mineral such as biotite, making it difficult to obtain information about any secondary minerals from XRD patterns of the whole precipitate. The results of the present study suggest that separating of thin fractions (especially, less than 1 μm) from the solid residue is a practicable approach. In addition, we draw the following two conclusions:

(1) In the biotic and abiotic experiments, hydrobiotite appeared in the fraction with grain sizes less than 1 μm on days 60 and 390, respectively. In other words, strain YM-1 of *Paenibacillus mucilaginous* evidently accelerated biotite weathering.

(2) Hydrobiotite is an irregularly interstratified mineral consisted of layers of neoformed vermiculite and relict biotite. In the biotic experiments, the vermiculite layer content in the hydrobiotite gradually increased with incubation time from about 30% on day 60 until to about 100% on day 420.

In other words, using enrichment method and XRD technology, researchers have the opportunity to get information about the formation of secondary minerals more clearly than the previous applied process. This method is very helpful for research on the weathering of minerals and rocks in the nature, especially, on the microbe-mineral interactions in laboratory.

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EXPRESSIONS OF GENES IN *Triticum aestivum* L. VARIETIES UNDER SOME ABIOTIC STRESSES

ŞENGÜL TORAMAN, P. – ERGÜN, N.*

Hatay Mustafa Kemal University, Science and Art Faculty, Biology Department, Antakya, Hatay, Turkey

**Corresponding author*
e-mail: ergun.nuray@gmail.com

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Abstract. Abiotic stresses caused by changing climatic conditions affect plants not only physiologically but also at the molecular level. This study, *Triticum aestivum* L. cv. Dağdaş-94 and Doğankent were divided into four groups at the end of the sixth day. The seedlings were exposed to flood, drought, and salt stress and harvested at the end of the twelfth day. Increased catalase enzyme, ascorbate peroxidase, and glutathione reductase activities were noted in Doğankent cultivar under drought stress conditions. Proline accumulation was mostly observed in Dağdaş-94 variety, exposed to drought stress. The chlorothricin (*CHL*) gene has been investigated in many plants and was examined first in *Triticum aestivum* in this study. *Triticum aestivum* salt-related hypothetical protein (TaSRHP) increased in both wheat varieties. This gene is dependent on salinity. Although the exact mechanism is not known exactly, the absence of amplification in *Triticum aestivum* zinc finger protein (TaZnFP) – which is known to occur under stress – suggests that it may be the result of reading on the twelfth day of the study, and this protein may appear as the first response to stress. It can be stated through this study; Dağdaş-94 variety is more drought-resistant than Doğankent variety.
Keywords: *stress, proline, antioxidant enzymes, CHL, TaSRHP, TaZnFP*

Introduction

Today, human and animal nutrition is largely based on cereals, especially wheat (*Triticum aestivum* L.). Turkey has a rich variety of plants due to soil structure and location. The wheat plant has a high nutritional value and workability in different ways. Turkey has become one of the largest gene centres. Wheat's resistance to stress conditions, diseases and pests is considered its quality and this diversity is important for wheat growing (Dirik et al., 2020).

The life of the plant is dependent on the soil. Due to global environmental changes, the soil is frequently exposed to abiotic stresses, such as salinity, drought, submerging, low and high temperature, ultraviolet radiation, nutrient deficiency, and metal toxicity (Hasanuzzaman et al., 2019).

Irregular water intake, caused by a lack of it for example, negatively affects the growth of the plant. An anaerobic environment forms in plant roots when it is flooding. This environment is divided into two categories according to the levels of oxygen: oxygen deficient and oxygen absent (Hossain and Uddin, 2011). Oxygen absence threatens the uptake of atmospheric gases necessary for respiration and photosynthesis in plants, causing the cessation of the citric acid cycle (TCA) cycle and oxidative phosphorylation in root mitochondria (Sairam et al., 2008). Studies in flooded fields have shown that oxygen is reduced by the accumulation of matter (Mittler et al., 2004). This activates the antioxidant system in plants (Ergün and Öncel, 2012; Özçubukçu et al., 2014).

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In arid conditions, the water potential of the soil decreases, followed by that of the plant and in later stages a, fall in turgor pressure, closure of stomata, decrease in leaf growth and decrease in photosynthesis take place (Bota et al., 2004).

Salinity is one of the major problems of soils worldwide. Salt stress directly affects some metabolic activities in plants; it can kill plants by limiting biological activity such as germination, cell division, growth and development. As the salt concentration in soil, the cleavage of chlorophylls, shrinkage of thylakoids, and accretion of adjacent grana membranes are observed, and the operability of the electron transport system in thylakoids decreases significantly under salty conditions (Şen and Alikamanoğlu, 2011).

Reactive oxygen species are produced as a result of abiotic and biotic stresses, such as salinity, drought, flood, and frost stress, and herbicide applications that plants are exposed to damage membrane lipids, DNA, macromolecules such as proteins, and photosynthetic pigments.

The chlorothricin gene (*CHL*; the gene encoding the light-harvesting chlorophyll *a*/*b*-binding protein) is a prerequisite for the formation of chlorophyll in plants and Mg-clamping of protoporphyrin IX into the tetrapyrrole branch (BioCyc Database Collection; Shimizu and Masuda, 2021). In this study, the presence of *CHL* gene in *T. aestivum* was examined for the first time, and gene data were obtained by Kolukırık (2014; *Table 1*). This gene is known to be important in the formation of the chloroplast organelle, which performs photosynthesis in plants and many species close to plants during the evolutionary process (Mayfield and Taylor, 1984). This gene found in different plants is named differently. In *Arabidopsis thaliana* sp., it is called *CHLI1-CHL11* (BioCyc Database Collection), and in *Zostera marina* L., it is named light-harvesting chlorophyll a/b-binding (*LHC*) proteins (Kong et al., 2016). *LHC* genes are highly conserved in the evolutionary process, and the classification of *ZmLhc* genes indicates that it is consistent with the evolutionary position of *Z. marina*. The real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that different ZmLhca and ZmLhcb members respond to stress in different patterns of expression, and salinity, temperature, light intensity, and light quality can affect the expression of most *ZmLhca* and *ZmLhcb* genes (Kong et al., 2016).

Table 1. *Names, code names, sequences, and product sizes of primers expressed in abiotic stress conditions used in this study*

Primer	Left primer (5')	Right primer (3')	Reference
CHL (chlorothricin)	CCT CGA CTACCT CGG CAA C	AAG AAG CCG AAC ATG GAG AA	Kolukırık (2014)
TaSRHP (*Triticum aestivum* salt-related hypothetical protein)	CGG CCG CGA CAC CTT CAT A	GGT CGC CGG GAA GCA CTT	Hou (2013)
TaZnFP (*Triticum aestivum* zinc finger protein)	GGA AGC CAA GCA ACC ATG TG	GGC TAC CGT GGC ACT AGG AG	Min et al. (2013)

T. aestivum salt-related hypothetical protein (*TaSRHP*), is a stress-related gene that can occur in plants exposed to stress (salinity, drought, cold, etc.) (Hou et al., 2013) (*Table 1*). The overexpression of TaSRHP in *A. thaliana* cv., the wild species, showed development by greater resistance against both salt and drought stress in Columbia (Hou et al., 2013). This study aimed to shed light on the *TaSRHP* gene.

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T. aestivum zinc finger protein (TaZnFP) is a transcription factor that is stimulated in cold, salinity, and drought stresses and as a result of the abscisic acid application (Min et al., 2013). Although the working mechanism is not yet fully known, the TaZnFP transcript factor shows an increase in control in drought and salinity stress conditions in the *Arabidopsis* plant.

Therefore, in this study, salt, flood, and drought stresses were applied to the wheat seedlings (*T. aestivum* L. cv. Doğankent and Dağdaş-94), and the physical changes that occurred were examined. Identifying the relationship between wheat varieties and various stress factors antioxidant enzymes (catalase enzyme [CAT] and glutathione reductase [GR] activities) and the amount of free proline were investigated by spectrophotometric method, some genes and proteins that are active under stress (*CHL*, TaSRHP and TaZnFP) the significance levels of inhibition were examined and clarified by PCR method. At the end of the study, it was aimed to determine the gene expression, enzyme, and other metabolic activities of wheat varieties having a high tolerance to flooding, salinity, and drought stresses. The most important pigment in plants chlorophyll which is the presence with the *CHL* gene expression and in other plants to what extent it affects the plant under which conditions it is our priority to investigate.

Materials and methods

Materials

As plant material, wheat seeds (*T. aestivum* L. cv. Doğankent and Dağdaş-94) were used in this study. The wheat seeds were obtained from the Eastern Mediterranean Agricultural Research Institute (Adana/Turkey). The Dağdaş-94 wheat variety is resistant to drought and cold stresses (Doğru and Ergün, 2021).

Plant growing conditions

Plastic pots with a diameter of 20 cm were used in plant cultivation. These pots were filled with a mixture of raw soil, organic fertilizer NPK (18:18:18), sand (2V/2V/1V), and air-dried soil. In each pot, 150 seeds were planted directly on the soil surface without any pre-processing and covered with a layer of soil about 1 cm thick. Immediately after sowing the seeds, all the pots were watered with 200 mL of water and taken to a plant-growing cabin. The plants were grown in the climate cabin at an average temperature of 24 ± 2°C, where the relative humidity was 66% and the light conditions were 1198 lux on average. Until the end of the sixth day, 100 mL of water was given every 2 days. On the third day, the wheat began to germinate. Each of these trials was performed jointly in different stress groups. After the sixth day, four different groups were formed, which are as follows:

1) *Normal water application (control)*: The application of 100 mL of water every 2 days from the planting of seeds was continued until the twelfth day, and growth was observed.
2) *Flood*: Sowing was done in pots supported by waterproof nylon bags. It was irrigated, as there was a 1 cm layer of water above the soil at the end of the sixth day. It was ensured that the layer of water remained above the ground level until the twelfth day.
3) *Drought*: Normal irrigation application was made until the sixth day. After the sixth day, there was no irrigation until the twelfth day.

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4) *Salinity (0.7% NaCl)*: The soil of the plants in this group was thoroughly mixed by adding 0.7% NaCl w/w before being filled in the pots. To prevent the inhibition of seed germination with salt effect, a 1-cm-thick layer of salt-free soil was formed on the surface, and the seeds were planted on this soil. Irrigation continued by the end of the twelfth day; it was watered as in normal water application. The plants were harvested by removing them from pots at the end of the twelfth day.

After harvesting, the shoot parts of the seedlings were cut into small pieces for analyses, labelled according to the group number of the seedlings, and stored at −80°C in a deep freezer. The GR activity was measured according to Çakmak and Marschner (1992) and Çakmak (1994). The CAT activity was measured at 240 nm using a spectrophotometer because of the fragmentation rate of H_2O_2. The reaction mixture (1 ml) consisted of; 50 mM phosphorus buffer (pH 7.6), 0.1 mM EDTA, 100 mM H_2O_2, and enzyme extract (Çakmak and Marschner, 1992). The ascorbate peroxidase (APX) activity was measured according to Asada (1992), taking into account the decrease in absorbance due to ascorbic acid oxidation at 290 nm wavelengths. Free proline extraction and determination was made according to Bates et al. (1973).

Measurement of gene expressions made with wheat leaf samples exposed to drought, flooding and salt stresses. Then, leaf samples were collected after harvesting and placed in liquid nitrogen. The samples were stored at −80°C until RNA analyses were performed. For total RNA isolation and reverse transcription, about 1 g of wheat leaves were divided into small pieces and taken into a 2-ml tube; these analyses were performed considering the study of Ergün et al. (2014).

In this study, *CHL,* TaSRHP ans TaZnFP gene expressions of wheat were performed by the service of Bio-eksen R&D. *Quantitative real-time PCR (Q-PCR) protocol*: The Biospeedy Relative Count Kit (Turkey) was used to determine the expression levels of genes in 12 wheat samples exposed to different stress factors. All the genes were studied in three replicates, and Livak and Schmittgen (2001) method were used for the experiment.

The kit included target genes of the *CHL* gene encoding the light-harvesting chlorophyll *a/b*-binding protein, TaSRHP, and TaZnFP. *EFA* was used as the housekeeping gene, which encodes a protein in wheat, to normalize the expression levels of target genes. The kit included primers specific to the housekeeping and target genes as well as the enzymes and buffers required for the RT-PCR analysis.

The Roche LC 96 RT-PCR instrument (Roche, Switzerland) was employed for all reactions. The reaction contained 1.5 mM $MgCl_2$, 0.2 mM Dinucleotittriphosphate (dNTP) mix, 1× reaction buffer, 0.1U FastStart Taq DNA polymerase, 1× EvaGreen, 4 ng/μL of template cDNA, and 0.5 μM of each primer. In the instrument, the annealing temperatures specific to the primer pairs (*Table 1*) and optimized reaction cycle temperatures were used. During Q-PCR, melt curve analysis was performed only between 65°C and 98°C to ensure only the desired product was amplified. Q-PCR data were analysed by Roche Lightcycler 96 software (v.1.1).

For statistical regression analysis, the related SPSS package program was used, and the variance analysis was performed using the MacAnova program. The Fisher least significant test was used to compare the mean of threshold cycles (Ct; $p < 0.05$). The differences between the results were determined by the Duncan test according to the homogeneity between variances (*Table 2*).

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Table 2. *Flood, drought, salt stresses (Triticum aestivum cv. Dağdaş and Doğankent) variance analysis results*

Parameters	CAT	GR	APX	PROLINE	*CHL*	TaSRHP
Wheat	0.000**	0.000**	0.44	0.038*	0.000**	0.000**
Stress	0.000**	0.001**	0.005*	0.000**	0.000**	0.000**
Wheat*stress	0.001**	0.014*	0.000**	0.000**	0.000**	0.000**

** Significant at 0.01 level, * Significant at 0.05 level

Results

Effects on the CAT, APX, and GR activities

The CAT activity was observed to cause a significant increase in Dağdaş-94 seedlings, especially those with high-stress tolerance (Yumurtacı and Uncuoğlu, 2012). It was noticed that drought and salt stress increase by approximately the same extent (by 50%) according to control ($p \leq 0.01$). Doğankent seedlings were found to decrease in flood and drought stress, whereas they increased in salt stress. The CAT activity of Dağdaş-94 seedlings, which is known to be resistant, increased more than that of Doğankent seedlings (*Fig. 1*).

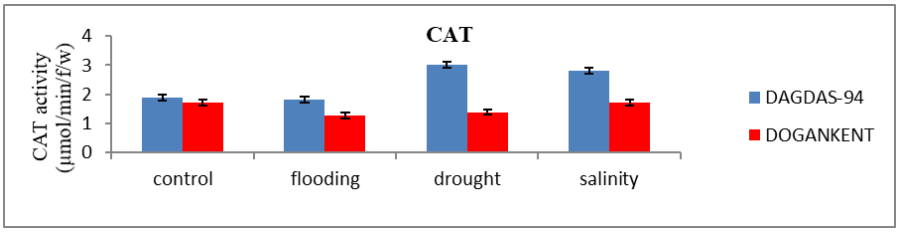

Figure 1. *Changes in the average catalase enzyme activities in the wheat seedlings (Triticum aestivum cv. Dağdaş-94 and Doğankent) grown under flood, drought, and salt applications (n = 3)*

In our study, during the GR activity, there was a noticeable increase in the seedlings of all two varieties in stress conditions, especially in Doğankent seedlings ($p \leq 0.01$). In flood and drought stress to the same degree and the GR activity in seedlings exposed to salt stress by 200% caused an increase ($p \leq 0.01$). In Dağdaş-94 seedlings, an increase in enzyme activity was observed in all three stresses according to control, and this increase in seedlings under flood stress increased by 100% ($p \leq 0.01$; *Fig. 2*).

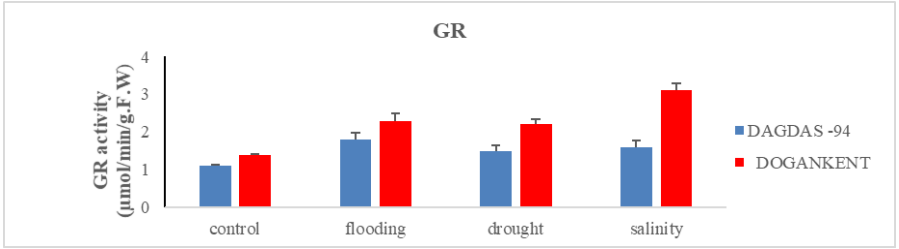

Figure 2. *Effects of the wheat seedlings (Triticum aestivum cv. Dağdaş-94 and Doğankent) grown under flood, drought, and salt applications to the average glutathione reductase (GR) activity (n = 3)*

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The APX activity increased in all three stresses applied according to control in the seedlings of both wheat varieties ($p \leq 0.01$). The maximum APX activity was noted in Dağdaş-94 seedlings exposed to flood and salinity stress ($p \leq 0.01$). In Doğankent seedlings, an increase in the APX activity was observed in all three stresses according to control, and the most increase was observed in salt stress (*Fig. 3*).

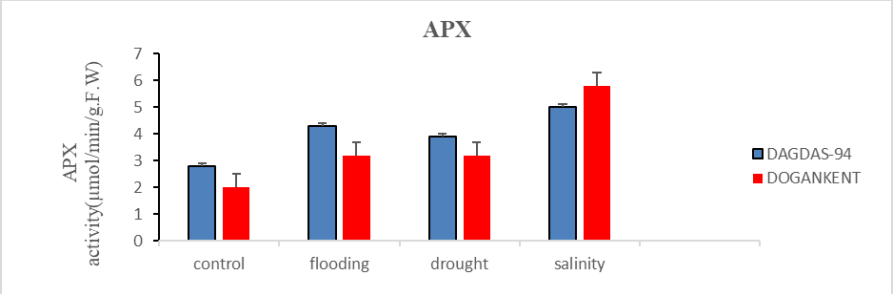

Figure 3. *Effects of the wheat seedlings (Triticum aestivum cv. Dağdaş-94 and Doğankent) grown under flood, drought, and salt applications to the average ascorbate peroxidase (APX) activity (n = 3).*

Proline content

Proline accumulation in drought, flood, and salt applications increased in Doğankent and Dağdaş-94 seedlings ($p \leq 0.05$). The maximum proline accumulation was observed in drought stress according to the control in Dağdaş-94 seedlings ($p \leq 0.001$). This is thought to be due to the proline being a hydrophilic amino acid. In Doğankent seedlings, the control rate is more than that in Dağdaş-94 seedlings. The maximum proline activity in this plant has been observed to increase equally in the least salinity, flood, and drought stresses (*Fig. 4*).

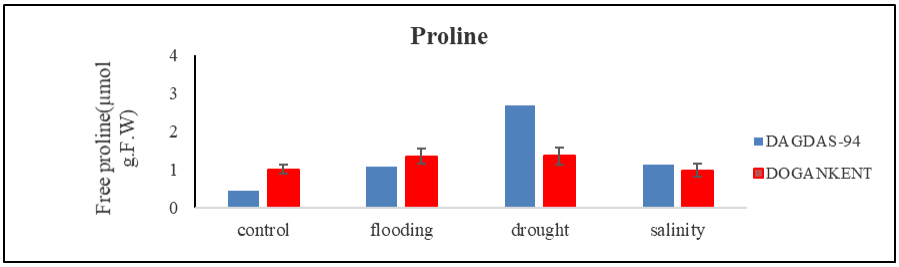

Figure 4. *Changes in the amounts of free proline in the wheat seedlings (Triticum aestivum cv. Dağdaş-94 and Doğankent) grown under flood, drought, and salt applications (n = 3)*

Gene expressions

In the study, the mRNA transcriptions in wheat seedlings exposed to flood, drought, and salinity stresses were studied in quantitative mRNA analyses performed by the real-time RT-PCR method. A total of three primers thought to be associated with abiotic stresses were used in the study, and the names and sequences of the genes are given in *Table 1*. The expression of these primers and relative values of the results of the RT-PCR analysis are shown in *Table 1*.

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CHL gene

The expression of *CHL* gene was more active in the control group Dağdaş-94 seedlings than in Doğankent seedlings (p<0.01). This gene activity has been found to decrease in both types of wheat seedlings exposed to abiotic stress (*Fig. 5*). In Doğankent seedlings, it was observed that the greatest reduction in this gene activity decreased according to control in salinity stress. In this case, chlorophyll biosynthesis can be said to be most affected by salinity stress.

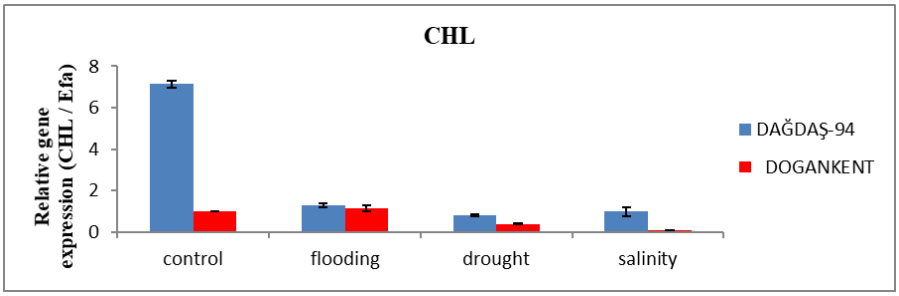

Figure 5. *Changes in the amounts of chlorothricin (CHL) gene in the wheat seedlings (Triticum aestivum cv. Dağdaş-94 and Doğankent) grown under flood, drought, and salt applications (n = 3)*

The *CHL* gene primer used in the study was made by Mustafa Kolukırık (2014) (Bioeksen R & D) by taking the *Arabidopsis* plant as an example. It has been stated that *CHL* gene primers play a role in chlorophyll syntheses and that the gene activity decreases under stress conditions. When both varieties studied were taken into account, it was determined that the expression of *CHL* gene decreased in all three abiotic stress conditions, but this decrease occurred more in the wheat seedlings of Doğankent variety than Dağdaş-94 variety ($p \leq 0.01$). In this case, it has been observed that the Doğankent variety is more sensitive than the Dağdaş-94 variety under all three stress conditions.

TaSRHP gene

The expression of *TaSRHP* gene showed amplification in the control group but did not cause any amplification in Doğankent seedlings ($p \leq 0.01$). It was observed that the *TaSRHP* gene activity decreased in the Dağdaş-94 seedlings exposed to abiotic stress and was not even active in salinity stress and increased in Doğankent seedlings because of abiotic stress (p <0.01). The *TaSRHP* gene expression activity was found to be maximum in salinity stress, as reflected in its name (*Fig. 6*). It has been observed that these gene values are statistically significant under different stress conditions and among wheat varieties (p < 0.01).

TaZnFP gene

Considering that the mechanism of *TaZnFP* is not yet fully determined, in this study, no amplification of the *TaZnFP* gene was found in the Doğankent and Dağdaş-94 seedlings under stress conditions. It was concluded that the stages of RNA isolation and cDNA synthesis from wheat samples were successful because of results in the housekeeping gene (*EFA*) and other genes and that there was no mRNA-encoding TaZnFP in the resulting cDNA pool. It has been observed that *CHL*, TaSRHP values are

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statistically significant under different stress conditions and among wheat varieties ($p < 0.01$).

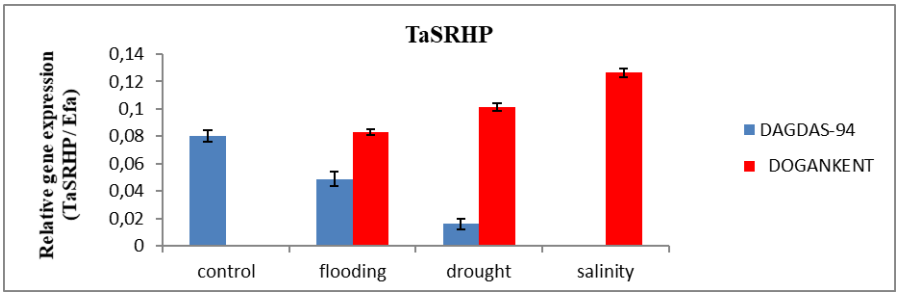

Figure 6. *Changes in the amounts of Triticum aestivum salt-related hypothetical protein (TaSRHP) genes in the wheat seedlings (T. aestivum cv. Dağdaş-94 and Doğankent) grown under flood, drought, and salt applications (n = 3)*

Discussion

In this study, the drought and salt applications of Dağdaş-94 and Doğankent seedlings showed increased activity of all three enzymes. It was determined that the CAT activity is higher in Dağdaş-94 seedlings, which is known to have high drought tolerance than Doğankent seedlings. Özçubukçu et al. (2014) reported that antioxidant enzyme activity significantly increased in flood-exposed wheat seedlings. Sen and Alikamanoğlu (2011) found that it is responsible for salinity stress in antioxidant enzyme activities such as superoxide dismutase (SOD), CAT, and APX and increases it. Çelik and Atak (2012) found that while there was no significant change in the CAT activity as a result of salt stress in tobacco plants, there were increases in the GR activity. When antioxidant enzymes were active under stress conditions, plants with higher tolerance showed a higher and more durable appearance in this case.

Proline accumulation and stress tolerance correlation have been reported in several studies (Ergün and Öncel, 2012; Özçubukçu et al., 2014). Keleş and Öncel (2002) applied salt to the wheat genotype and determined a decrease in the amount of proline. In their study, Ergün et al. (2014) observed that proline accumulation was created in response to plants under various stress conditions. In our study, there was an increase in proline accumulation in both varieties. The greatest increase was observed in Dağdaş-94 seedlings subjected to drought stress. This indicates that the Dağdaş-94 variety is drought resistant.

It was determined that the *CHL* gene played a role in chlorophyll synthesis and that this gene activity decreased under abiotic stress conditions (Grafe et al., 1999). Parallel to our study, we have shown that the light-harvesting chlorophyll *a*/*b*-binding (LHCB) proteins are positively involved in protective cell signalling in drought stress (Liu et al., 2013). Because the expression of *CHL* gene can be lead to tolerant of Dağdaş-94 seedlings, it has been observed that it is more active in Dağdaş-94 seedlings than in Doğankent seedlings. In Doğankent seedlings, this gene activity decreases according to control, and this decrease was observed to be at maximum salinity stress.

TaSRHP is specific to the species of *T. aestivum* and is a protein that plays a role in salt stress and has emerged as a hypothesis under stress conditions. This stress gene is known to differ according to the variety and region of the plant (Hou et al., 2013). In our study, the expression of *TaSRHP* gene showed amplification in Dağdaş-94 seedlings in

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the control group, but it was observed that it did not cause any amplification in Doğankent seedlings. In the seedlings exposed to abiotic stress, this protein activity decreased in Dağdaş-94 seedlings and increased in Doğankent seedlings. The increase of TaSRHP, a protein specific to salt stress, in Doğankent seedlings suggests that Doğankent seedlings are more resistant to salt stress. The work we have done may shed light on other studies that can be done in this area.

It has been determined that Doğankent and Dağdaş-94 seedlings under stress do not express the *TaZnFP* gene. Min et al. (2013) showed that TaZnFP overexpressed under stress conditions in *T. aestivum* cv. However, the CCCH-type zinc finger proteins to which TaZnFP belongs are a large family, and there has been reported to be insufficient information on the diversity and function of these proteins. They estimated that there are about 70 CCCH-type zinc finger proteins in *A. thaliana* (Wang et al., 2008). Although many of these proteins have been predicted to be active in abiotic and biotic stress resistance, the reaction of very few of them to stress under different conditions has been studied. One reason why the expression of *TaZnFP* could not be determined in this study is that the wheat varieties used could activate another CCCH-type zinc finger protein in response to stress. Another possibility is that Min et al. (2013) found that this protein is expressed in the twelfth hour. We showed that in the samples taken at the end of the sixth day, the *TaZnFP* gene was not expressed. Accordingly, it is thought that *TaZnFP* can be used as the first response mechanism created against stress in the short term and that it may lose its effect in the long term. It is thought that the presence of this protein in the wheat genome can be thoroughly investigated and used in breeding studies to determine the level of its effect under stress conditions.

Conclusion

This study is important in terms of determining answers to the molecular factors of drought, flood, and salinity stresses in two types of bread wheat seedlings (Dağdaş-94 and Doğankent), which are widely produced in our country.

It has been determined that plants subjected to abiotic stress – especially flood, drought, and salinity stress – may differ depending on the type and age of the plant and duration of stress. Because plants can develop tolerance to stress factors, it is critical to know the physiological and molecular mechanisms that enable this situation. It is aimed to use the genes we used in our study, especially the *CHL* gene, as a source in future studies and to be present in studies on wheat breeding. And shed light on the studies to be carried out by stating that the *CHL* gene plays a role in abiotic stresses and can lead to reductions in chlorophyll biosynthesis. The corresponding identification and mapping of genes in wheat breeding are necessary to improve the availability of them.

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OCCURRENCE OF NOXIOUS WEEDS UNDER DIFFERENT SOIL MANAGEMENT SYSTEMS

MELO, A. K. P.[1] – ALBUQUERQUE, J. A. A.[1*] – SIQUEIRA, R. H. S.[2] – SILVA, E. E.[3] – MEDEIROS, R. D.[3] – SOUZA, K. T. S.[1] – SOUZA, L. T.[1] – GONÇALVES, A. C. M.[1] – SOARES, M. B. B.[4]

[1]Federal University of Roraima (UFRR), BR 174, Km 12, S/Nº, Campus do Cauamé, Bairro Monte Cristo, Boa Vista, Roraima, CEP 69310-250, Brazil (phone: +55-95-3627-2573)

[2]Federal Institute of Roraima (IFRR), Rodovia Antonino Menezes da Silva, Km 03, S/Nº, Campus do Amajari, Amajari, Roraima, CEP 69343-000, Brazil (phone: +55-95-3593-1119)

[3]Brasilian Agricultural Research Corporation (EMBRAPA-RR), Rodovia BR 174, Km 8, Distrito Industrial, Boa Vista, Roraima, CEP 69301-970, Brazil (phone: +55-95-4009-7100)

[4]Researcher at Agribusiness Technology Agency of São Paulo (APTA), Rodovia Washington Luiz, Km 372, Pindorama, São Paulo, CEP 15830-000, Brazil (phone: +55-17-3572-1592)

[*]Corresponding author
e-mail: anchietaufrr@gmail.com

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Abstract. The aim of this work was to identify weed species in areas under different soil management systems. The research was carried out in the Brazilian Agricultural Research Corporation experimental area, in the 2017/2018 harvest, in the city of Boa Vista, Roraima State, Brazil. The experimental design was randomized blocks and the treatments included of five soil management systems (native vegetation, minimum tillage, no-tillage, conventional tillage and conventional tillage with crop rotation). Weed collection, identification, counting and drying were performed and then the phytosociological indices (Relative density, relative frequency, relative abundance, importance value index, relative importance value index and dry mass) were calculated. Variance analysis with means compared by Tukey test ($P < 0.05$), group analysis by hierarchical and non-hierarchical method, and principal component analysis were performed. Correlation coefficients of the variables were estimated. No and minimum tillage systems had the lowest weed rates per square meter. The botanical families Poaceae, Fabaceae and Rubiaceae are the most representative in the studied systems. Concerning relative frequency, the species *Cyperus flavus* stood out in all treatments other than native vegetation.
Keywords: *no-till, spontaneous plants, phytosociological parameters, cover plant, native vegetation*

Introduction

The use of the same weed control methods every year in the cultivation area is not recommended because their efficiency is diminished due to selection of species resistant or adapted to such management and, therefore, over time, this practice becomes economically unviable. According to Junior et al. (2019) the lack of proper management in modern agriculture results in low productivity, which is a characteristic problem in crops.

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Conservationist systems such as no-tillage and minimum tillage have been re-studied and adopted in order to improve soil physical, chemical and biological conditions and reduce production costs, such as weed control.

Prior knowledge of the main noxious weeds that could affect the next production cycle would help in targeted and precise control (Délye et al., 2013; Alcântara Neto et al., 2019; Menezes et al., 2019) aiming at system sustainability.

Phytosociology is the floristic and structural study of plant communities that requires the identification of species and classification of the most important ones. Phytosociological data contains relevant information for proper weed management in agricultural systems. This science covers one of the most commonly used methods for floristic recognition in agricultural or non-agricultural areas (Lima et al., 2014).

Phytosociological surveys in cultivated areas promote knowledge on weed populations, as well as knowledge of their morphological characteristics, such as: propagation type, life cycle, growth habit and photosynthetic route. The data obtained, when analyzed together, will indicate the most appropriate control method to be used (Cruz et al., 2010; Albuquerque et al., 2013, 2014).

The article aimed to analyze noxious weeds found under different soil management systems in Boa Vista, Brazil, in 2017-2018, through a randomized block design of five treatments, in order to collect phytosociological data for proper weed management in the region.

Material and methods

Research location and period

The research comprised an experiment conducted in the 2017/2018 agricultural years harvests, under different soil management systems. The experiment was conducted at the Brazilian Agricultural Research Corporation [EMBRAPA] "Água Boa" Experimental Field, located at 30 km from Boa Vista, Roraima, Brazil, on the left bank of BR-174, toward the city of Manaus, Amazonas, Brazil. The experimental area has 1,200 ha and is situated between the geographic coordinates 02°39´00" and 02°41´10" north latitude and 60°49´40" and 60°52´20" west longitude from Greenwich.

The vegetation is represented by graminosa and arboreal species (Vale Júnior and Schaefer, 2010), with predominance of creeping species of the genera *Trachypogon spp* and *Andropogon spp*.

Soil classification

The soil of the area is classified as Medium Texture Yellow Argisoil distrocoeso (consistent). The soil physico-chemical analysis is presented in *Table 1*.

Table 1. *Physical and chemical analysis of the soil in the 0 - 20 cm layer of the study area not incorporated in the production system, in the savannah of Roraima, Brazil*

Chemical Properties													
Depth	pH	OM	K	Ca	Mg	H+Al	P	S	Cu	Fe	Zn	Mn	B
0 - 20 cm		g dm^{-3}	----cmolc dm^{-3}-------				---------- Mg dm^{-3} ----------						
	4.1	1.1	0.01	0.3	0.2	0.8	3.0	3.0	0.1	30	0.2	0.6	0.39
Physical Properties (g kg^{-1})													
Depth	Clay						Sand				Silt		
0 - 20 cm	200						760				40		

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Description and conduct of the experiment

The experimental design was randomized blocks with five treatments and four replications. The treatments consisted of five soil management systems (native vegetation, minimum tillage, no-tillage system, conventional tillage system and conventional tillage system with crop rotation). The dimensions of the experimental plots were 10 x 10 (100 m^2) and the treatment areas were 101 x 131 m.

Related crops with year of cultivation: no-till (soybean + brachiaria), minimum tillage (soybean + brachiaria), conventional tillage with rotation (soybean + brachiaria), conventional planting (soybean) and native vegetation (fallow).

The phytosociological survey was performed following the inventory square method (Braun-Blanquet, 1979) with the aid of a cast iron square with dimensions of 0.50 x 0.50 m, randomly thrown four times in each of the treatments. Weeds were cut close to the ground, stored in plastic bags and taken to the laboratory, where they were quantified and identified with the aid of specialized literature (Lorenzi, 2000, 2014; Moreira and Bragança, 2010). Initially weeds were identified at class, family, scientific name, common name and described the type of propagation, growth habit, life cycle, photosynthetic route and coding of the European and Mediterranean Plant Protection Organization [EPPO].

Descriptive analysis of the following phytosociological parameters was performed: 1. Relative density [RD] = Species density x 100 / total density of all species; 2. Relative frequency [RF] = Species frequency x 100 / Total frequency of all species; 3. Relative abundance [RA] = Species abundance x 100 / total abundance of all species; 4. Importance Value Index [IVI] = RD + RF + RDo; 5. Relative Importance Value Index RIV = IVI x 100 / total importance value index of all species (Brandão et al., 1998) and weed dry mass.

Statistical analysis

Data were submitted to analysis of variance by the F test and, Tukey test, at 5% probability, was used to compare means when there were significant differences. Weeds were classified according to hierarchical cluster analysis using the Ward method and the Euclidean distance dissimilarity coefficient. The importance value index of weed species was analyzed by the non-hierarchical grouping and the groups were ordered in relation to the type of soil management used, considering the grouping of means (K-means). The correlation coefficients of the variables were estimated and principal component analysis was performed to identify the change factors associated with each soil management system.

Results

Table 2 summarizes the variance analysis of the data obtained for the number of weeds in the five different soil management systems. It was observed a significant effect on the number of weeds at the different management systems adopted.

It can be noted in *Figure 1* that no-till and minimum tillage systems provided the lowest number of weeds, 75 and 98 plants per m^2, respectively. The conventional system in turn presented the highest degree of weed infestation and was not effective for weed control.

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Table 2. *Summary the variance analysis for the number of weeds in the five soil management systems*

SV	Number of Weeds			
	DF	MS	TEST "F"	P
Soil management systems	4	17771.20	78.77**	<0,0001
BLOCK	3	441.60	1.96	0,1743
ERROR	12	225.60		
VC (%)	9.99			

*,**, ns - significant at 5%, 1% and not significant, respectively, by the 'F' test

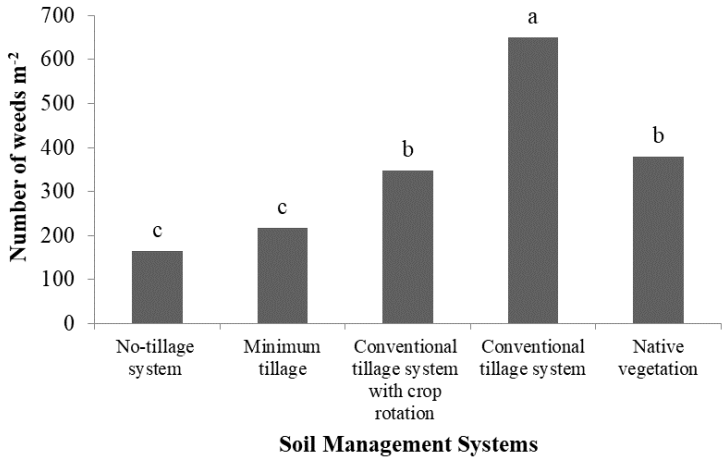

Figure 1. *Average number of weeds per square meter in different soil management systems*

The phytosociological survey allowed the identification of 10 weed species infesting the experimental area, with predominance of dicotyledonous, distributed in 5 botanical families, being Poaceae the most representative, with 3 species, followed by the families Fabaceae (2) and Rubiaceae (2), representing 30% (Poaceae), 20% (Fabaceae) and 20% (Rubiaceae) (*Table 2*).

Regarding the characteristics: propagation type, growth habit, life cycle and photosynthetic route there was a higher incidence of sexual, herbaceous, annual and C3 photosynthetic pathway (*Table 3*), respectively.

Regarding the phytosociological study, at the native vegetation system the highest relative frequencies [RF] were observed for the species *Trachypogon plumosus* (38,82), *Spermacoce capitata* (21,42) and *Cenchrus echinatus* (17,49) (*Table 3*). At the minimum tillage system, the highest relative frequencies (Frr) were from *Cyperus flavus* (25.19%), *Sida spinosa* (15.73%) and *Trachypogon plumosus* (15.08%) species (*Table 3*). For conventional tillage system, the highest relative frequencies [RF] were observed for *Cyperus flavus* (28.43%), *Cenchrus echinatus* (19.77%) and *Digitaria horizontales* (17.39%) species (*Table 2*).

In the no-tillage system area, the highest relative frequencies [RF] were observed for *Cyperus flavus* (25.85%), *Cenchrus echinatus* (18.58%) and *Borreria verticillata* (15.08%) (*Table 3*).

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Table 3. *Botanical class, family, scientific name, common name, propagation type, growth habit, life cycle, photosynthetic route and EPPO codes of species collected in the cerrado areas of Roraima, Brazil, 2017/2018*

Class	Family	Scientific Name	Common Name	Propagation Type	Growth Habit	Life Cycle	Photosynthetic Route	[EPPO] code[1]
Dicotyledonous	Pedaliaceae	*Sesamum indicum*	Sesame	Seeds	Herbaceous	Yearly	C_3	SEGIN
	Malvaceae	*Sida spinosa*	Evil	Seeds	Herbaceous	Perennial	C_3	SIDSP
	Fabaceae	*Mimosa pudica*	Sleeping Flower	Seeds	Bush	Yearly or Perennial	C_3	MIMPU
		Desmodium tortuoso	Bug	Seeds	Herbaceous	Yearly	C_3	DEDTO
	Rubiaceae	*Spermacoe capitata*	Beach poaia	Seeds	Herbaceous	Yearly	C_3	SPCTN
		Borreria verticillata	Friar's Cord	Seeds	Herbaceous	Yearly	C_3	BOIVE
Monocotyledonous	Poaceae	*Cenchrus echinatus*	Burr grass	Seeds	Herbaceous	Yearly	C_4	CCHEC
		Trachypogon plumosus	Borehole	Seeds	Herbaceous	Perennial	C_4	TRNPL
		Digitaria horizontalis	Grass mattress	Seeds	Herbaceous	Perennial	C_4	DIGHO
	Cyperaceae	*Cyperus Flavus*	Tiririca	Seeds/ Rhizome	Herbaceous	Perennial	C_4	CYPFW

[1][EPPO] code: Also known as the Bayer Code, it is a coding system used by the European and Mediterranean Plant Protection Organization (EPPO) to designate important agricultural plants, pests and pathogens, 2019

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For the conventional tillage system with crop rotation, the highest relative frequencies [RF] were for *Cyperus flavus* (27.92%), *Cenchrus echinatus* (19.77%) and *Sesamum indicum* (15.79%) species (*Table 4*).

Table 4. *Scientific name, dry mass [DM], relative density [RD], relative frequency [RF], relative abundance [RA], importance value index [IVI] and relative importance value index [RIV] of species in native vegetation, minimum tillage, conventional tillage system, no-tillage system and conventional tillage system with crop rotation on Roraima cerrado, Brazil, 2017/2018*

Scientific name	DM (g)	RD (%)	RF (%)	RA (%)	IVI (%)	RIV (%)
Native Vegetation						
Cyperus flavus	2.33	4.6	11.83	3.55	19.98	6.66
Cenchrus echinatus	2.65	57.57	17.49	5.35	80.41	26.80
Sesamum indicum	4.65	6.64	10.44	6.57	23.65	7.88
Spermacoce capitata	12.54	9.91	21.42	7.65	38.98	12.99
Trachypogon plumosus	96.7	21.28	38.82	76.88	136.98	45.66
Total		100.00	100.00	100.00	300.00	100.00
Minimum Tillage						
Mimosa pudica	3.66	2.49	8.91	6.94	18.34	6.11
Cyperus flavus	4.23	43.74	25.19	17.98	86.91	28.97
Desmodium tortuoso	2.57	3.59	13.87	2.80	20.26	6.75
Borreria Verticullata	1.78	2.59	9.37	2.60	14.56	4.85
Digitaria horizontalis	3.61	33.68	11.85	14.70	60.23	20.00
Sida spinosa	2.79	3.76	15.73	5.26	24.75	8.25
Trachypogon plumosus	6.78	10.15	15.08	49.72	74.95	24.98
Total		100.00	100.00	100.00	300.00	100.00
Conventional Tillage System						
Mimosa pudica	1.78	3.94	9.76	1.96	15.66	5.22
Digitaria horizontalis	2.67	15.71	17.39	12.93	46.03	15.34
Sida Spinosa	3.56	4.57	3.87	2.63	11.07	3.69
Cyperus flavus	3.89	49.75	28.43	8.73	86.91	28.97
Sesamum indicum	2.57	4.47	4.65	14.44	23.56	7.85
Cenchrus echinatus	4.79	8.82	19.77	54.44	83.03	27.67
Spermacoce capitata	1.57	12.74	15.86	4.87	33.47	11.15
Total		100.00	100.00	100.00	300.00	100.00
No-tillage System						
Digitaria horizontales	2.58	16.77	15.72	16.94	49.43	16.47
Cenchrus echinatus	3.18	34.76	25.19	27.98	87.93	29.31
Thachypogon plumosus	3.71	13.59	13.87	17.78	45.24	15.08
Borreria Verticullata	1.67	10.23	19.37	12,60	42.20	14.06
Cyperus flavus	2.89	24.65	25.85	24.70	75.20	25.06
Total		100.00	100.00	100.00	300.00	100.00
Conventional Tillage System with Crop Rotation						
Sesamum indicum	5.13	4.65	15.78	15.40	35.83	11.94
Digitaria horizontalis	2.68	17.43	15.43	18.93	51.79	17.26
Desmodium tortuosum	1.60	6.87	12.47	12.63	31.97	10.65
Cyperus flavus	3.47	39.86	27.92	23.73	91.51	30.50
Thachypogon plumosus	2.76	15.76	9.82	14.44	40.02	13.34
Cenchrus echinatus	2.44	20.43	18.58	14.87	53.88	17.96
Total		100.00	100.00	100.00	300.00	100.00

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The results of the hierarchical cluster analysis are represented in *Figure 2*. Considering in the dendrogram a break point in the distance of 120, 3 groups were identified. The result of the non-hierarchical cluster analysis is described in *Figure 3*. Considering the grouping of means [K-means], being K = 3, it was found that group 1, formed by the species *Trachypogon plumosus*, presented the highest importance value index [IVI] values in the plots under native vegetation and minimum tillage, but with no importance at the conventional soil management system.

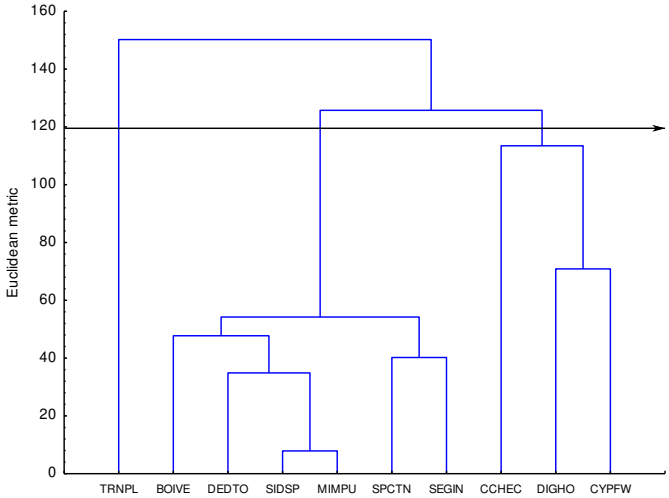

Figure 2. *Weed classification by hierarchical cluster analysis by the ward method*

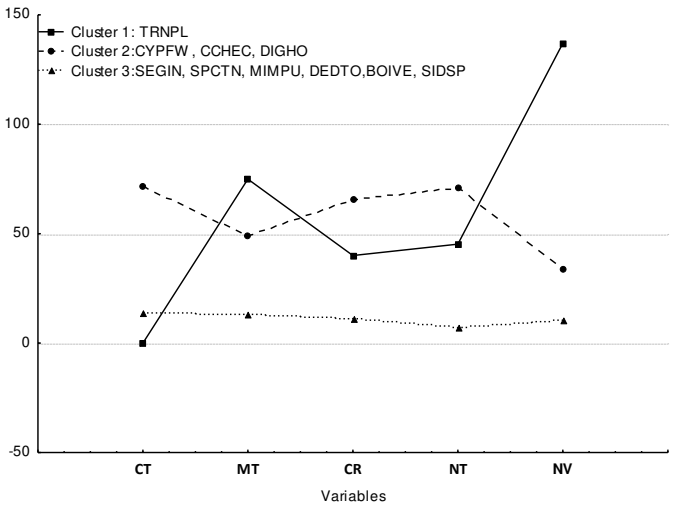

Figure 3. *Non-hierarchical cluster analysis containing the ordering of groups in relation to the Weed Species Value Index for each soil management used: Conventional Tillage System [CT], Minimum Tillage [MT], Conventional Tillage System with Crop Rotation [CR], No-Till System [NT] and Natural Vegetation [NV]*

Group 2, formed by the species *Cyperus flavus*, *Cenchrus echinatus* and *Digitaria horizontalis*, was characterized by the presence in all treatments, with a high degree of

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[IVI], standing out at the conventional tillage, conventional tillage with crop rotation and no-tillage systems. However, Group 2 presented lower [IVI] in natural vegetation areas.

The first two groups with higher Importance Value Indexes contained species belonging to the botanical families Cyperaceae and Poaceae, and in the second group were the species considered difficult to control in agricultural areas. The third group characterized by greater variability of species and botanical families, presented lower [IVI] than groups 1 and 2. These results stress the need to identify the weed community in an area and the role of soil management in decision making about weed management.

Principal component analysis was performed to obtain an overview of soil management systems. According to the analysis, 76.78% of the variation present in the original data was explained by the first two main components [CP1 and CP2] (*Table 5*). The first major component explained 57.55% of the total variance and the second 19.23%. According to Teixeira et al. (2012), the first major component always contains the most information from the original values, representing the most relevant results.

Table 5. *Coefficient of correlation of variables*

Factor	PC1	PC2	PC3	PC4	PC5
IVI – Conventional Tillage	0.778370	-0.493632	0.300139	-0.110642	-0.219411
IVI – Minimum Tillage	0.655918	0.263033	-0.691035	0.048281	-0.143960
IVI – Conventional Tillage with Crop Rotation	0.918269	-0.133543	-0.143805	-0.261253	0.223640
IVI – No-Till	0.895766	-0.019263	0.196783	0.387594	0.090987
IVI – Natural Vegetation	0.442834	0.793942	0.396137	-0.119326	-0.048901
Exploratory variance	2.8778	0.9614	0.7839	0.2472	0.1295
Total variance ratio (%)	57.55	19.23	15.68	4.95	2.59

Obs.: Variables in bold have significant importance for the respective factor. Equal signs indicate direct, and opposite, indirect relationship

Most soil management systems, with the exception of IVI of weeds from natural vegetation, showed high discriminatory power in the first major component [PC1] (values close to 1) (*Table 5*). As the factors evaluated have a positive sign, they indicate that rightmost units in the graph (*Figure 4*) are more influenced by them.

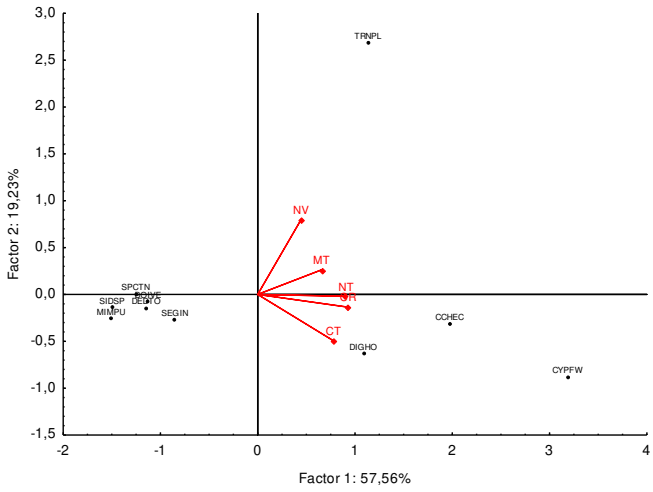

Figure 4. *Two-dimensional dispersal of major weed components in different soil management systems: Conventional Tillage System [CT], Minimum Tillage [MT], Conventional Tillage System With Crop Rotation [CR], No-Till System [NT] And Natural Vegetation [NV]*

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Figure 4 shows the two-dimensional dispersion of the main components 1 and 2 and the soil management systems. The *Trachypogon plumosus* species is strongly associated with the natural vegetation soil management system, confirming its segregation verified in cluster analysis and analysis of variance (*Figure 3* and *Table 5*). The species *Digitaria horizontalis*, *Cenchrus echinatus* and *Cyperus flavus* were strongly associated with conventional tillage soil management, either in monoculture or in rotation.

Discussion

Differentiated soil management practices change the frequency and species of weeds in cultivated areas (Lacerda et al., 2013; Lima et al., 2014). According to Correia et al. (2006), the composition and population densities of weed communities are influenced by soil management systems.

The number of seeds in the soil seed bank of no-tillage system is considered high, but the percentage of seeds that germinate and become competitive can be considered very low (Gomes-Junior and Christoffoleti, 2008).

In phytosociological studies performed, the families Poaceae, Fabaceae and Rubiaceae, respectively, were the ones that prevailed in researches with cultivated species (Marques et al., 2010). The predominance of weed species from the Poaceae family is justified by their high amount of diaspores, propagules that facilitate their spread and hamper their control (Lorenzi, 2008). According to Jakelaitis et al. (2003), for species that reproduce by seeds, the use of agricultural implements provides their spread as well as their distribution in the area.

Among the diversity of Poaceae species that grows in the native areas of Roraima state, *Trachypogon plumosus* is one of the most important, representing between 70 and 90% of its botanical composition (Costa et al., 2014). According to Costa et al. (2013), in a native pasture area of Roraima, the species *T. plumosus* appears with great representativeness, between 70 and 90% of botanical composition. Studies have shown that forage yield of *T. plumosus* is variable and directly influenced by management practices and climatic conditions (Costa et al., 2011, 2013, 2014).

These species were favored by the early practices of ploughing and harrowing, which promoted the spread of their seeds. According to Santiago et al. (2007), some weeds are favored by the cultivation system, such as the morning glory (*Ipomoea grandifolia*).

Regarding the Fabaceae family, similar studies performed in the Cerrado of Roraima concluded that 87% of the diversity of species found belongs to this family (Flores and Rodrigues, 2010). Several other works on identification of weeds report the Fabaceae family as the most representative in diverse cultivated areas (Albuquerque et al., 2013, 2014; Gomes et al., 2014; Lima et al., 2014; Evangelista et al., 2015).

The floristic composition of the weed community and its distribution can be altered based on the dynamics between species, climatic conditions, cultural methods and soil management.

It is generally believed that C_4 plants are more efficient than C_3, however it depends on several aspects (Silva et al., 2007). C_4 plants require higher energy levels for the production of photoassimilates, as they have two carboxylative systems and therefore need to recover two enzymes for a new photosynthetic cycle. All energy comes from light; if C_4 plants do not have access to adequate levels of irradiance, they tend to be more affected by light energy competition than C_3 plants (Concenço et al., 2013).

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According to Ferreira et al. (2007), the liming practice generally favors the increase of noxious weeds in no-tillage systems, due to higher pH and calcium content in the topsoil.

According to Albuquerque et al. (2013), for the agrarian sciences professional to recommend the appropriate management system on a farm, he must have basic knowledge on several weed characteristics, including propagation type, life cycle, growth habit, photosynthetic route, as well as identification of species in the young phase.

Conclusion

No-till and minimum tillage systems have the lowest number of weed infestations.

Among the species collected in the area, the botanical families Poaceae, Fabaceae and Rubiaceae are the most representative.

The type of soil management system adopted has a direct influence on the weed community.

About relative frequency, the species *Cyperus* flavus does not stand out in native vegetation only.

Future studies are needed to improve weed control efficiency even in soil management systems with lower weed infestations.

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GENOTOXIC POTENTIAL OF SOILS IRRIGATED WITH TREATED WASTEWATER: CASE OF THE CEBALA-BORJ TOUIL IRRIGATED PERIMETER (TUNISIA)

SOUGUIR, D.[1*] – HÖRMANN, G.[2] – HALIS, S.[3] – JATOI, B. W.[4] – HACHICHA, M.[1]

[1]*Institut National de Recherches en Génie Rural, Eaux et Forêts, Université de Carthage, Ariana, Tunisia*

[2]*Department of Hydrology and Water Resources Management, Kiel University, Kiel, Germany*

[3]*Department of Agricultural and Biosystems Engineering, North Dakota State University, Fargo, ND, USA*

[4]*Chemistry Institute, Shah Abdul Latif University, Khaipur, Pakistan*

**Corresponding author*
e-mail: souguir.dalila@yahoo.com

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Abstract. In order to assess the effects of treated wastewater (TWW) irrigation on soil, a study was conducted in the TWW irrigated perimeter Cebala-Borj Touil, using the *Vicia*-micronucleus test and soil leachates. The study was carried out in 4 sub-perimeters (C_3^+, C_{10}^+, C_3^- and C_{10}^-) with different secondary effluent irrigations histories. The salinity of the soil leachates developed in two directions: (i) salinity decreased with increasing duration of TWW irrigation for C_3^+ and C_{10}^+; (ii) salinity increased with the longer time since the last TWW irrigation for C_3^- and C_{10}^-. The heavy metal contents differed between the studied leachates. Growth parameters and membrane integrity did not show significant differences between the plants exposed to the leachates and controls (C_0). However, the root tips treated with the leachates showed changes in mitotic activity, induction of micronuclei and appearances of chromosomal and nuclear abnormalities. The genotoxicity may be attributed to (i) individual effects of contaminants, and/or (ii) synergetic or antagonist interactions of different contaminants contained in the leachates. The micronucleus test seems to be a possible early warning system that allows the detection of soil genotoxicity, whereas growth parameters and membrane integrity did not show any variation during the 2-day exposure period.
Keywords: *abnormalities, heavy metals, micronucleus test, non-conventional water, salinity*

Introduction

Agriculture in arid and semi-arid Mediterranean regions relies mainly on irrigation. Since the water resources in these regions are scarce, the use of non-conventional water like wastewater effluent for an irrigation purpose is increasing more and more. Tunisia is a typical example for Mediterranean region with about 12 million inhabitants, a semi-arid climate and only 4.8 billion m^3 of renewable water resources (less than 450 m^3/inhabitant/year). An additional constraint in the country is salinity. About 10% of the soils and 50% of the water resources are impacted from the salinity with the concentration of 1.5 g/l or more (Hachicha, 2007).

Thus, to fight water scarcity, Tunisia has adopted a strategy to preserve its water sources, which includes the use of alternative water sources and the increased control of water demand in all socioeconomic sectors. Tunisia has a long experience in using so-called "non-conventional" water resources such as treated wastewater (TWW). Standards for the Tunisian wastewater treatment plant effluent discharge (TN 106.02

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revised) (JORT, 2018) and reuse in agriculture (TN 106.03, under revision) (INNORPI, 1989) have been established. In these standards, salt, heavy metal, and pathogen limits in the wastewater effluent were determined. In addition, a list of the plants that can be irrigated using wastewater effluent was set.

Reuse of effluents in agriculture can be a reliable alternative to cope with the irrigation water scarcity for farmers. However, long-term irrigation with non-conventional water may result in long-term gradual changes of soil physical properties and an accumulation of contaminants like salts and heavy metals. For the risk assessment of contaminated soils, guidelines based on physicochemical analyses were developed. However, this approach only covers the direct toxic effects of contaminants on living organisms, but not the complex synergetic or antagonistic effects. Therefore, simple physical and chemical analyses do not provide enough information about the complex effects of pollutants on organisms. Thus, biological tests using higher plants are necessary to test toxicity, the genotoxicity and the genome disruption. The use of plant bioassays for testing and monitoring soil and water pollution has many advantages: these organisms are easy to handle, do not require maintenance, have a wide applicability and are inexpensive (Grant, 1993).

The micronucleus assay using *Vicia faba* is a common genotoxicity test for environmental contaminants. It provides detailed information about abnormal cell division, micronucleus formation and chromosomal and nuclear damages. Chromosomal aberrations include chromosome structural aberrations (clastogenesis) and spindle malfunction affecting the chromosome number (aneugenesis). This bioassay has been used to assess the genotoxicity of contaminated soil, sediment, organic material, water, and industrial effluent (Smaka-Kincl et al., 1996; Sta et al., 2012; Renjana et al., 2013; Cotelle et al., 2015; Corrêa et al., 2016). It was used for several stress conditions such as heavy metals (Souguir et al., 2008, 2011; Yi et al., 2010), olive mill wastewater (El Hajjouji et al., 2007), pesticide (Sta et al., 2012) and diesel exhaust particules (Corrêa et al., 2016).

This study is part of a research program evaluating the impact of non-conventional water application on soil properties, which resulted in many research papers of Tunisian scientists. The most important subjects were the development of soil salinity, and the distribution of heavy metals after irrigation with both saline water and TWW, e.g. in Sfax, Zaouit Sousse, etc. (Klay et al., 2010; Kallel et al., 2012; Belaid et al., 2012; Ferjani et al., 2013). Other studies focused on xenobiotic organics, polycyclic aromatic hydrocarbons, polychlorinated biphenyles and organochlorinated pesticides in soils near Nabeul (Mahjoub et al., 2009; Haddaoui et al., 2016). However, as of our knowledge, this is a first study that use bioassays to assess the soil state after irrigation with wastewater effluent. The aim of our work was the use of the *Vicia*-micronucleus test to assess genotoxic effects of short and long-term irrigations with secondary effluents on the soil of Cebala-Borj Touil, the oldest and biggest irrigated perimeter in Tunisia.

Materials and methods

Study area

The study was carried out in the Cebala-Borj Touil area (*Fig. 1*), the largest irrigation area in Tunisia, with 3,200 ha of total 8,100 ha irrigated area (about 40%) with treatment plant effluent. It is located 8 km north of Tunis with an average rainfall of 450 mm/year; the rainy season extends from October to March. The groundwater is

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very shallow and unsuitable for irrigation due to high salinity levels. The effluents were supplied by the outflow of three wastewater treatment plants (WWTPs) of Great Tunis (Choutrana, Cherguia and Cotière Nord). These plants treat almost 75% of the total urban, domestic and industrial discharges of the City of Tunis. The WWTPs are equipped with primary and secondary treatment units to remove organic pollution.

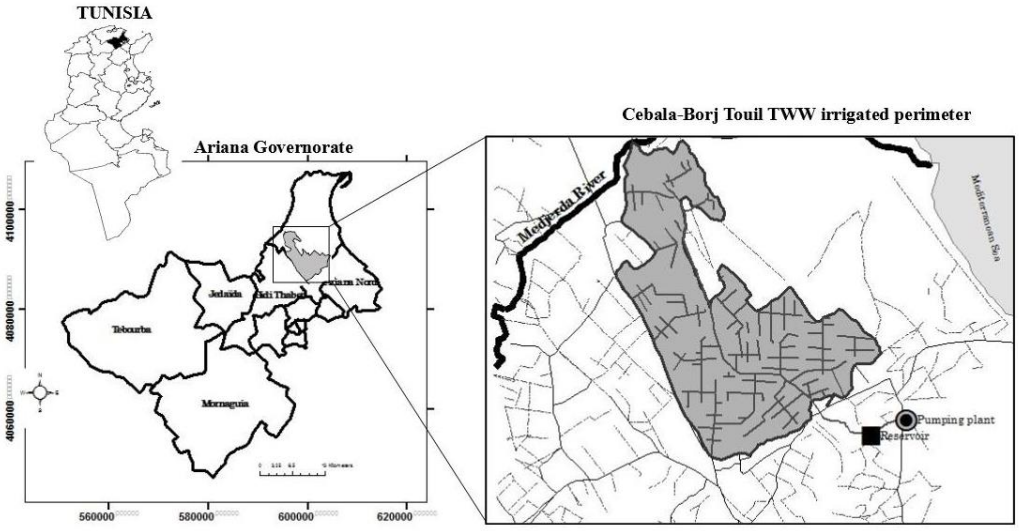

Figure 1. *Location of the Cebala-Borj Touil TWW irrigated perimeter*

Effluents from these plants are discharged to the Mediterranean Sea through the ONAS and Khelij canals. The wastewater effluent is pumped 4 km downstream of Choutrana to a buffer reservoir located 120 meters higher than the irrigated farms. Each year, only a small part of the water is used to irrigate the Cebala area, the rest flows directly to the sea. Irrigation with treated wastewater started in 1989. The method used is gravity irrigation, which causes a prolonged stagnation of water in the depressions, especially in the areas with high groundwater level. The fields are used for winter farming to grow cereals (wheat, barley, and hay) and fodder crops (berseem, green barley, and vetch-hay).

The characterization of treatment plant effluent

Table 1 lists annual averages of 2010, 2015, and 2017 of pH, salinity and chemical elements of the TWW used for irrigation and the Tunisian standard thresholds for the reuse of secondary effluents in irrigation (NT106.03). In 2017, the TWW was characterized by a basic pH (7.91) and an average salinity, measured by the electrical conductivity (EC) of about 5.03 dS/m with high contents of sodium and chloride. The content of trace elements does not exceed the values of the Tunisian standard, except for Cr. A comparison of the different years shows an increase in salinity and metal concentrations.

Soil sampling and extraction

The Cebala-Borj Touil perimeter is composed of many sub-perimeters with silty-clayey soils. The fine particles (clay + silt) exceed 80% and sand is about 17%. The

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content of organic matter is very low (about 0.7%) compared to high content of total carbonate of 41%, determined by ISO 10693.

Table 1. *Characteristics of the TWW used for irrigation in the Cebala-Borj Touil irrigation perimeter*

Parameter	Average			Standard[c]
	2010[a]	2015[b]	2017	NT106.03
pH	8.2	7.82	7.91	6.5-8.5
EC (dS/m)	3.70	4.02	5.30	7.0
Cl^- (me/l)	-	20.84	39.95	56.6
SO_4^{2-} (me/l)	-	8.86	8.72	-
HCO_3^- (me/l)	-	10.11	8	-
Na^+ (me/l)	-	21.78	28.61	-
Ca^{2+} (me/l)	-	3.17	12.16	-
Mg^{2+} (me/l)	-	5.33	9.33	-
K^+ (me/l)	-	0.92	1.07	-
Cd (mg/l)	0.002	0.010	0.002	0.01
Co (mg/l)	0.008	0.020	0.011	0.1
Cr (mg/l)	0.003	0.020	0.117	0.1
Cu (mg/l)	0.008	0.020	0.010	0.5
Fe (mg/l)	0.048	0.210	0.078	5.0
Mn (mg/l)	0.006	0.080	0.036	0.5
Ni (mg/l)	0.022	0.030	0.038	0.2
Pb (mg/l)	0.08	0.030	0.037	1.0
Zn (mg/l)	0.032	0.030	0.009	5.0

[a]Technical report (2010-2014)
[b]Dahmouni et al. (2019)
[c]Tunisian standard for TWW reuse in irrigation (NT106.03)

For this study, 4 sub-perimeters were chosen. The parcels have different TWW irrigation histories: The C_3^+ and C_{10}^+ parcels have been irrigated with TWW for 3 and 10 years, respectively. The C_3^- and C_{10}^- parcels were initially meant to be irrigated with TWW for 12 years, but the supply has stopped 3 and 10 years ago, respectively. Irrigation was stopped for several reasons: the guidelines prohibited cultivation of vegetables and cash crops in TWW irrigated areas and young people were not interested any more to work in the agricultural sector. For a correct assessment of the soil TWW effect, a non-irrigated field C_0, with silty-clayey soils and without any irrigation with secondary effluents, was selected near the studied fields. It was used to determine the background values for pH, electric conductivity (EC), heavy metals, and genotoxicity. Soil samples were taken in February 2017 after a rainy period (14 mm) from 0 - 40 cm using a Dutch auger. A composite sample was obtained from three sub-samples collected from each plot. The 6 kg mixed soil sample was air-dried and sieved at 2 mm. The leachate was produced with deionized water with a liquid-to-soil ratio of 1:1. After shaking with 120 rpm for 8 h, the soil solution was centrifuged with 3,500 rpm for 10 min and then the supernatant was filtered. The leachate was immediately used for the physicochemical analysis and the plant treatments.

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Leachate analysis

pH and electric conductivity (EC) have been respectively measured with a pH-meter Lutron pH 211-type and a conductimeter Cond./TDS, AZ 8361-type. Chloride was measured with the silver-nitrate method and sulfate with the nephelometric method using barium chloride. Bicarbonate was determined after titration with sulfuric acid. Sodium and potassium were analyzed by flame emission spectroscopy (Jenway, PFP7) and calcium and magnesium were determined with the EDTA-complexometric titration method. Heavy metals in the leachate were analyzed by atomic absorption spectrometry (Perkin Elmer).

V. faba treatments

V. faba seeds (Tunisian Chahbi variety) were surface sterilized with 10% sodium hypochlorite, rinsed several times with water and placed on moistened filter paper at 25 °C for 3 - 4 days. Subsequently, the roots were exposed to the 5 soil leachates with three replicates. After 48 h of exposure, the seedlings were rinsed a few times with distilled water and divided into three groups: the first was used for growth measurement; the second to determine membrane integrity and the third for the micronucleus test.

Growth parameters

Control and treated seedlings were separated into roots and hypocotyls for the measurement of root length, fresh and dry matters. Root length was determined by measuring the length of the whole root after the leachate treatments. For each treatment, the length of, at least, 10 roots were measured. Dry matter was determined after drying the fresh parts of 27 plants in an oven at 65 °C for 2 to 3 days until constancy weight.

Assessment of the loss of integrity of the plasma membrane

The loss of the plasma membrane integrity was evaluated with three root tips by a spectrophotometric test with Evans blue as described by Souguir et al. (2011). The first centimeter of the roots was incubated in 0.025% Evans Blue (m/v) for 30 min, rinsed for 15 min and squashed in a 800 µl solution of 50% MeOH (v/v) and 1% sodium dodecyl sulphate (SDS). The root extracts were then incubated for 15 min at 50 °C. The homogenate was centrifuged at 14,000 rpm for 15 min. The optical density of the supernatant was determined spectrophotometrically at 600 nm. 9 roots were used to assess the plasma membrane integrity under each treatment.

Genotoxicity assessment

The genotoxicity of the leachate was tested with the micronuclei of the meristematic roots of *V. faba*. The root tips were cut, placed overnight in Carnoy's fixation solution containing ethanol and glacial acetic acid (3:1), and stored in the dark in 70% of ethanol before being hydrolyzed with 1N HCl as described by Souguir et al. (2008). For each treatment, at least 12 roots were used. The root meristematic tissues were stained with orcein, squashed between a slide and a coverslip, and finally examined under a research microscope (Leica DM2500) with 40 or 100 times magnification.

Three slides were prepared for each of the three replicates and a total of 9,000 cells were observed from nine separate slides per treatment. Mitotic cells were expressed as

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the number of dividing cells in 1,000 observed cells. Micronuclei were observed in both interphasic and mitotic cells. Only micronuclei observed in interphases were counted and expressed in terms of micronuclei per 1,000 interphase cells.

The cells were also scored for micronuclei in dividing cells, clastogenic (bridge, fragment and stickiness,) and aneugenic (non-disjointed and isolated chromosomes) abnormalities. The number of each abnormality was expressed per 100 cells. The preparations were photographed with a digital camera (Canon EOS 1100) attached to the microscope.

Statistical analysis

The results are presented as the means ± standard deviation (SD) obtained from three replicates. All the treatments were compared to the leachate of the site C_0. Significant differences were determined by Tukey's test at the 0.05% confidence level with the SPSS software (IBM SPSS statistics, v20).

Results

Leachate characterization

pH, salinity, and ionic composition of the leachates are shown in *Table 2*. The pH was basic from 7.75 to 8.19 with no difference between irrigated and non-irrigated soils (8.04). The salinity of soils irrigated with secondary effluents was higher compared to the 0.69 dS/m of the control soil (C_0). Two trends are visible: (i) On soils with continuous irrigation, the salinity decreased with increasing irrigation duration. The soil with 3 years of continuous TWW irrigation showed an $EC_{1:1}$ (2.03 dS/m) higher than soils receiving TWW for 10 years (1.07 dS/m); (ii) Salinity increased in periods without a TWW irrigation from about 3.5 dS/m on C_3^- to 5.03 dS/m after 10 years on C_{10}^-.

Table 2. *pH, salinity and ionic composition of leachates*

Parameters	C_0	C_3^+	C_{10}^+	C_3^-	C_{10}^-
pH	8.04 ± 0.02a	8.0 ± 0.00a	8.19 ± 0.00a	8.03 ± 0.01b	7.75 ± 0.03a
EC (dS/m)	0.69 ± 0.02a	2.03 ± 0.00c	1.07 ± 0.00c	3.50 ± 0.01b	5.03 ± 0.02d
Ionic composition					
Cl^- (me/l)	3.29 ± 1.62a	8.46 ± 0.00b	1.88 ± 0.81b	9.40 ± 0.81a	36.19 ± 4.07c
HCO_3^- (me/l)	4.66 ± 0.28b	8.00 ± 1.32ab	5.83 ± 0.28bc	5.50 ± 0.50c	3.0 0 ± 0.50a
SO_4^{2-} (me/l)	7.46 ± 0.62ab	8.64 ± 1.85b	5.50 ± 2.22b	9.82 ± 0.56a	25.98 ± 0.52c
Na^+ (me/l)	3.75 ± 0.22a	11.90 ± 3.90b	5.55 ± 0.34ab	8.71 ± 0.09ab	36.30 ± 5.28c
Ca^{2+} (me/l)	2.66 ± 0.28a	4.33 ± 2.02a	2.33 ± 0.28a	3.00 ± 0.50a	10.66 ± 2.75b
Mg^{2+} (me/l)	3.66 ± 1.25ab	2.83 ± 1.52ab	1.83 ± 0.76b	5.66 ± 0.28a	10.33 ± 2.02c
K^+ (me/l)	0.02 ± 0.00a	0.35 ± 0.06d	0.05 ± 0.00d	0.14 ± 0.00b	0.07 ± 0.01c

C_0: never irrigated; C_3^+: irrigated for 3 years; C_{10}^+: irrigated for 10 years; C_3^- not irrigated for 3 years and C_{10}^-: not irrigated for 10 years. Values are mean ± SD. Different letters indicate significant differences at $P < 0.05$ according to Tukey's test

The leachates were also analyzed for trace elements (*Fig. 2*). For the soil C_0, the Co and Cd contents were lowest, the concentrations of Cu ranged from 0.005 to 0.009 mg/l, for Ni and Pb from 0.000 to 0.007 mg/l, and for Cr from 0.001 to 0.014 mg/l. The Mn

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and Fe contents were highest in C_0 reaching 0.018 mg/l Mn and 0.5 mg/l Fe. Heavy metal contents in irrigated soils were higher than in C_0. The highest ranges of Cd, (0.010 - 0.017 mg/l) and Fe (0.762 - 0.872 mg/l) were observed in C_{10}^+ while the highest concentrations of Cu, Co, Pb, Ni and Cr were detected in C_{10}^-. In this soil leachate (C_{10}^-), the heavy metal contents varied between 0.010 and 0.017 mg/l for Cu, between 0.005 and 0.013 mg/l for Co, between 0.021 and 0.044 mg/l for Pb and Ni, and between 0.124 and 0.161 mg/l for Cr.

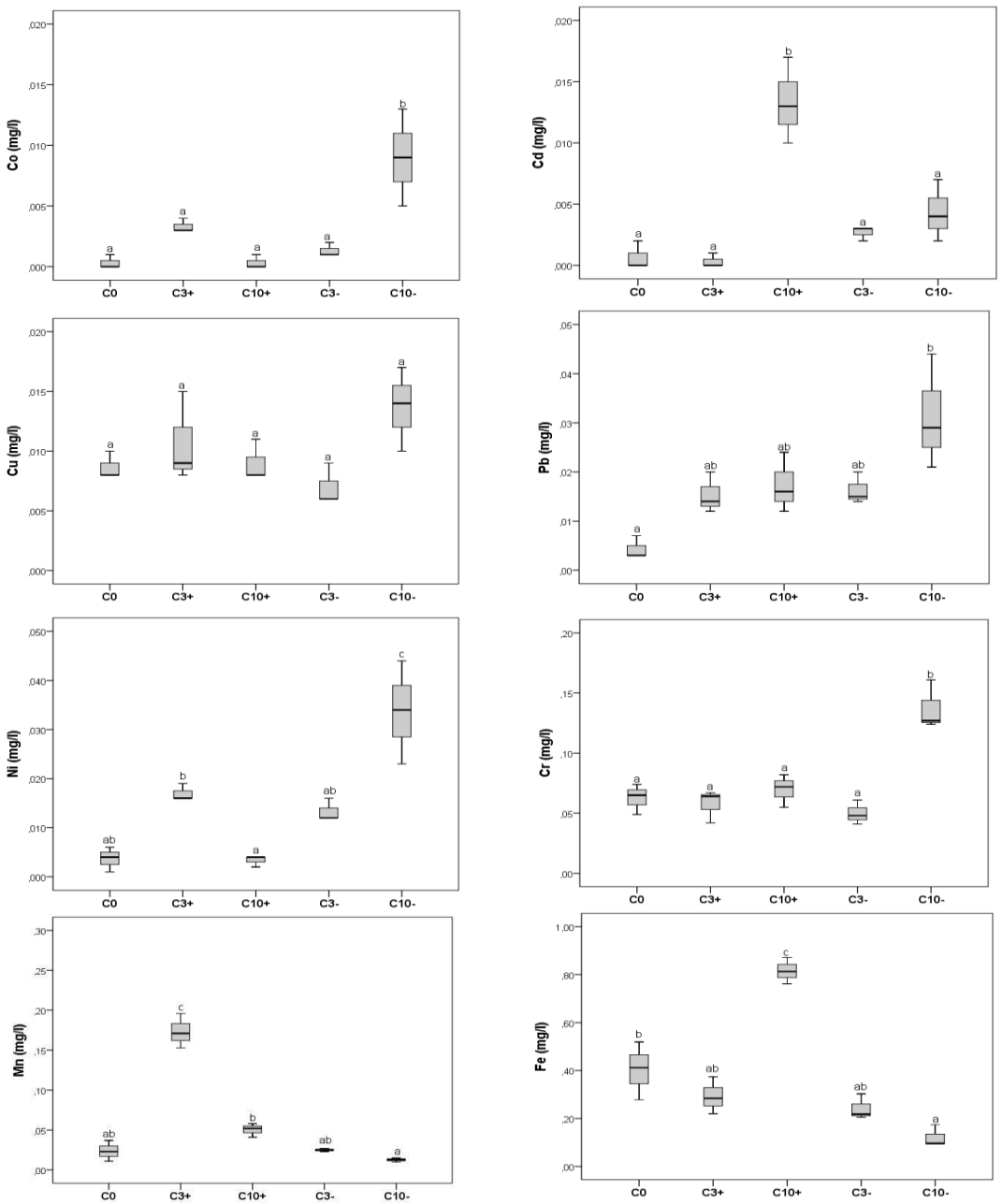

Figure 2. *Boxplots for the heavy metal compositions in soil leachates. The horizontal lines within the boxes represent the median values. C_0: soil never irrigated with the TWW; C_3^+ and C_{10}^+: soils irrigated for 3 and 10 years, respectively; C_3^- and C_{10}^-: soils not irrigated for 3 and 10 years, respectively. Different letters indicate significant differences at $P < 0.05$ according to Tukey's test*

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Growth parameters and membrane integrity

The *V. faba* growth parameters were recorded after 48 h of exposure to the leachates. The root length measurements did not show significant differences between control and treated plants (*Table 3*). Likewise, fresh and dry matter and the area of the roots were not different from the control.

Table 3. *Growth parameters of the V. faba seeds exposed to different leachates*

Parameters	C_0	C_3^+	C_{10}^+	C_3^-	C_{10}^-
Root length (cm)	3.10 ± 0.60a	2.80 ± 0.50a	3.07 ± 0.29a	2.70 ± 0.50a	3.60 ± 0.50a
Fresh matter (g)					
Root	0.41 ± 0.05a	0.45 ± 0.00a	0.40 ± 0.04a	0.46 ± 0.03a	0.42 ± 0.05a
Hypocotyl	0.25 ± 0.06a	0.20 ± 0.07a	0.23 ± 0.06a	0.23 ± 0.05a	0.17 ± 0.04a
Dry matter (g)					
Root	0.02 ± 0.00a	0.02 ± 0.00a	0.02 ± 0.00a	0.03 ± 0.00a	0.02 ± 0.00a
Hypocotyl	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a

C_0: soil never irrigated with the TWW; C_3^+ and C_{10}^+: soils irrigated for 3 and 10 years, respectively; C_3^- and C_{10}^-: soils not irrigated for 3 and 10 years, respectively. Values are means ± SD. Different letters indicate significant differences at $P < 0.05$ according to Tukey's test

The leachates did not influence the plasma membrane integrity (*Fig. 3*), measured by Evans blue absorption.

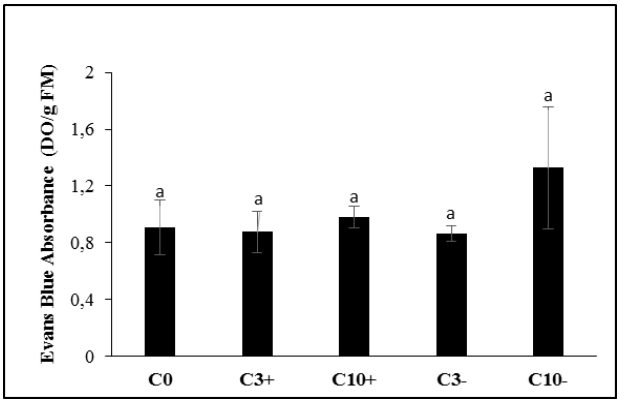

Figure 3. *Plasma membrane integrity as determined by the Evans blue absorption of the V. faba root extracts while exposed to the soil leachate. C_0: soil never irrigated with the TWW; C_3^+ and C_{10}^+: soils irrigated for 3 and 10 years, respectively; C_3^- and C_{10}^-: soils not irrigated for 3 and 10 years, respectively. Values are means ± SD. Same letters indicate no significant differences at $P < 0.05$ according to Tukey's test*

Genotoxicity assessment

Figure 4 depicts the effect of the leachates on cell division and micronucleus induction. The response of the mitotic cycle to the leachates was different. C_0 cells examined under microscope showed 37 divided cells per 1,000 counted cells. The frequencies of the mitotic phases increased significantly in C_{10}^+ and C_3^-. The highest value was observed in C_{10}^+ (53 divided cells per 1,000 counted cells). However, in

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soils receiving irrigation for 3 years (C_3^+) and those without TWW irrigation for 10 years (C_{10}^-), the values dropped to less than 25 divided cells per 1,000 counted cells in C_{10}-.

Concerning micronucleus formation, only cells in interphase with one micronucleus were scored (*Fig. 4*). The control soil showed an induction of 30 micronuclei per 1,000 cells. The micronuclei formation in C_3^+, C_{10}^+ and C_3^- was increased by a factor of 2 to 3 compared to C_0. C_{10}^- was the only treatment with a micronuclei formation similar to C_0.

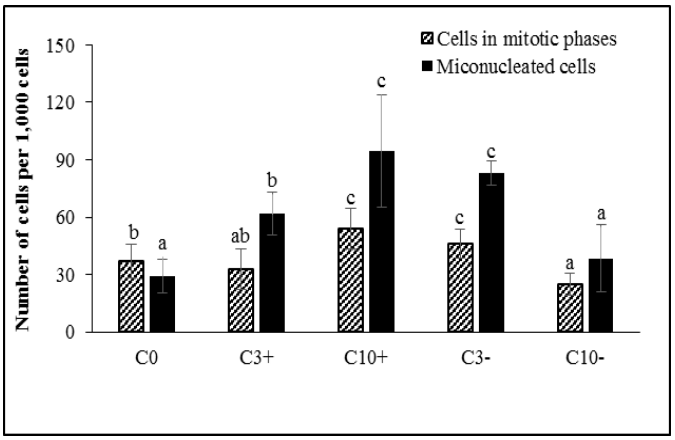

Figure 4. *Number of cells in mitotic phases and micronucleated cells recorded between 1,000 cells in V. faba roots under exposure to the leachates. C_0: soil never irrigated with the TWW; C_3^+ and C_{10}^+: soils irrigated for 3 and 10 years, respectively; C_3^- and C_{10}^-: soils not irrigated for 3 and 10 years, respectively. Values are means ± SD. Different letters indicate significant differences at $P < 0.05$ according to Tukey's test*

Simultaneously to the micronucleus formation, more or less similar types of aberrations were noticed as a response to the treatment with leachates (*Fig. 5*). The clastogenic aberrations include chromosome bridges, fragments and stickiness. Bridges were the most common structural aberration found in all treatments with values from 0.6 to 1.1 aberrations in 100 counted cells. Fragments were also noted in the anaphases and the telophases (*Fig. 5*) and stickiness, the less common clastogenic aberrations (0.04 - 0.37%), were only observed in the prophases and the metaphases.

Aneugenic aberrations linked to the disfunction of the mitotic spindle included vagrant chromosomes, multipolar division and lagging chromosomes. In our study, we presented only lagging chromosomes aberrations, enclosing non-disjointed and isolated chromosomes (*Fig. 5*). The non-disjointed chromosomes frequency varied between 1 and 1.54%, while isolated chromosomes ranged between 0.7 and 1.3%.

The nuclear abnormalities like buds, lobulated nuclei, irregular shapes and pyknoses were also observed in cells exposed to the leachate (*Fig. 6*).

Discussion

In order to assess the effect of irrigation with secondary effluents on soils, 4 parcels with 4 different irrigation histories were chosen: 3 and 10 years of continuous irrigation (C_3^+ and C_{10}^+, respectively) and 3 and 10 years after TWW irrigation arrest (C_3^- and C_{10}^-, respectively). In addition, a soil near the studied parcels never irrigated

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with TWW was used as control (C_0). All analyses were based on the leachate extracted from the surface layer of the soils. The leachate method allows the detection of the genotoxic and/or mutagenic chemicals which are not adsorbed by the solid particles (Cotelle et al., 2016).

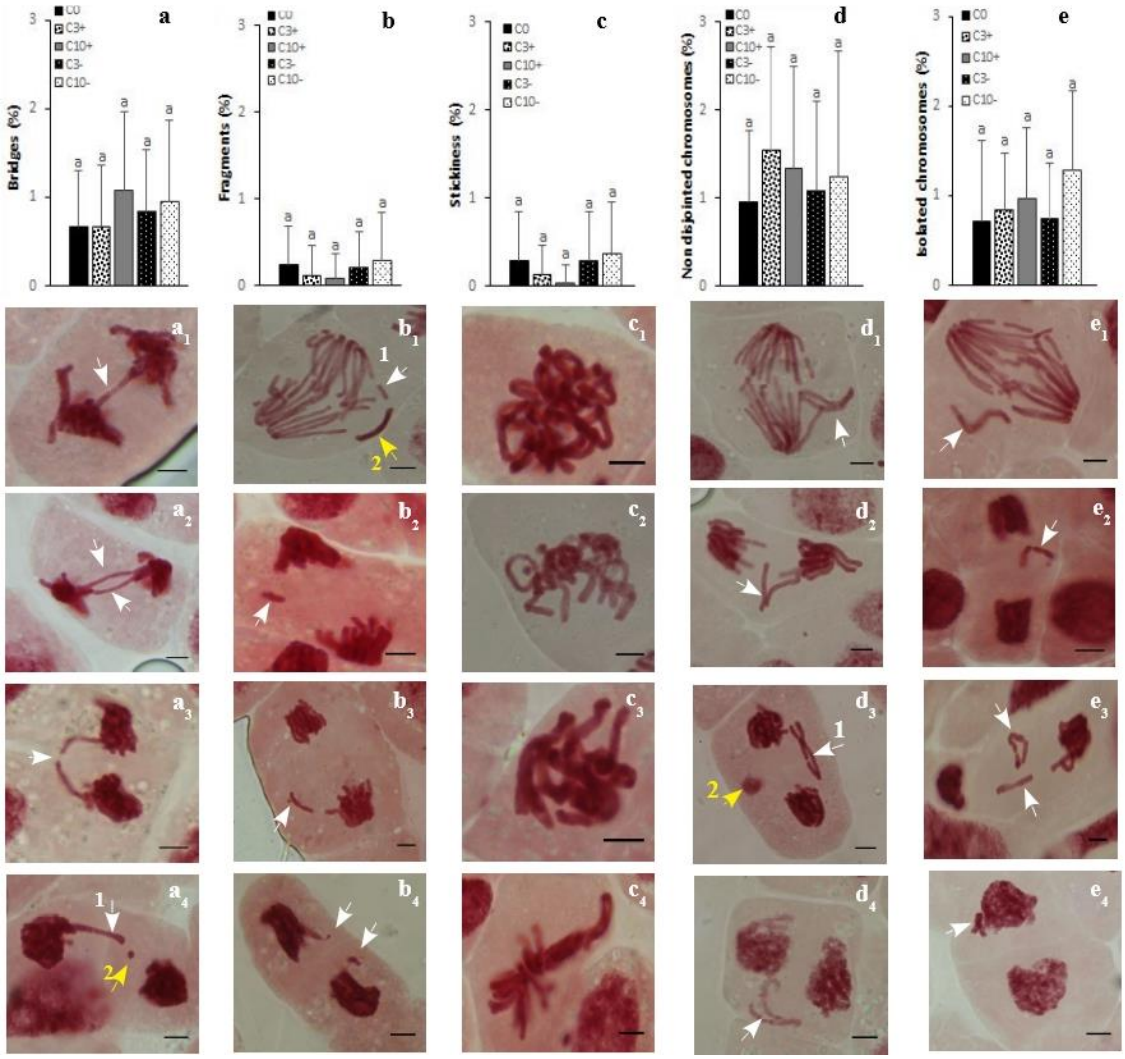

Figure 5. *Abnormalities induced in the root-meristematic zone of the V. faba while exposed to the leachates of soils irrigated with the TWW in the Cebala-Borj Touil perimeter. Clastogenic abnormalities include bridges, fragments and stickinesses. (a) the percentage of bridges; (a_1) the bridge in anaphase; (a_2) two bridges in anaphase; (a_3) the bridge break; (a_4) the bridge break (1) with a chromosome fragment (2). (b) The percentage of fragments; (b_1) the fragment (1) and the isolated chromosome (2) in the anaphase; (b_2-b_3)– the fragment in telophase; (b_4) two fragments in the telophase. (c) The percentage of stickiness; (c_1) the stickiness in prophase; (c_2) the stickiness in the beginning of the telophase; (c_3) the stickiness in the metaphase. The aneugenic abnormalities were represented by laggings. (d) The percentage of the non-disjointed chromosomes; (d_1-d_2) the non-disjointed chromosomes in the anaphase, (d_3) the non-disjointed chromosome (1) and the micronucleus (2) in the telophase; (d_4) the non-disjointed chromosome. (e) The percentage of the isolated chromosomes; (e_1) the isolated chromosome in anaphase; (e_2) the isolated chromosome in the telophase; (e_3) two isolated chromosomes in the telophase; (e_4) the isolated chromosome in the telophase ending. In a, b, c, d, and e, values are means ± SD. Different letters indicate significant differences at $P < 0.05$ according to Tukey's test*

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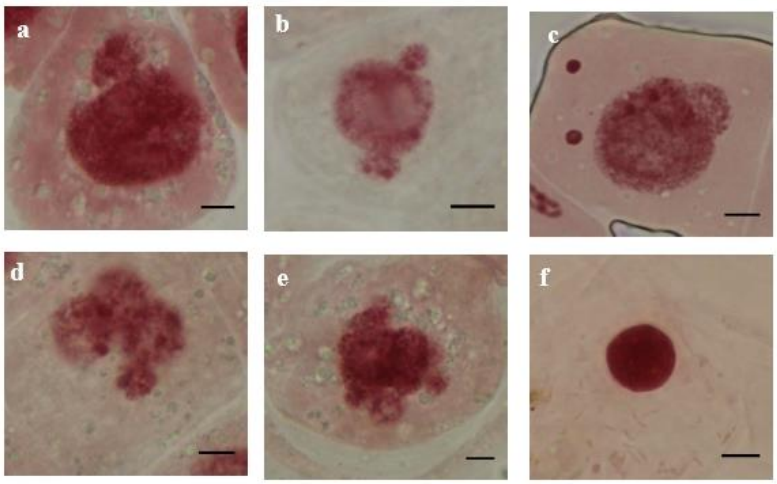

Figure 6. *Nuclear abnormalities induced in the root-meristematic zone of V. faba under exposure to the leachates of soils irrigated with the TWW in the Cebala-Borj Touil perimeter. (a) The nucleus with a bud; (b) the nucleus with two buds; (c) the bimicronucleated cell with a budding nucleus; (d-e) the lobulated nuclei with irregular shape; (f) the pyknosis (magnification: 1000x)*

Most Tunisian studies showed an increase of salinity after irrigation with wastewater effluents (Klay et al., 2010; Belaid et al., 2012). The high salt content of Tunisian TWW is also an effect of the high initial salinity of the water used for irrigation. The water used for domestic purposes (drinking water) reached already 2.3 dS/m (1.5 g/l). The salinity of the secondary effluent is also influenced by side effects like the treatment plant location which may be near a salty lake and the sea or the infiltration of salty groundwater into the sewer network (Bahri, 2002). The long-term effects of irrigation with secondary effluents on calcisol fertility were analyzed by Belaid et al. (2012) for a soil near Sfax. They found that the 15-year irrigation period added a significant amount of ions and increased the soil salinity up to 4 dS/m, even after the improvement of the TWW quality. Another investigation near Zaouit Sousse also revealed an increase of the soil salinity during the irrigation period (Klay et al., 2010). The depth profile showed an increase of salinity from top to the bottom (120 cm) with high levels in the deep horizons due to the downward salt transport caused by irrigation and winter rainfall.

In the irrigated perimeter Cebala-Borj Touil, the surface layer salinity is different. Despite the sampling after a rainy period with salt leached to the bottom of the soil, the $EC_{1:1}$ measurements exhibited different trends between soils with continuous irrigation and others where irrigation was stopped. Even irrigation with high salinity of TWW (5.26 - 5.36 dS/m) decreased the $EC_{1:1}$ of the leachate from 2.03 dS/m after 3 years of TWW irrigation to 1.07 dS/m after 10 years. On the plots where TWW irrigation was stopped 10 years ago, salinity increased to 5 dS/m. These salinity trends are probably related to the shallow and very saline groundwater with levels of 12 and 14 dS/m (2014 - 2015) (Dahmouni et al., 2019) and an average groundwater level of less than 150 cm. During rainfall events, it can reach the surface because of insufficient drainage. Soil salinity may be significantly reduced after irrigation, because of leaching of salts to the lower horizons. In our case, the TWW reuse seems to decrease the soil salinity. This theory was supported by the second salinity tendency, which highlighted increases of the EC after TWW irrigation was stopped.

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In addition to salt, secondary effluents can contain significant amounts of toxic metals which can accumulate up to critical levels in the soil. The heavy metals of the leachates were compared to the C_0 soil used as control. We found an increase of heavy metals in soils under long-term irrigation with TWW compared to the non-irrigated soil (C_0). Our results also confirm the findings of Khaskhoussy et al. (2015) about the effect of summer irrigation with TWW on Cebala soils. They found a significant increase of Zn, Co, Cu, Cd, Pb and Ni contents in 0 - 120 cm depth after the application of TWW. Klay et al. (2010) carried out another investigation of the development of the total load of heavy metals after long-term irrigation with the Sousse secondary effluent. The heavy metal contents of this effluent, used for irrigation during 14 years, were relatively low except for Cd and Pb, which exceeded the Tunisian guideline values (TN106.03) and therefore accumulated preferentially in the deep horizons. The mobility of the heavy metals and their vertical distribution are closely related to the soil physicochemical parameters such as pH and salinity and to the quality of the irrigation water. In our study, the highest contents of Cd and Fe were found in the leachate of the soil with the longest TWW irrigation (C_{10}^+), while the highest contents of Cu, Co, Pb, Ni and Cr were found in the leachate of the soil where irrigation with TWW stopped 10 years ago (C_{10}^-), and where salinity was highest. It has been reported that increasing salinity reduces the organic complexation of the majority of metals as an effect of the increasing competition for available sites (Mantoura et al., 1978). This induces the solution from the solid phase and the leaching to the groundwater. The contamination of the Cebala groundwater and drainage water with heavy metals has been reported. Works, conducted by Dahmouni et al. (2018) and Dahmouni (2019), showed higher levels of Cd, Cr, Co and Pb levels compared to the TN106.02, Tunisian standard relative to the discharge of the wastewater effluents. This standard fixes the maximal levels of Cd, Co, Ni and Pb on 0.005 mg/l, 0.01 mg/l, 0.2 mg/l and 0.1 mg/l, respectively.

In this work, the analysis of the physicochemical proprieties of the leachates was completed by a genotoxic study with a *V. faba* root-micronucleus assay. This test is a widespread and reliable technique in ecotoxicology to assess the contamination of matrices like waters and soils (Zhang et al., 2004; Song et al., 2007; Cotelle et al., 2016; Souguir et al., 2019). The micronucleus test can be performed with pH values ranging between 3.5 and 9.0 for a more accurate evaluation of the chemical genotoxicity (Dyèvre et al., 2014). Up to now, no test of the TWW effect on the soil has been carried out with this method in Tunisia. Contamination risks were only evaluated based on physicochemical properties (Klay et al., 2010; Belaid et al., 2012). First, *V. faba* growth parameters and membrane integrity were measured. Root length, weight of fresh and dry matters of the plant organs and the Evans blue absorption did not show significant differences between the plants exposed to leachates and the control C_0. However, the root tips showed genotoxic effects of the leachates, like changes of cell division, micronuclei, and abnormal chromosomes and nuclei.

The mitotic activity increased significantly in the roots exposed to C_{10}^+ and C_3^- compared to the control C_0. Such effects were observed under a low concentration of the NaCl by Radic et al. (2005) who found that a salt concentration of 150 mM markedly increased the mitotic activity in the cell-root tips of *Centaurea ragusina.* However, Teerarak et al. (2009) proved that all NaCl concentrations between 40 and 160 mM inhibited cell division in the root tips of *Allium cepa*. A decline of the mitotic activity was also noticed especially in C_{10}^- compared to C_0, which may be attributed to

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the mitotic inhibition by contaminants in the leachate. They prevent or block the formation of various metabolites necessary for a normal sequence of mitosis. The reduction could also be caused by the blocking of the G2-phase in which tubulin is required for the formation of the mitotic spindle (Mahoney et al., 2006).

The C_0 genotoxicity may result from the whole (antagonist/synergic) effects of contaminants present in this soil. The TWW irrigation seems to increase the micronucleus formation in soils with 3 and 10 years of TWW irrigation 2 to 3 times higher than the C_0 soil. The highest level of micronucleus was observed after 10 years of irrigation in soil with low salinity and high concentrations of Cd and Fe. The end of TWW irrigation 3 years ago still maintained genotoxic character of the soil through a high induction of micronucleus. However, the end of TWW irrigation 10 years ago did not influence the micronucleus formation and no significant difference was found between C_0 and C_{10}^-, apparently because of the low level of cells in mitotic division which also decreased the formation of micronuclei. The C_{10}^- leachate had the highest concentrations of Cu, Co, Pb, Ni and Cr. The sensitivity of the micronucleus assay to the heavy metals is well studied and many authors reported that the micronucleus frequency increased with increasing heavy metal concentrations and until it reaches a maximum beyond which fewer micronuclei were formed (Godet et al., 1996; Zhi-gang and Qiao-gu, 2009; Foltête et al., 2012). Wang (1999) found a positive correlation between the micronucleus frequency and Cr contents in Cr-contaminated soils. Other positive correlations were found between the micronucleus induction and Cu, Cd, Co and Fe concentrations in paddy soils taken from an area where electronic waste was deposited (Jun-hui and Hang, 2009). C_{10}^- was also the leachate with the highest salinity (5.03 dS/m). The genotoxic character of salts has been previously studied by Teerarak et al. (2009) who showed the existence of micronucleus and chromosomal abnormalities under NaCl treatments. Recently, the higher incidence of micronuclei and the various types of chromosomal and nuclear aberrations were also investigated under either direct exposure to NaCl solution or indirectly through the leachate of a salty soil in the *V. faba* root tips (Souguir et al., 2017, 2018).

The slides prepared for the mitotic activity assessment and the micronucleus formation were used to detect the chromosomal and nuclear abnormalities in the root tips exposed to the leachates. The clastogenic aberrations included chromosome bridges, fragments and stickiness. The bridge was the most important structural chromosomal abnormality. It was found in all treatments and it is lethal for the cell (Hall and Garcia, 2006). It may be induced by some clastogenic substances in the leachates. Stickiness reflects a highly toxic and usually irreversible effect leading to cell death (El Ghamery et al., 2000). It is caused by DNA depolymerization, DNA condensation and the physical adhesion of the chromosomal proteins (Osterberg et al., 1984; Patil and Bhat, 1992). In our study, stickiness was the less abundant clastogenic aberration with values between 0.04 and 0.37%.

In addition to the clastogenic abnormalities, the contaminants in the leachates seem to interrupt or cause a malfunction of the spindle function, an inappropriate chromosomal segregation when daughter cells are formed. In our study, the spindle alteration was detected through the appearance of lagging chromosomes in the anaphase and the telophase. Lagging chromosomes included non-disjointed and isolated chromosomes. The daughter cells enclosing a lagging chromosome may be formed with unequally sized or irregularly shaped nuclei during the interphase (El Ghamery et al., 2003).

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Nuclear abnormalities are characterized by morphological alterations in interphasic nuclei. These alterations were observed in *V. faba* under treatments and considered as nuclei carrying nuclear buds, lobulated nuclei with irregular shape and pyknosis. Cells with nuclear buds contain nuclear bodies connected to the main nucleus by a thin nucleoplasmic bridge (Bolognesi et al., 2013). Nuclear buds may emerge from the elimination of exceeding genetic material derived from polyploidization (Fernandes et al., 2007; Bolognesi et al., 2013). Irregular nuclei were also observed by Fernandes et al. (2007), who suggested that nuclear altered morphology occurred before the nuclear buds formation. Such nuclear alterations (irregularly shaped nuclei and nuclear buds) were attributed to NaCl (Souguir et al., 2018), Pb, Cr and Cu ions (Liu et al., 1992, 1994; Abdel Migid et al., 2007). Pyknosis detected in cells exposed to the leachates was an irreversible condensation of chromatin in a nucleus of dying cells (Thomas et al., 2008).

The micronucleus test with soil leachates seems to be well suited to detect the genotoxicity, whereas growth parameters and the membrane integrity did not show any variation during the 2-day exposure period. A study of the sequential effects of Cd on genotoxicity and lipoperoxidation of *V. faba* roots during 48 h (Souguir et al., 2011) showed that the genotoxicity events occurred 12 h after Cd exposure and the prior plasma membrane integrity and the lipid peroxidation. The same metal was used in the study of Foltête et al. (2012) who found an early genotoxicity effect of a Cd-spiked soil, while the first signs of toxicity appeared in the *V. faba* after a 2- month exposure period.

The genotoxicity detected in the Cebala leachates may be attributed to (i) the individual effects of contaminants, and/or (ii) the effects of synergic or antagonist interactions. Contaminants included salts, heavy metals and many other substances contained in TWW but not identified in our study. In addition to the effects of salts and heavy metals on cell division, micronucleus induction and chromosomal and nuclear abnormalities, salinity strongly enhances the uptake of heavy metals by plants due to the increasing mobility in the soil solution, even in an agricultural soil with very low concentrations of metals. The increased mobility of heavy metals depends on the total amount in the soil and the type of salt. It is attributed to the complexation of the salt-derived anions with heavy metals and to the competition between salt-derived cations with heavy metal ions for sorption sites on the solid phase (Hatje et al., 2003; Acosta et al., 2011). Such an effect has been mainly studied with Cd. This element is one of the most dangerous metals due to its high mobility and to the low concentration at which it affects plants (Benavides et al., 2005; Rasheed et al., 2020).

The irrigation with TWW does not only add salts and heavy metals to soils, but also other contaminants from both domestic and industrial discharges. The transfer of the organic pollutants from the secondary effluents to the soils and their relative genotoxic effects has been broadly studied (Mahjoub et al., 2009; Haddaoui et al., 2016).

The genotoxicity observed in the root tips may also be due to the indirect effects of contaminants through the generation of free oxygen radicals or the alteration of the calmodulin synthesis or function (Ünyayar et al., 2006; Souguir et al., 2011). Calmodulin is specifically located in the mitotic spindle, it is involved in the movement of chromosomes and the control of polymerization and depolymerization of microtubules (Means and Dedman, 1980).

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Conclusion

This study investigates the effects of irrigation with TWW on cultivated soils using the micronucleus assay. The leachates from the first soil layer (0 - 40 cm depth) have different irrigation histories and exhibit different salinity levels, and various heavy metals. The genotoxicity was not accompanied by changes of growth parameters or membrane stability, which may confirm the role of the micronucleus test as a predictive biomarker of the harmful effects of long-term irrigation with the TWW on plants.

Two lessons can be learned from this work. The first concerns the agricultural use of TWW in semi-arid Mediterranean regions and the second relates to the use of genotoxicity biotest. Concerning the use of TWW, continue irrigation leads, even if standards are respected, to an increase in soil salinity and contamination by heavy metals which varies according to the metal. This evolution depends on the management of irrigation, whether continuous or intermittent. Despite the fact that, in our case, soil contamination would not be serious, more effort should be given to improve the quality of the secondary effluents at the outlet of the treatment plants and control soil and drainage system. For the use of the biotest, it proves very useful to evaluate in a thorough way, the risks on the crops before irrigation and during the irrigation cycles. It thus appears to be a powerful tool for the detection of anomalies that it would be recommended to integrate it into the control programs of the soils irrigated by TWW.

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STATISTICAL RESEARCH ON RAINFALL AND RIVER DISCHARGE PATTERNS OVER TIME FROM A HYDROLOGICAL PERSPECTIVE

MALARVIZHI, R.[1*] – RAVIKUMAR, G.[2]

[1]*Department of Civil Engineering, College of Engineering Guindy, Anna University, Sardar Patel Road, Chennai 600025, Tamilnadu, India.*

[2]*Centre for Water Resources, Department of Civil Engineering, College of Engineering Guindy, Anna University, Sardar Patel Road, Chennai 600025, Tamilnadu, India*
(e-mail: grk6617@gmail.com; phone: +91-98-4025-7426)

**Corresponding author*
e-mail: babloomalar15@gmail.com; phone: +91-80-1246-0783

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Abstract. Climate signals are the indicators of climate change evidence in a particular region or area, which can be witnessed through an erratic rainfall pattern showing less variability than usual which simultaneously affects the hydrological cycle of water systems in major. The behaviour of any weather pattern can be visualized by statistical analysis in combination with trend patterns. This study was made to draw the rainfall and river discharge variability of the Thamirabarani River, Tamilnadu, India through statistical analogy. The trend analysis was carried out by non-parametric Mann-Kendall, Spearman's Rho and Linear Regression Test for ten Rain Gauge stations and one Stream Gauge station from 1980 to 2018 (39 years). The results were interpreted for annual and seasonal patterns where seasonal includes South-west monsoon, North-east monsoon, Winter and Summer periods respectively. This statistical analysis of weather parameters is a preliminary investigation for further research, through hydrological simulations, predictions and scenarios a better management and planning of water resources can be achieved in the Thamirabarani River basin, Tamilnadu, India.
Keywords: *weather parameters, river basin, statistical test, trend analysis, temporal variation, spatial distribution, climate signals*

Introduction

Across the world, scientists now agree that there is climate change due to anthropogenic activities. The phrase climate change has to be a priority on the list while developing planning and management of natural resources. It creates inequitable burden to the poor and developing countries as well as impacting the world's water resources. The projected future climatic scenarios indicate the rise in global temperatures from 1.4 to 5.8 degrees Celsius. Therefore, the slight shift in climate patterns of the 21st century may be significant and disruptive whereas if it will be on the higher end of the spectrum it could be catastrophic. All the above framed contexts are the guidelines by IPCC SR15 report to be followed when it comes to research and investigations.

Rainfall is the major water resources for the earth which is collected in various forms as precipitation in various shapes and structures on the surface as well as ground sources without major losses. It is the fact that water is a naturally recycled resource but the spatial and temporal scales of its pattern has been changed in wide-range. For the sustainable development of planning and management of water resources primarily, the rainfall patterns have to be analyzed in different perspective. The accuracy in rainfall measurement have been increased by the scientific methods like remote sensing and

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automatic measurements etc. Before the validation, simulation, calibration and prediction of hydrological parameters such as rainfall and temperature, it is very necessary to understand the behavior of raw observed data. This real-time dataset statistical study has to be given precedence in any methodology with respect to water resources and its management. Because of the statistical analysis and trend prediction, the behavior of any weather parameter could be well understood in accordance with its periodical manner, extremities and also the causes. Rainfall and river discharge are interconnected as the water discharge is a dependent factor on the rainfall pattern, though both are separately observed in terms of data measurements. The impact of rainfall amount on river discharge shown through statistical analysis and trend prediction is the study to be discussed below along with the help literature review.

The precipitation trends of rainfall give immense results about the increase or decrease of trend pattern with the help of conventional Mann-Kendall, Mann-Kendall and Sen's slope estimator statistical hypothesis testing (Gajbhiye et al., 2016; Gocic and Trajkovic, 2013; Hussien et al., 2019). The trend prediction which can be attempted successfully through Mann-Kendall and Spearman's Rho Test of statistical analysis used to correlate the data in the form of monthly, seasonal and annual time series (Ahmad et al., 2015; Palanichamy and Sankaralingam, 2020). The spatial and temporal variation can be scaled down with the long-term rainfall records by the Mann-Kendall test that shows the trend patterns as well as the extremities (Anand and Karunanidhi, 2020). The statistical analysis can also be done with various number of hypothetical testing such as Linear Time series analysis, Mann-Kendall Z-statistics, Sen's slope estimator and Linear growth model along with combination of weather parameters for better understanding of the climate strategies (Bello et al., 2020; Pandit, 2016). The rainfall records or patterns of measurement not only show a trend in its variation but also conclude the abnormal conditions of the climate as signals for the long time period (Das and Tripathy, 2020). The prediction, fluctuations and anomalies can be investigated through trend patterns of rainfall which tends to accelerate the river discharge inferred through this spatio-statistical analysis of Auto Regressive Integrated Moving Average (ARIMA) approach (Dawood et al., 2020). The analysis of spatio-temporal trends of rainfall is done by Theil and Sen's Slope estimator test for rainfall magnitude as well as Inverse Weight Distance (IDW) through ArcGIS (Geographic Information System) software to predict the variation in trend patterns (Diop et al., 2016). The rainfall trend prediction through non-parametric Mann-Kendall and Sen's slope estimator testing shows significant variation that portraits the spatial scale by interpolation techniques in Quantum GIS (Geographic Information System) software for the management studies (Meshram et al., 2018).

The rainfall trend pattern sometimes helps us to understand the efficiency of result in terms of quantity like its impact on regional or global climate scales by the methods such as Mann Kendal's rank correlation statistics and wavelet analysis (Nikhil Raj and Azeez, 2012). Rainfall and temperature are the dependent factors that never fails to show their impacts on each other, which is evident through the geo-statistical and descriptive statistics and also the clear signature of change in climate through observed data will be making the adaptation and mitigation measures (Savo et al., 2012; Talib et al., 2021). The annual and seasonal trends have been predicted for the temperature of long-term dataset using Modified Mann-Kendall and CUMSUM statistical testing by 95% of confidence interval suggesting mitigation measures for the recovery of water resources (Singh et al., 2015). An accuracy rate increases when there is an update in dataset likewise the satellite data using Artificial Neural Network (ANN) will improve the trend prediction and spatio-

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temporal variations in different aspects in statistical testing (Sobral et al., 2020). The trend analysis of rainfall will be more informative when it is compared with statistical and graphical methods, also in parallel used to make decisions in terms of trend as increasing or decreasing pattern (Rathnayake, 2019). Presence of two or more variable predicts widely where at least one independent and two or more dependent variables will be leading to proper construction of trend patterns as well as the variation and changes happening in time-scales of climatic data (Nyokabi et al., 2017). The precipitation trend variation gradually affects the water discharge trends which are noticeable in the case of Mann-Kendall and Pettit abrupt statistical testing and then leads to double mass curve and regression analysis for the prediction of quantitative impacts of climate change (Li et al., 2020). With the one parameter like stream flow it is possible to predict the trend pattern through the statistical analysis, which will be helpful in decision making when it comes to spatial and temporal scales of urbanized watersheds (Bhaskar et al., 2020). The trend analysis of stream flow prediction with respect to monthly, seasonal and annual pattern of mean values showed the variations and also used in change point analysis (Kale and Sönmez, 2019). The spatio-temporal trends, variability and teleconnections are analyzed using both parametric and non-parametric statistical testing with the help of gridded rainfall data. As per the inference, the study will draw down the large scale impacts of Sea Surface Temperature (SST) through this trend analysis (Sah et al., 2021). The Rainfall and its runoff plays a major role with other hydro-climatic variables while the trend patterns of rainfall and runoff using Mann-Kendall test and Sen's slope estimator has taken into an account primarily for the prediction of annual and seasonal variations and its impacts on other hydrological parameters respectively (Solaimani et al., 2021; Alifujiang et al., 2021). Therefore, this study has been framed by authors to predict the trend pattern in spatio-temporal scales for Rainfall and River discharge in Thamirabarani River, Tamilnadu, India using Mann-Kendall, Spearman's Rho and Linear Regression test in reference to highlighted literature.

Study area

Thamirabarani River was selected as the focus element in this study. This river is one of the oldest systems in Tamil Nadu, India. The river is short but it is a perennial source in the Southern part of Tamilnadu, India. Irrigation development is a major source of income for the people living adjoining the Thamirabarani river. Hence, Thamirabarani River was chosen to examine the behavior of extremities with a reference to statistical perspective. It originates from the peaks of Pothigai hills on the Eastern slopes of the Western Ghats at an altitude of 2000 m and confluences with the Bay of Bengal at Gulf of Mannar, India. It is the lifeline for the people of Tirunelveli and Thoothukudi districts, Tamilnadu, India. The total area of the basin is 5,650 km^2, of which hilly portion is 688 km^2. The basin is situated between 8°21'N and 9°13'N Latitudes and 77°10'E and 78°8'E Longitudes. It enjoys the benefit of both the Southwest and Northeast monsoons as it receives supply from the rainfall over Western Ghats, India. The river traverses about 125 km through Thirunelveli and Thoothukudi districts in Tamil Nadu, India.

The catchment area is divided into seven sub-basins as Upper Thamirabarani, Lower Thamirabarani, Chittar, Gadananadhi, Uppodai, Manimuttar and Pachaiyar as presented on the map (*Fig. 1*). It has 33 rain gauge stations throughout its basin at present. Amongst that only 10 out of 33 rain gauge stations (*Fig. 2*) are selected for an analysis, due to the data reliability in observed rainfall measurements for a long-term period of 39 years. The

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selected rain gauge stations were spatially distributed throughout the basin without any bar adjustment in its topographic features which plays vital role in rain gauge station placements. In addition to this, there is presence of two full climatic stations one at upstream (Cheranmadevi), the other at the middle of the basin (Kalampatti) as well as one stream gauge station (Murappanadu) at downstream (*Fig. 2*). The one major critical point station named Murappanadu stream gauge was selected as it joins two major stream order at this point before the outlet of basin enters the Bay of Bengal. Also, the block map for Thamirabarani basin (*Fig. 3*) is represented below for the study reference.

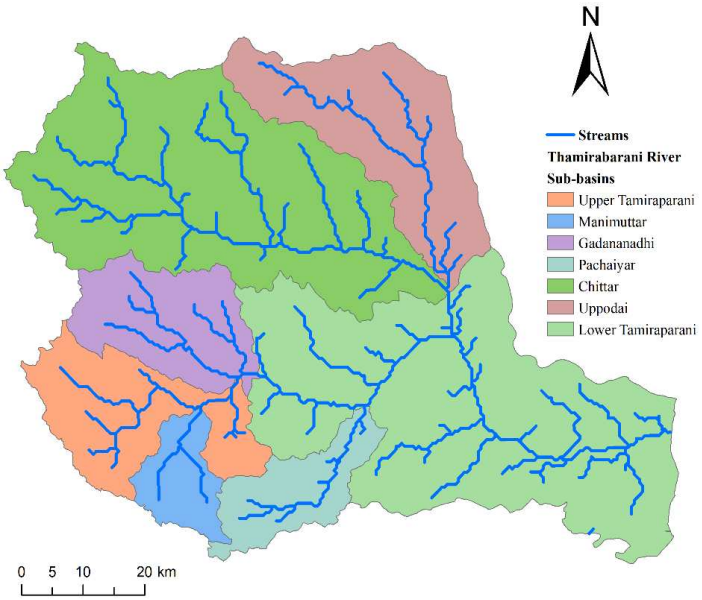

Figure 1. *Thamirabarani River and its sub-basins*

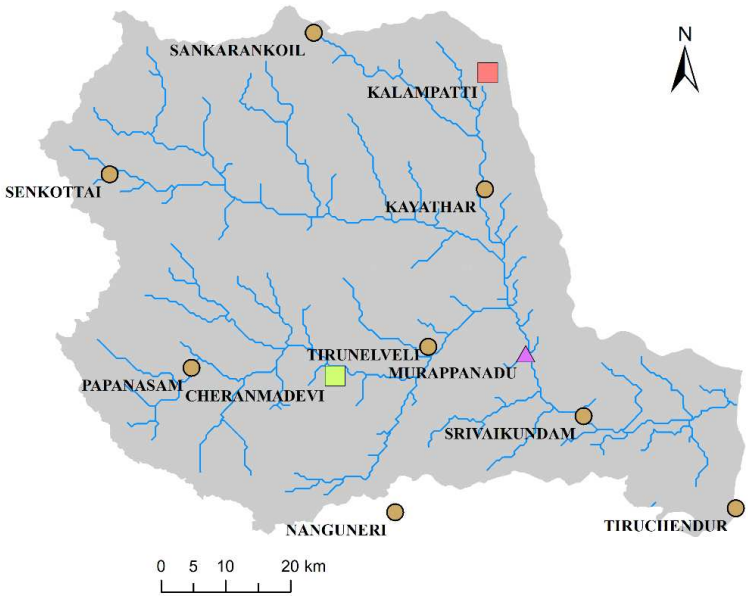

Figure 2. *Rain gauge station locations in Thamirabarani River basin*

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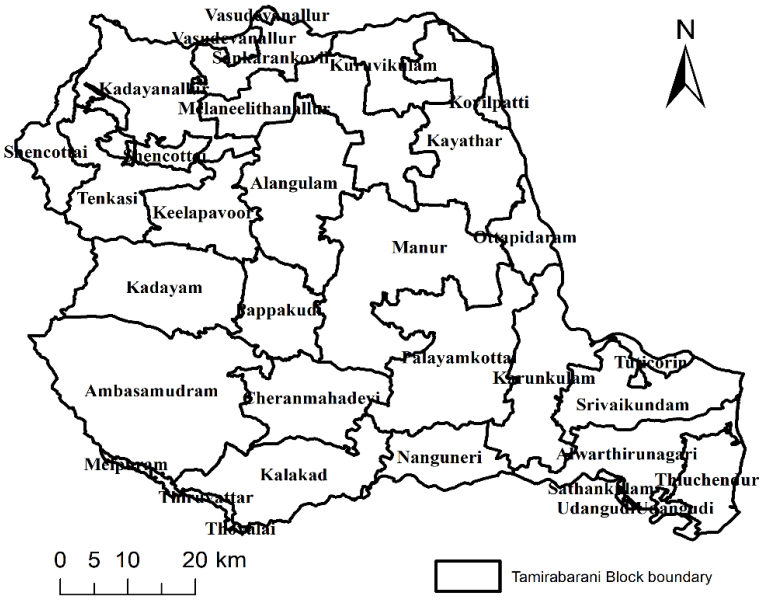

Figure 3. *Block boundaries in Thamirabarani River basin*

The rain gauge stations under respective sub-basins are listed out in *Table 1* and its salient features for the study area of Thamirabarani river basin is compiled in *Table 2*. As per the availability of raw observed data obtained from Central Water Commission (CWC), India and Institute for Water Studies (IWS), Taramani, and Chennai for 39 years (1980-2018), the rain gauge stations were chosen in accordance with the installation period and utilized below for the methodology.

Table 1. *Stream gauge and rain gauge stations under sub-basin zones*

Zones	Sub-basin	Rain gauge station
1	Upper Thamirabarani	Papanasam
2	Lower Thamirabarani	Murappanadu – Stream gauge station Srivaikundam Tiruchendur
3	Gadananadhi	Cheranmahadevi – Full Climate Station (FCS)
4	Manimuttar	Nanguneri
5	Pachaiyaar	Tirunelveli
6	Chittar	Senkottai
7	Uppodai	Sankarankoil Kalampatti - Full Climate Station (FCS) Kayathar

The Cheranmahadevi and Kalampatti are the only weather stations observing all the possible weather parameters for thamirabarani river basin maintained by Central Water Commission (CWC), India and the Papanasam station is installed with automatic rainfall measurements by Institute for Water Studies (IWS), Taramani and Chennai, India.

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Table 2. *The geographic details of Thamirabarani River basin*

S. No	Rain gauge station name	District	Tasil/Taluk	Latitude	Longitude	Altitude (masl)
1	Cheranmahadevi	Tirunelveli	Cheranmahadevi	08°41'17"	77°33'49"	63
2	Kalampatti	Thoothukudi	Kayathar	09°08'48"	77°47'23"	78
3	Kayathar	Thoothukudi	Kayathar	08°56'50"	77°46'33"	62
4	Nanguneri	Tirunelveli	Nanguneri	08°29'48"	77°38'47"	106
5	Papanasam	Tirunelveli	Ambasamudram	08°42'02"	77°21'42"	8
6	Sankarankoil	Tirunelveli	Sankarankoil	09°10'04"	77°32'12"	138
7	Senkottai	Tirunelveli	Senkottai	08°58'18"	77°14'54"	156
8	Srivaikundam	Thoothukudi	Srivaikundam	08°37'45"	77°54'44"	19
9	Tiruchendur	Thoothukudi	Tiruchendur	08°29'56"	78°07'30"	14
10	Tirunelveli	Tirunelveli	Manur	08°43'40"	77°41'41"	33
11	Murappanadu	Thoothukudi	Srivaikundam	08°43'01"	77°49'54"	26

Materials and methods

Rainfall and river discharge analysis

With the available monthly data, the rainfall is categorized for four seasons faced by the study area i.e. (I) Pre-monsoon (March – May) which is also referred as summer or mango showers, (II) Monsoon (June-September) as South-west monsoon, (III) Post-monsoon (October-December) as North-east monsoon and (IV) Winter (December – February). Therefore, these four seasons are grouped into Seasonal pattern and altogether as an Annual Rainfall pattern. This rainfall pattern is subjected to be applied for both rain gauge and stream gauge stations where the former measures rainfall in mm and the latter does river discharge in m^3/s. The preliminary factors of statistical test such as maximum, minimum, mean, standard deviation, variance, coefficient of variation, skewness and kurtosis were determined for both annual and seasonal datasets to study the behavior of spatial as well as temporal scale changes in the rainfall amount and pattern for the river basin. The statistical testing of river discharge data will predict the hydrological causes through the trend occurrence in the monthly, seasonal and annual scenarios.

Trend prediction

Mann-Kendall test

The Mann-Kendall test is a non-parametric (distribution-free) test which means there is no requirement of assumptions unlike linear regression analysis. This test is to assess whether the trend pattern is upward or downward monotonically over the period of time and is best viewed as exploratory analysis. It is most appropriately used to identify stations where changes are significant or of large magnitude and to quantify these findings. According to literature review, the best fitted method to do statistical analysis of weather parameters is Mann-Kendall as the major test done at first further acts as a dependent variable for other detection tests. The observed measurements over time are arranged in an order of x_1, x_2 … x_{n-1} as x_i and x_2, x_3 … x_n as x_j where the number of times represented as 1, 2, 3 … n respectively. The sign of all possible differences (x_j -

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x_i) where j > I are determined. The sgn (x_j - x_i) is an indicator function and the values are compared using *Equation 1:*

$$sgn\left(x_j - x_i\right) = \begin{cases} +1, if\, x_j > x_i \\ 0, if\, x_j = x_i \\ -1, if\, x_j < x_i \end{cases} \quad \text{(Eq.1)}$$

The Mann-Kendall's statistic (S) was determined as the difference between the number of positive and negative differences by *Equation 2:*

$$S = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} sgn\left(x_j - x_i\right) \quad \text{(Eq.2)}$$

where n is the number of collected data samples. The S-test have to be computed with variance if n > 10. Hence, the variance of S was calculated by *Equation 3:*

$$Var(S) = \frac{1}{18}\left[n(n-1)(2n+5) - \sum_{p=1}^{g} t_p\left(t_p - 1\right)\left(2t_p + 5\right)\right] \quad \text{(Eq.3)}$$

where 'g' is the number of tied groups i.e. a set of data sample having the same value and t_p is the number of data samples in the p^{th} tied group. The MK test statistic (Z) was determined by *Equation 4:*

$$z_{MK} = \begin{cases} \frac{S-1}{\sqrt{Var\,(S)}} if\, S > 0 \\ 0\ if S = 0 \\ \frac{S+1}{\sqrt{Var\,(S)}} if\, S < 0 \end{cases} \quad \text{(Eq.4)}$$

The positive (negative) value will show the trend to increase (decrease) over time and the trend is detected eventually as per the classification requirements.

Spearman's rho test

Spearman's rank correlation coefficient is the statistical measure between the two datasets to ensure the strength of its link. This test comes under non-parametric methods and also used to correlate two variables in a group of data. As said here, the observed data include one with time in years and the other as rainfall time series data in month per millimeters. Similar to Mann-Kendall test, the numbers of time series data are replaced by ranks in order. The test statistic (ρ_s) was designed by Siegel and Castellan framed as *Equation 5:*

$$\rho_s = \frac{S_{xy}}{\sqrt{S_x S_y}} \quad \text{(Eq. 5)}$$

where ρ_s is the correlation coefficient, $S_x = \sum_{i=1}^{n}(X_i - \bar{X})^2$, $S_y = \sum_{i=1}^{n}(Y_i - \bar{Y})^2$, $S_{xy} = \sum_{i=1}^{n}(X_i - \bar{X})^2\,(Y_i - \bar{Y})^2$ and X_i (time), Y_i (variable of interest), x and y refer to the ranks (x, y, S_x, and S_y have the same values in analyzing the trend). For long-term samples, the quantity $\rho_s = (n\text{-}1)^{1/2}$ is approximately normally distributed with mean of 0

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and variance of 1 (critical test statistic values for various significance levels can be obtained from probability tables).

Linear regression test

Regression analysis also comes under statistical technique that attempts to explore and model the relationship between two or more variables. The model here designed and tested are simple linear regression in which one as independent variable (regressors or predictors) and the other as dependent variable (response) tends to produce straight line with the help of residuals formed. The linear regression formula is represented below in *Equation 6:*

$$E(Y) = \beta_o + \beta_1 x \quad \text{(Eq.6)}$$

where β_o = intercept and β_1 = slope of the regression coefficients followed by x as independent variable and Y as dependent variable. The slope, β_1, can be interpreted as the change in the mean value of Y for a unit change in x. The random error term, ϵ, is assumed to follow the normal distribution with a mean of 0 and variance of σ^2. Since Y is the sum of this random term and the mean value, E(Y), which is a constant, the variance of Y at any given value of x is also σ^2. Therefore, at any given value of x, say x_i, the dependent variable Y follows a normal distribution with a mean of $\beta_o + \beta_1 x_i$ and a standard deviation of σ.

This linear regression is an inbuilt test for both Mann-Kendall and Spearman's Rho test and the main purpose of this test is to produce the trend line for the given data samples with the help of mean and variance. It is the basic method for all statistical analysis and models. Therefore, this method of hypothetical testing is applied to the weather parameters in correlation namely, Rainfall and River discharge will draw the trends. Further, its prediction has been projected for the deep down micro-level studies and its measures in the hydrological aspects.

Results and discussion

Statistical analysis of rainfall

The statistical characteristics are calculated for the best fit of models which is considered to be the preliminary stage in statistical testing. There are various mathematical statistic formulations available in that only eight factors namely, minimum, maximum, mean, standard deviation, variance, co-efficient of variation, skewness and kurtosis are selected as the most suited parameters in statistics for hydro-meteorological variables such as rainfall.

The graphical representation of basic factors calculated as descriptive statistics such as minimum, maximum, mean and standard deviation of annual rainfall data are presented (*Fig. 4*). From the graph, it has been clearly recorded that the maximum annual rainfall is at Papanasam station (6131 mm) which is located at upstream side and the minimum annual rainfall is at Kayathar station (12.2 mm) at downstream side of the basin. Though, the annual mean rainfall varies from 1511.1 mm for Senkottai station to 573.2 mm for Tirunelveli station whereas the fluctuation is too great in terms of spatial and temporal scales. Similarly, the standard deviation with lesser value is closer to mean

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at Srivaikundam station (221.6 mm) and the larger value is the most normally distributed one at Papanasam station (913.8 mm).

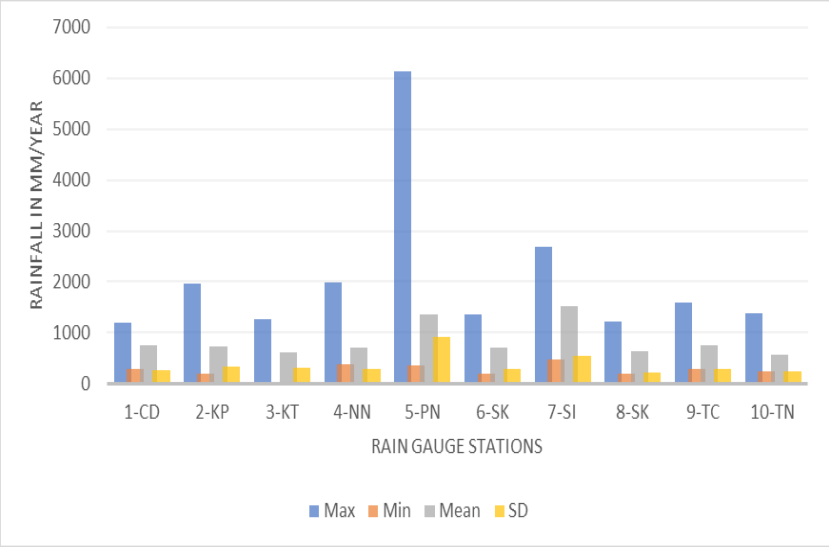

Figure 4. *Annual rainfall of ten rain gauge stations and its four statistical factors*

The confined eight statistical factors are formulated and calculated as parameters of statistical testing for all the ten rain gauge stations with respect to annual and seasonal pattern, the values are presented in *Table 3*.

The other four minor parameters such as variance, coefficient of variation, skewness and kurtosis are all dependent variables of the former statistics calculations which are explained above (*Fig. 4*). Variance is the square of the standard deviation the results in *Table 3* show that higher variance values are far away from the mean and the lower one is closer to it.

As per *Table 3,* the coefficient of variation for annual rainfall amount varies from 66.7% in Papanasam station to 33.1% in Cheranmahadevi station. This statistical property is used when the data set is widely distributed by its mean then it can replace standard deviation while the CV values less than one are represented as low-variance and greater than one as high-variance that are exponentially distributed system. So, the annual rainfall amount is within the range of low-variance for both the stations. Almost all the stations and rainfall patterns both annual as well as seasonal are under positive values except for two, which indicates that they are skewed to the right in the normal distribution curve. The rainfall during annual as well as north-east monsoon (NEM) period in Papanasam station and rainfall during summer (SMR) in Nanguneri station were noticed as more skewed one. Most of the skewness coefficient values are identical to or nearly identical to zero representing that the data falls under normal distribution case. In case of kurtosis coefficient, the values lesser than three tends to be the flattened curve in normal distribution and greater values than three are replaced to Laplace distribution. The kurtosis property purely depends on the data set and here, the Papanasam station exhibits the higher value says that the peak values are the outliers to normal distribution and can be flattened by Laplace distribution. So, the peak values greater than three are in 13 out of 50 station points as outliers which can be corrected in Mann-Kendall test.

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Table 3. *Statistical characterization of rainfall data*

Sl. No	Rainfall stations	Time series	Max (mm)	Min (mm)	Mean (mm)	Standard deviation (mm)	Variance	Coefficient of variation	Skewness	Kurtosis
1	Cheranmahadevi	ANNUAL	1190.6	278.3	750.97026	255.08247	65067.06	33.96705	-0.26387	-0.68641
		SWM	219.2	0	70.90436	46.88552	2198.25	66.12501	0.84964	1.19033
		NEM	913.64	195.8	485.86564	203.53673	41427.2	41.89157	0.37464	-0.82444
		WNT	269.5	0	55.38795	67.14279	4508.15	121.22275	1.82416	2.90625
		SMR	556.8	13	138.81231	96.28804	9271.39	69.36564	2.30288	8.45497
2	Kalampatti	ANNUAL	1959.63	183.06	739.60821	325.47771	105935.74	44.00677	1.41522	4.46048
		SWM	861.15	36.72	147.36897	165.44794	27373.02	112.26782	3.2734	11.67695
		NEM	848.63	110.42	413.26256	163.5359	26743.99	39.57191	0.23367	0.01874
		WNT	251.33	0	51.4841	63.28029	4004.39	122.91229	1.64741	2.13664
		SMR	357.58	12.95	127.49256	84.83762	7197.42	66.54319	1.26933	1.02199
3	Kayathar	ANNUAL	1267.7	12.2	621.39795	307.12609	94326.44	49.42502	-0.21749	0.08799
		SWM	383	0	85.63077	84.83089	7196.28	99.0659	1.40267	2.40541
		NEM	802	0	399.48	232.81809	54204.26	58.28029	0.1674	-0.66574
		WNT	118.5	0	21.01282	29.78485	887.14	141.7461	2.04252	4.41538
		SMR	454	0	115.27435	93.46954	8736.56	81.08442	1.23594	3.0657
4	Nanguneri	ANNUAL	1983.6	381	697.53333	280.78571	78840.61	40.25409	2.63262	10.85948
		SWM	251.5	2.1	79.44615	46.84578	2194.53	58.96544	1.17935	3.52142
		NEM	1129.5	147	437.92308	196.63616	38665.78	44.90199	1.3202	3.00585
		WNT	224	0	45.61026	51.63969	2666.66	113.21946	1.74823	3.4378
		SMR	751	7.2	134.55385	132.46313	17546.48	98.44619	3.28104	12.92621
5	Papanasam	ANNUAL	6131	362	1369.53846	913.81791	835063.17	66.72452	3.86418	19.65916
		SWM	780	6	186.84615	153.60573	23594.72	82.20974	2.07961	5.94568
		NEM	3130	190	850.58205	511.00197	261123.01	60.07674	2.65563	9.82523
		WNT	2070	0	156.04359	334.37772	111808.46	214.28482	5.22784	29.9223
		SMR	784	42	176.06667	139.74534	19528.76	79.37069	2.6712	9.17811
6	Sankarankoil	ANNUAL	1369	196.9	699.86704	292.13715	85344.13	41.74181	0.73928	0.16106
		SWM	288	9.2	76.30331	55.73158	3106.01	73.03954	1.64517	4.18178
		NEM	1181	76	427.06317	241.54348	58344.63	56.55953	1.4156	2.29228
		WNT	202.6	0	45.77409	53.22803	2833.2	116.2832	1.52113	2.01334
		SMR	387.2	20	150.72255	89.13748	7945.46	59.13995	0.66235	0.03501
7	Senkottai	ANNUAL	2689.7	474	1511.08979	552.54031	305302.67	36.56586	0.57437	-0.28072
		SWM	1070.5	148.9	413.98569	224.80045	50535.53	54.30155	1.23589	1.10439
		NEM	2013	151	700.35572	333.48423	111211.65	47.61636	1.7108	5.13112
		WNT	450	0	101.44179	126.54623	16013.95	124.74762	1.49288	1.57487
		SMR	734.33	47.9	295.29992	185.61111	34452.54	62.85582	0.95804	0.22008
8	Srivaikundam	ANNUAL	1209.8	201.3	645.35442	221.60722	49109.79	34.33883	0.33953	0.20565
		SWM	179	0	64.22861	53.47743	2859.83	83.26103	0.74839	-0.55635
		NEM	953.7	109.7	429.31662	213.34664	45512.21	49.68902	0.61916	-0.20956
		WNT	239.2	0	47.49992	61.61936	3796.93	129.72571	1.72065	2.42218
		SMR	347.8	2	104.28427	76.18057	5803.47	73.05083	1.17828	1.5342
9	Tiruchendur	ANNUAL	1599.4	275.6	748.00056	285.09504	81277.75	38.11383	1.03674	1.33091
		SWM	109	0	30.67999	28.4335	808.44	92.67761	1.19992	0.76773
		NEM	1360.4	170.8	560.65897	267.16539	71377.35	47.65203	0.87972	0.7007
		WNT	297.7	0	65.82564	73.07422	5339.72	111.00622	1.51907	2.11038
		SMR	372.2	0	90.70459	84.68192	7179.62	93.36625	1.50382	2.43855
10	Tirunelveli	ANNUAL	1391	248.8	573.19958	232.93238	54258.65	40.63784	1.1619	2.51419
		SWM	184.4	2	67.12051	49.18344	2419.01	73.27632	0.77042	-0.04373
		NEM	876	112.2	361.83077	159.54201	25453.65	44.09299	0.92276	1.26563
		WNT	305.4	0	37.1641	65.17043	4247.18	175.35854	3.15812	10.45069
		SMR	316	2	107.08462	79.61844	6339.1	74.35096	1.10089	0.92111

*SWM = south-west monsoon; NEM = north-east monsoon; WNT = winter; SMR = summer

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It is interpreted from the statistical properties that the rainfall amount contributed to the annual pattern in maximum by north-east monsoon (NEM) rainfall and minimum during winter (WNT) rainfall. The inference in whole from the descriptive statistics are the clear signature of rainfall data fluctuations and is now evident to proceed with the statistical testing using non-parametric methods for the trend analysis.

Trend analysis

Annual rainfall trends

With the analysis done through Mann-Kendall and Spearman's Rho test, annual rainfall data have predicted trends with the limit of confidence intervals such as 90, 95 and 99%. The projected trends are correlated with both Mann-Kendall and Spearman's Rho test as one prediction for each rain gauge stations. This statistical hypothesis testing produced significantly both increasing (+ve) and decreasing trend (-ve) while there are also results with non-significant trends. The obtained annual rainfall trend results for Mann-Kendall and Spearman's Rho test are tabulated below in *Table 4* and the visualization of results in terms of spatial scale with respect to rain gauge stations is shown in *Figure 5*.

Table 4. *Statistical analysis on annual rainfall pattern*

S. No	Rain gauge station	Annual statistics		
		Z	ρ	Trend
1	Cheranmahadevi	1.331	0.201	No trend
2	Kalampatti	0.363	0.024	No trend
3	Kayathar	1.887***	0.298***	Increasing trend
4	Nanguneri	0.073	0.025	No trend
5	Papanasam	0.847	0.164	No trend
6	Sankarankoil	1.766***	0.281***	Increasing trend
7	Senkottai	2.032**	0.331**	Increasing trend
8	Srivaikundam	0.992	0.142	No trend
9	Tiruchendur	0.847	0.146	No trend
10	Tirunelveli	-1.694***	-0.289***	Decreasing trend

Level of significance: *** = 0.10, ** = 0.05, * = 0.01

Table 4 shows the positive (increasing) trends, the Senkottai station showed significantly increasing trend at 5% of significance level wherein the Kayathar and Sankarankoil station had 10% of significance level in its increasing trend. Also, when it comes to negative (decreasing) trend, the only station subjected to significantly decreasing trend at 10% confidence level was Tirunelveli station in the whole basin for rainfall annually. The stations showing no trend are indicated with pictorial representation (*Fig. 5*).

With the same statistical testing of Mann-Kendall and Spearman's Rho test, the monthly rainfall data grouped under four seasons such as south-west and north-east monsoon, winter and summer, experienced by the study area were also applied season-wise to detect the trend values. Hence, in *Table 5* the results are analyzed for both

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monsoon seasons while in *Table 6* results are recorded for both winter and summer seasons.

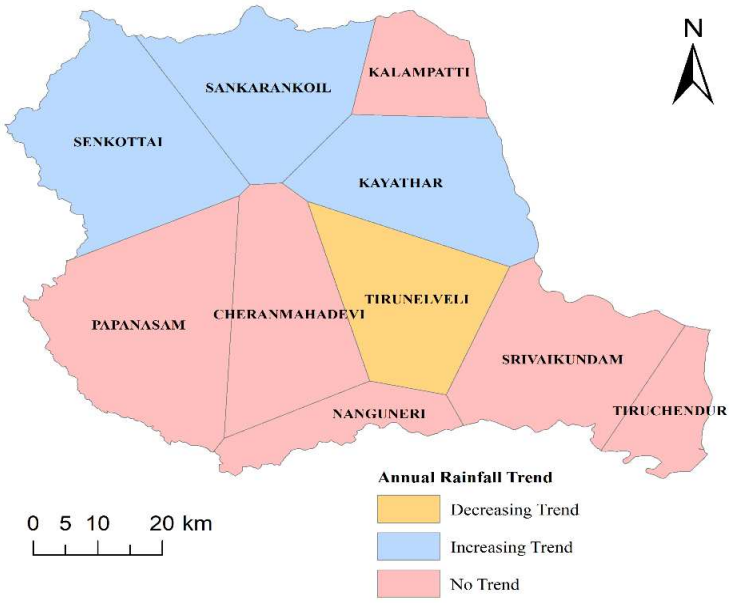

Figure 5. *Spatial variation of annual trend distribution in Thamirabarani River basin seasonal rainfall trends*

Table 5. *Statistical analysis on seasonal patterns of SWM and NEM*

S. No	Rain gauge station	Seasonal statistics					
		South-west monsoon (SWM)			North-east monsoon (NEM)		
		Z	ρ	Trend	Z	ρ	Trend
1	Cheranmahadevi	0.919	0.15	No Trend	1.694***	0.267***	Increasing Trend
2	Kalampatti	-1.113	-0.239	No Trend	0.242	0.009	No Trend
3	Kayathar	0.423	0.104	No Trend	2.468**	0.382**	Increasing Trend
4	Nanguneri	-0.823	-0.151	No Trend	0.774	0.113	No Trend
5	Papanasam	1.21	0.217	No Trend	0.895	0.176	No Trend
6	Sankarankoil	1.089	0.184	No Trend	0.774	0.103	No Trend
7	Senkottai	0.871	0.141	No Trend	1.839***	0.282***	Increasing Trend
8	Srivaikundam	-0.448	-0.084	No Trend	0.992	0.151	No Trend
9	Tiruchendur	-0.23	-0.03	No Trend	0.968	0.152	No Trend
10	Tirunelveli	-1.125	-0.175	No Trend	-1.125	-0.177	No Trend

Level of significance: *** = 0.10, ** = 0.05, * = 0.01

From the seasonal analysis, it is clearly shown that only increasing trend exists when it comes to seasonal basis of rainfall. The annual rainfall pattern of positive (increasing) trends have got an impact from seasonal rainfall, which is proven by this statistical analysis of Mann-Kendall and Spearman's Rho testing. With reference to *Tables 5* and *6*, the test results show no variation in trend i.e. non-significant trend was noticed during south-west monsoon and winter season meaning that the rainfall amount in all rain

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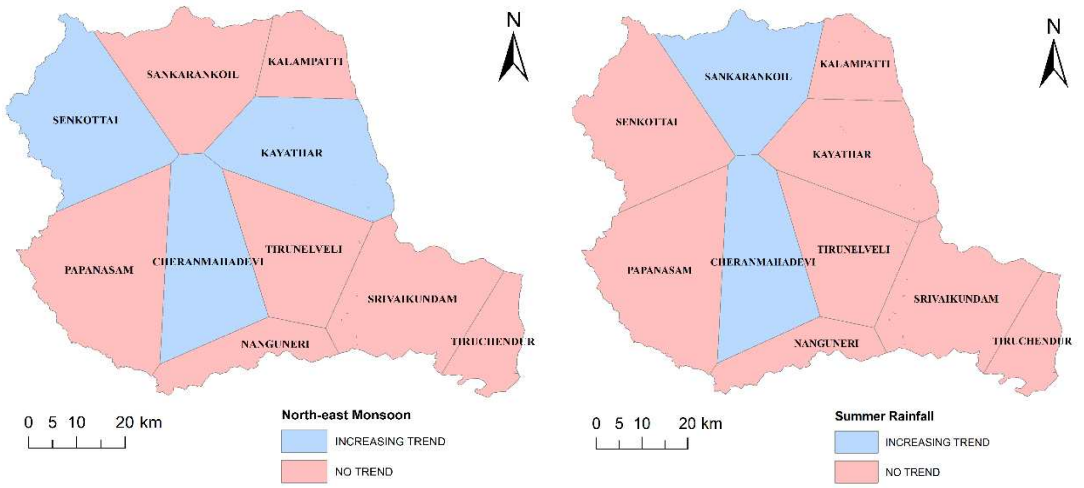
gauge stations are in normal range. In the case of north-east monsoon season, the Cheranmahadevi and Senkottai station on the upstream side of the basin gets increasing trend for 10% confidence level while the Kayathar station got 5% confidence level of increase in trend that must be taken into consideration. Similarly, during summer period there is an evidence of positive (increasing) trends with the same Cheranmahadevi station at 10% confidence level and the only noted rain gauge station of Sankarankoil have got increasing trend at 1% confidence level. The spatial scale representation of analyzed results showing only trends which are easier to interpret are mapped as north-east monsoon (*Fig. 6a*) and summer season (*Fig. 6b*) in two figures for better understanding of trend patterns in wide range throughout the basin.

Table 6. *Statistical analysis on seasonal patterns of winter and summer*

S. No	Rain gauge station	Seasonal statistics					
		Winter			Summer		
		Z	ρ	Trend	Z	ρ	Trend
1	Cheranmahadevi	0.46	0.122	No Trend	1.79***	0.309***	Increasing Trend
2	Kalampatti	0.254	0.078	No Trend	0.798	0.108	No Trend
3	Kayathar	-0.302	0.169	No Trend	0.992	0.175	No Trend
4	Nanguneri	-0.073	0.067	No Trend	0.327	0.049	No Trend
5	Papanasam	0	0.048	No Trend	1.198	0.212	No Trend
6	Sankarankoil	0.593	0.16	No Trend	3.097*	0.496*	Increasing Trend
7	Senkottai	0.532	0.13	No Trend	1.21	0.148	No Trend
8	Srivaikundam	-0.254	0.041	No Trend	0.169	0.024	No Trend
9	Tiruchendur	-0.593	-0.009	No Trend	0.835	0.138	No Trend
10	Tirunelveli	-1.04	-0.078	No Trend	-0.302	-0.052	No Trend

Level of significance: *** = 0.10, ** = 0.05, * = 0.01



(a) North-east monsoon (October to December) (b) Summer (March to May)

Figure 6. *Spatial variation of seasonal trend distribution in Thamirabarani River basin annual, seasonal and monthly trends of river discharge*

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River discharge is the stream flow or surface runoff of the basin. It totally depends on the amount of rainfall and it is one of the direct dependent variable of an independent parameter rainfall in terms of hydrology. So, the statistical characteristics can be overviewed through rainfall statistical analysis and the trend prediction is made through the Mann-Kendall and Spearman's Rho test for annual, seasonal and monthly trends considered to undergo deep down predictions.

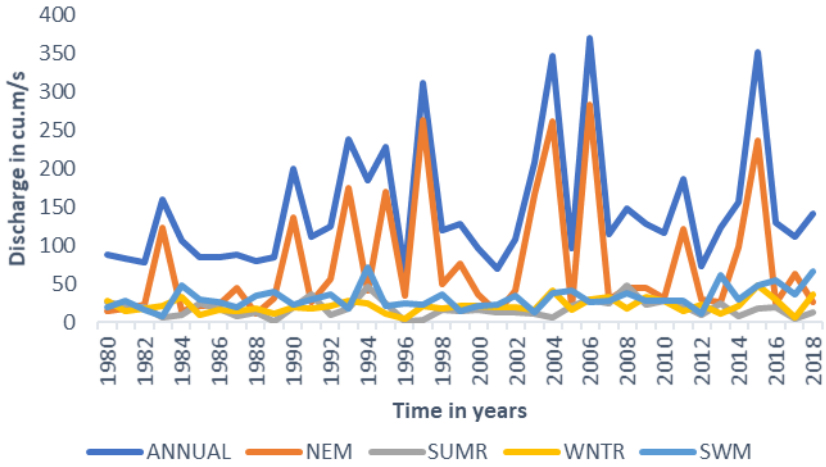

Figure 7. *Temporal variation of Murappanadu station river discharge*

Murappanadu stream gauge station is the only recorded discharge data in the basin located at the major stream order outlet. Due to its large scale contribution at that point of the basin, the recorded stream gauge data was analysed starting from annual to monthly discharge pattern for better trends in observation. The temporal scale representation of river discharge data (*Fig. 7*) from 1980-2018 concludes that the north-east monsoon impacts the discharge rate to increase and influence the annual discharge rate in intensive manner. The obtained test results and trend patterns for annual, seasonal and monthly time scales are tabulated in *Tables 7* and *8.*

Table 7. *Annual and seasonal trend distribution for river discharge*

S. No	Murappanadu stream gauge station	Statistical analysis		
		Z	ρ	Trend
1	Annual rainfall	2.298**	0.37**	Increasing trend
2	South-west monsoon	2.226**	0.339**	Increasing trend
3	North-east monsoon	1.621***	0.271***	Increasing trend
4	Winter	1.379	0.237	No trend
5	Summer	-0.073	0.013	No trend

Level of significance: *** = 0.10, ** = 0.05, * = 0.01

From *Tables 7* and *8,* the river discharge trend prediction shows an increasing trend in both annual and seasonal pattern. It is evident from the trend values that the impact of south-west and north-east monsoon seasonal pattern creates the weather event by

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increasing (positive) in trends further, and affects the annual discharge amount from the river. The increasing (positive) trends for annual and monsoon seasonal pattern has been predicted with 5% of confidence level. Obviously, it is a must to view the monthly trends as it contributes in seasonal pattern of river discharge. The results of predicted monthly trend values show increase in trends (positive) for the month of January, March and September with 10% of confidence level whereas the month of July, August, November and December shows the increasing (positive trends) along with the 5% of confidence level.

Table 8. *Monthly trend distribution for river discharge*

S. No	Murappanadu stream gauge station	Monthly statistical analysis		
		Z	ρ	Trend
1	January	2.758***	0.392***	Increasing trend
2	February	0.012	0.027	No trend
3	March	1.633***	0.305***	Increasing trend
4	April	0	-0.009	No trend
5	May	-0.23	-0.053	No trend
6	June	-1.5	-0.241	No trend
7	July	2.395**	0.353**	Increasing trend
8	August	2.419**	0.38**	Increasing trend
9	September	1.802***	0.311***	Increasing trend
10	October	-0.012	-0.004	No trend
11	November	2.117**	0.345**	Increasing trend
12	December	2.298**	0.363**	Increasing trend

Level of significance: *** = 0.10, ** = 0.05, * = 0.01

Comparative result analysis of rainfall and river discharge

According to the non-parametric Mann-Kendall, Spearman's Rho and Linear Regression statistical testing in the annual rainfall analysis, the upstream side of the river basin has one station named Senkottai that is affected by increase (positive) in trend and in the middle of basin with two stations namely; Sankarankoil and Kayathar with increase (positive) in trends while the decreasing (negative) trend have been noticed in downstream side of the basin with station named Tirunelveli has to be investigated with its hydrological parameters for sustainable management of water resources. Simulatneously, the Murappanadu stream gauge station river discharge is facing the positive (increasing) trends due to the impact of rainfall points at its upstream rain gauge stations such as Cheranmahadevi, Senkottai, Kayathar, and Sankarankoil predicting increase (positive) in trends. Therefore, the analysis shows that there is variation in the climate of the basin therefore, the anthropogenic activities responsible for the cause and effect of rainfall have to be inspected in all the five rain gauge stations.

From the analytical point of view, it is observed that the rainfall in all three patterns such as annual, seasonal and monthly have been altered over the long period of time scale i.e., 39 years (1980-2018) which is indicating that there is a slight change in climate that is taking place slowly throughout the basin. The actual morphology of the basin is perennial having sufficient surface and ground water flow in varied flooding condition during its monsoon periods. But this flood conditions are now changing its nature as per

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the observed data due to an erratic rainfall making its impact on the heavy discharge towards the river banks. Therefore, the quantity of rainfall getting its impact in rainfall pattern simultaneously affects the river discharge as extreme events like floods. The change in climate is somewhat visible through the survey that there is an increase in deforestation along the Western Ghats, India (upstream side) as well as industrialization along the downstream side of the basin in terms of local scale. The global increase in Land Surface Temperature (LST) and Sea Surface Temperature (SST) impacts this river basin as it is subjected to both land and sea surfaces in terms of global scale which can be predicted appropriately through climate modelling as scenarios for past present and future conditions.

Micro-level impact study on trend pattern

To have a clear picture on the trend pattern from the whole basin to block level impact of trend can be predicted through Theissen polygon interpolation method with help of ArcGIS software. This interpolation method helps us to predict the rate of climate change in block level to the whole basin and able to make decision on the management level studies. The spatial representation of block level trend prediction for annual (*Fig. 8*), and seasonal such as north-east monsoon (*Fig. 9a*) and summer (*Fig. 9b*) is shown below and *Table 9* indicates the rate of trends with respect to basin blocks respectively. This pictorial representation (*Fig. 8*) shows that the rainfall stations subjected to the significant trends i.e. positive (increasing) and negative (decreasing) as well as non-significant trends in the whole basin has the capability to calculate the trend rate in percent. Hence, the spatial scale variation as figures for annual (*Fig. 8*) as well as seasonal (*Fig. 9*) patterns and the temporal scale of annual trend rate determination tabulated as *Table 9* the rate of climate change for the whole basin of Thamirabarani river has been concluded.

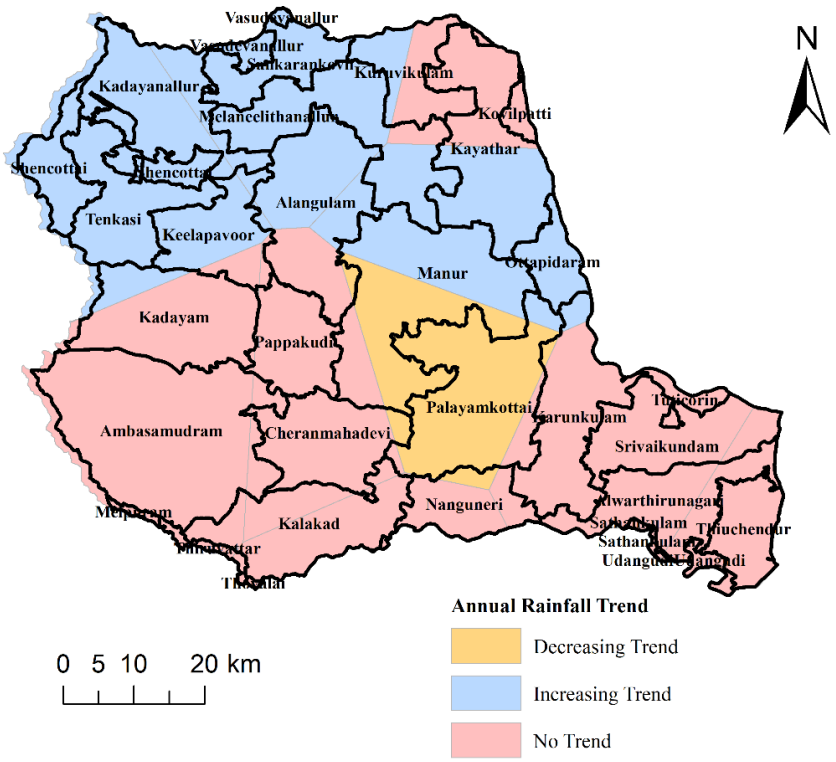

Figure 8. *Spatial representation of blocks influenced by annual rainfall trend*

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Table 9. *Block-level trend analysis on Thamirabarani River basin*

Sl. No	Name of the blocks	Area (km^2)	Trend (%)		
			Increasing trend	Decreasing trend	No trend
1	Alwathirunagari	211.64	-	-	100
2	Karunkulam	260.54	6.53	1.98	91.47
3	Kayathar	330.70	56.73	-	43.27
4	Kovilpatti	24.31	-	-	100
5	Ottapidaram	80.05	100	-	-
6	Sathankulam	7.16	-	-	100
7	Srivaikundam	227.85	-	-	100
8	Tiruchendur	117.27	-	-	100
9	Tuticorin	31.70	-	-	100
10	Udangudi	22.41	-	-	100
11	Alangulam	326.32	68.57	2.28	29.15
12	Ambasumdram	589.74	-	-	100
13	Cheranmadevi	218.34	-	5.99	94.01
14	Kadayam	291.48	15.70	-	84.30
15	Kadayanallur	263.97	100	-	-
16	Kalakad	271.91	-	-	100
17	Keelapavoor	176.79	78.74	-	21.26
18	Kuruvikulam	146.32	36.19	-	63.81
19	Manur	473.76	43.81	43.42	12.77
20	Meelaneelithanallur	308.69	91.93	-	8.07
21	Nanguneri	140.50	-	12.16	87.84
22	Palayamkottai	388.17	4.93	84.91	10.17
23	Papakudi	157.68	-	-	100
24	Sankarankoil	124.83	100	-	-
25	Senkottai	169.67	100	-	-
26	Tenkasi	203.44	100	-	-
27	Vasudevanallur	330.34	100	-	-
28	Melpuram	7.81	-	-	100
29	Thiruvattar	19.35	-	-	100
30	Thovalai	2.15	-	-	100
Total blocks		**5624.90 km^2**	**33.44%**	**5.02%**	**61.54%**

From *Table 9,* it is interpreted that these blocks in Thamirabarani River basin are involved in trend analysis. The thirty blocks along with ten rain gauge stations made its interpolation and determined each and every block trend patterns with respect to area of the basin. Therefore, as a result the increasing trend of 33.44% and decreasing trend of 5.02% was subjected to significant rate of change in climate along with remaining 61.54% of non-significant zero trends. This calculated rate of change by trend analysis will be leading to take measures and management in particular sub-watersheds of the whole basin whichever under severe cause.

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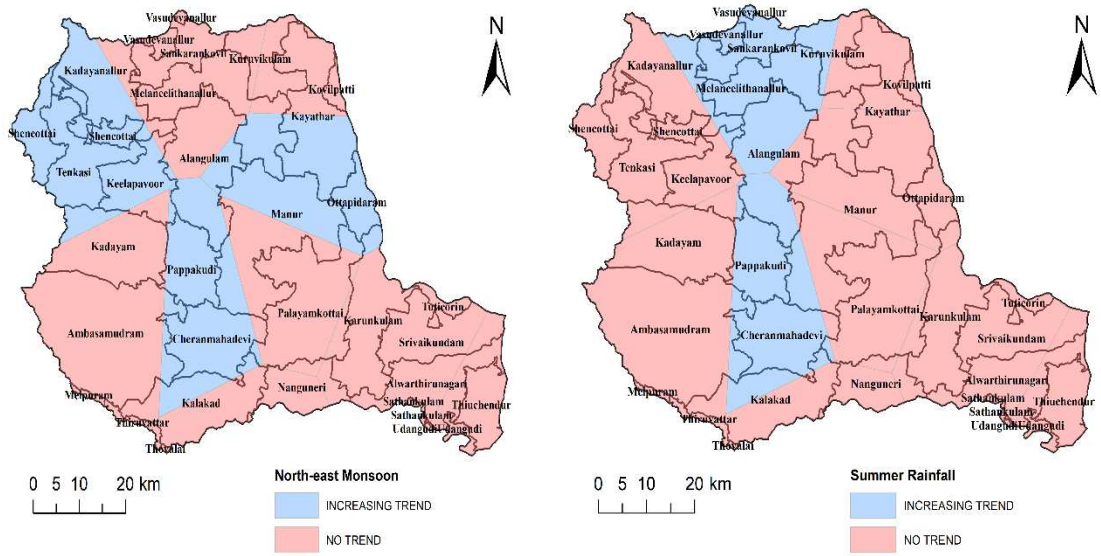

(a) North-east Monsoon (October to December) (b) Summer (March to May)

Figure 9. *Spatial representation of blocks influenced by seasonal rainfall trend*

Conclusion

With ten rain gauge stations and one stream gauge station, the analysis was done for annual and seasonal rainfall variability as well as annual, seasonal and monthly river discharge variation study in comparison with rainfall and its river discharge for the period of 39 years (1980-2018) in Thamirabarani river basin, Tamilnadu, India. The annual, seasonal and monthly trends are predicted by Mann-Kendall (MK) test, Spearman's Rho (SR) test and Simple linear regression analysis with respective confidence intervals. Out of ten rain gauge stations, the five rain gauge stations are showing both increasing (positive) and decreasing (negative) trends during annual and seasonal periods while in the stream gauge station, the river discharge is showing only increasing (positive) trends throughout the annual, seasonal and monthly patterns. The trend patterns as well as temporal and spatial variability of rainfall and its river discharge clearly shows that the Thamirabarani river basin is under climatic impacts over the period of time. The comparative result of rainfall and river discharge shows that the fluctuations in rainfall range leads to rate of change in river discharge of the basin. With the micro-level study, the blocks taken with respect to rain gauge station throughout the basin by interpolation helps us to understand the trend patterns in terms of climate change rate. Hence, the study concludes that the 33.44% of increasing trend will make changes in extreme events like flood and 5.02% of decreasing trend have the probability of creating drought to the basin. The statistical analysis is the preliminary assessment for any river basin study which helps in better hydrological modelling, sustainable water management, agricultural development and economic prosperity of the region. The present study can be initiated as one of the major objective for future work regarding any analysis of hydrological parameters in modelling and software. Also, the statistical results will be oriented towards finding the significance of trends using worse likelihood test for the prediction of return period floods and auto-correlation modelling for weather forecasting.

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HARVEST INDEX OF PEA PLANT AND SOIL PROPERTIES INFLUENCED BY A TWO-YEAR AMENDMENT OF BIOCARBONS UNDER MUNICIPAL WASTEWATER IRRIGATION IN ARID CLIMATE

MANZOOR, M.[1,2*] – GUL, S.[1,3] – BIBI, S.[4] – GUL, I.[3,5] – AKBAR, A.[6] – ALI, I.[7,8] – REHMAN, G. B.[1]

[1]Department of Botany, University of Balochistan, Quetta, Balochistan, Pakistan

[2]Department of Plant Sciences, Sardar Bhadur Khan Women's University, Quetta, Balochistan, Pakistan

[3]Department of Natural Resource Sciences, Macdonald Campus, McGill University, 21 111 Lakeshore Road, Ste-Anne-de-Bellevue, H9X 3V9 Quebec, Canada

[4]Institute for Ecology, Technical University, Berlin, Germany

[5]Institute of Environmental Sciences and Engineering, School of Civil and Environmental Engineering, National University of Sciences and Technology (NUST), Islamabad, Pakistan

[6]Department of Microbiology, University of Balochistan, Balochistan, Pakistan

[7]Department of Biochemistry, University of Balochistan, Quetta, Pakistan

[8]School of Life Sciences and Engineering, Southwest University of Science and Technology, 621010 Mianyang, China

**Corresponding author*
e-mail: Alfa_botany@yahoo.com

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Abstract. In this study, the influence of biochars, produced from cow manure and wood, on the harvest index of pea plants (*Pisum sativum* L.) and soil properties under groundwater and municipal wastewater irrigation was investigated. Biochars were applied at 5, 10 and 15 t ha^{-1} rates for two years. Yield biomass (pods) was higher under groundwater irrigation. As compared to control, amendment of biochar did not influence harvest index for both years, under irrigation treatments; however, as compared to wastewater irrigation, harvest index tended to be higher under groundwater irrigation. Soluble phosphorus level was higher in response to manure derived biochar under groundwater irrigation and nitrogen level was higher in response to lower rate of manure derived biochar under wastewater irrigation as compared to groundwater-irrigated soil. Under wastewater, macroaggregate stability was significantly increased within the soil as compared to groundwater irrigation, while; macroaggregates stability was observed in response of wood-derived biochar at higher rate under groundwater irrigation. Bacterial diversity was two-fold higher in the soil irrigated with groundwater as compared to the soil irrigated with wastewater.
Keywords: *bacterial diversity, soil aggregation, biochar, pea plants, biomass*

Introduction

Baluchistan has arid to semi-arid climate and diversified precipitation. It consists of an area larger than other provinces of Pakistan, this has significant importance for agricultural activities (Ahmad and Islam, 2011). Inadequacy of water and poor quality of soil is the main hurdle for crop productivity in this province. However, urban areas

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have more practice of wastewater than groundwater irrigation and short-term studies demonstrate its significant positive influence on crop growth (Haider et al., 2018; Hameeda et al., 2019). Generally; overpopulation, economic development and urbanization have increased the wastewater effluent production from industries, commercial and domestic sources. This effluent is continuously contaminating the groundwater resources, environment and polluting the soil (Elgallal et al., 2016; Libutti et al., 2018). Irrigation of agricultural lands with wastewater can be a mean of its utilization as a bioresource and can be an effective way to reduce its effect on the environment. However, as wastewater can contain toxic chemicals (e.g., heavy metals) and excess amount of nutrients, continuous irrigation can have a negative influence on crop growth and soil quality (Murtaza et al., 2010).

The amendment of biocarbon, which is also known as biochar is found to have a positive influence on crop growth and soil quality under wastewater irrigation and crop (Kamran et al., 2019; Nzedigwu et al., 2019). For this, wastewater with biochar amendments can be the best substitute of groundwater for agricultural purposes and to improve crop yield and soil quality (Akoto-Danso et al., 2018; Haider et al., 2019). Concurrently, biochars amendment are in common practice in Pakistan to overcome nutrients deficiency in soil of arid climatic region; however, influence of biochar types, biochar production is associated with soil physiochemical properties, and climatic region of that area (Jeffery et al., 2011; Gul et al., 2015; Yadav et al., 2017) (meta-analysis). Besides, wood derived biochar, animal manure is the best option to produce biochar for being cost effective and frequently available and it is the rich source of nutrients. Interestingly, manure and wood derived biochars are reported for increase in yield and improvement of soil quality (Hameeda et al., 2019).

Pea plant has high agronomic importance and it is a common vegetable in Pakistan. Pea seeds (pods) consumed as a food and stover is used as a forage; however, after harvesting the above ground crop, under-ground part increases the nutrients availability in soil. Pea plant is the rich source of dietary fiber, micronutrients and protein; this is a healthy and economically affordable source of food for poor population. For this, pea plant is being extensively cultivated in Pakistan and in all over the world (Manzoor et al., 2019). In Pakistan pea is being cultivated in an area of 26569 ha and has 1465411 t production. Punjab has the highest production, followed by KPK, Balochistan and Sindh. Sindh has the lowest production of pea (Fruit, Vegetables and Condiments Statistics of Pakistan 2017-2018).

In the arid climate of Quetta, Balochistan pea production was about 12369 t from 2015 to 2018. Above mentioned statistics showed that climate of Quetta, Baluchistan is suitable for the cultivation of pea crop but there is need to bring changes in agricultural practices. Higher nutrients availability in soil can bring a significant increase in yield under wastewater irrigation. Generally, biochar amendments can improve the soil quality by increasing soil pH, EC, organic matter and soil microbial diversity and in results, increase the soil aggregates stability under wastewater irrigation. Higher crop production of pea plant under wastewater irrigation is linked to an increase in soil fertility. Uncertainties remained regarding interaction between biochar and wastewater irrigation.

For this, present study evaluated the influence of two types of biochar (manure and wood) on harvest yield index, soil properties (pH, EC, organic matter, C: N ratio, mineral nitrogen, soluble phosphorus and aggregates stability), and bacterial diversity under groundwater and wastewater irrigation treatments for two years. Our hypothesis

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is (1) wastewater irrigation positively influence harvest index of pea plant and improves soil quality (2) amendment of biochar further improves harvest index of pea soil quality under wastewater irrigation.

Materials and methods

Biochar and manure source and preparation

Wood biochar was purchased from the local timber market and cow manure was collected from a dairy farm. Both biochar types were processed to slow pyrolysis by using local kiln and temperatures was about 350-500 °C (Manzoor et al., 2019). Wood derived biochar was crushed, passed through 2 mm and 0.65 mm sieves to obtain two particle-sizes of 2 mm and 0.65 mm and only 2 mm particle-sized was selected for manure derived biochar; however, fresh manure was evenly added to the plots. Properties of biochar (wood and manure) and irrigation treatments (groundwater and wastewater) are described as follows (*Table 1*).

Table 1. *Biochars (wood and cow manure) and wastewater properties are listed*

Factors	pH	EC	Organic matter (%)	Nitrogen (g kg^{-1})	Phosphorus (g kg^{-1})
Wood biochar	7	5	71	10	11
Cow manure biochar	8	3	35	11	14
Wastewater	3	1720	-	-	-

In May soil sampling was done at 10 cm depth from each plot, soil was sealed in the bags and was air dried at room temperature in the lab.

Field area and experimental design

Field trial was established in the Botanical Garden of University of Baluchistan, Quetta, Pakistan. 60 plots (2 × 2 m^2) were plotted in the open field; 30 plots were irrigated with groundwater and 30 were irrigated with wastewater irrigation. Biochar (wood and cow manure) types, application rates and irrigations treatments were analyzed by three factorial experimental design. Plots of both irrigation treatments were plotted separately. The experiment was established for the years of 2016-2017 in the early spring. Ten different treatments of biochars were applied for each irrigation treatment (*Table 2*). Each treatment was followed by three replicates under groundwater and wastewater irrigation (*Fig. 1*). Cow dung was evenly mixed in the sixty plots at the rate of 5 t h^{-1}. Plots were irrigated twice before the cultivation of pea plant in the first week of October. Finally, in January about 100 seeds were sown in each plot and were regularly irrigated until harvested. In April, plants were on flowering stage and harvested in the first week of May.

Harvest index

After harvesting the crop, plants were air dried and on the basis of plant dry weight, aboveground biomass and harvest index (Unkovich et al., 2010) was estimated by *Equation 1:*

$$\text{Harvest index (\%)} = \text{Grain yield} / \text{Biological yield} \times 100 \quad \text{(Eq.1)}$$

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Table 2. *Treatments, their application rates and abbreviations*

Biochar types	***Particles size***	***Application rate***	***Abbreviations***
Control	--	0 kg m^{-2}	GW/WW
Wood biochar	2 mm	0.5 kg m^{-2}	BW .5
	2 mm	1 kg m^{-2}	BW 1
	2 mm	1.5 kg m^{-2}	BW 1.5
	0.65 mm	0.5 kg m^{-2}	BW (.65) .5
	0.65 mm	1 kg m^{-2}	BW (.65) 1
	0.65 mm	1.5 kg m^{-2}	BW (.65) 15
Manure biochar	2 mm	0.5 kg m^{-2}	BM .5
	2 mm	1 kg m^{-2}	BM 1
	2 mm	1.5 kg m^{-2}	BM 1.5

Figure 1. *Growth of pea plant in the field, plot and pods under arid climate*

Ph, EC and organic matter in soil

Crop was harvested in May and soil was taken 10 cm in depth from each plot. Soil was sealed in zip bags and was air dried in the lab at room temperature. For pH and EC of soil was analyzed in a (1:5) soil: distilled water ratio. Soil was mixed with stirrer and after 30 min reading was recorded by using Jenway 3520 pH meter and Jenway 4150 EC meter, respectively. Organic matter in soil was assessed by following the Estefen et al. (2013) protocol.

Mineral nitrogen (N) and soluble phosphorus (P)

Mineral nitrogen was assessed (NH_4^+ and NO_3) in soil by following the Sims et al. (1995) protocol, by using a spectrophotometer (Schimazu UV-vis 160) as an alternate of microplate reader. Soluble phosphorus content was assessed by using a spectrophotometer (Schimazu UV-vis 160) and followed the D, Angelo et al. (2001) protocol.

Macro and microaggregates stability

Soil aggregates were analyzed on the basis of size distribution of soil particles. Stack of sieves were arranged according to the sieves size followed by 2000 µm, 650 µm, 85 µm, 10.6 µm. Large sized sieve was set at the top and the smallest sized was set at the bottom of the stack. 20 g of soil was kept on the top of the stack (2000 µm). Stack was immersed carefully in the bucket full of water. Stack was continuously moved up

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and down (jerk) for 30 times within 2 min. Soil on the top of each sieve was collected separately and oven dried at 150 °C for 24 h and final reading was recorded (Demisie et al., 2014).

Bacterial diversity

Whole community of DNA was extracted from 250 mg of fresh soil by using DNeasy PowerSoil® Kit (QIAGEN, Hilden, Germany) through manufacturer's instruction. The DNA was sent to MACROGEN (USA) for sequencing and homology. The primer with the sequence 518F (CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGGTATCTAATCC) were used for the detection of microbes in the soil samples. Obtained sequences were analysed by BLAST (Basal Local Alignment Search Tool) from NCBI. Phylogenetic trees were reconstructed by the neighbour joining method using Editseq (DNASTAR Lasergene; DNASTAR, Madison, WI), Clustal X ver. 1.81 (Thomson et al., 1997) and MEGA ver. 6.0.2 (Tamura et al., 2007).

Statistical analysis

Data was statistically analyzed by using Minitab18 software for Analysis of variances and LSD.

Results

Harvest index

In the first year, yield biomass (pods) was significantly increased in response of control, manure derived biochar applied at the rate of 10 t ha^{-1}, large particles sized wood derived biochar applied at a lower rate of 5 and at a higher rate of 15 t ha^{-1}, however at small particles sized wood derived biochar applied at a lower rate of 5 t ha^{-1} as compared to small sized particles applied at a higher rate of 10 and 15 t ha^{-1} under groundwater. While under wastewater irrigation, there was not any significant difference between the treatments. Besides non-significant differences between the treatments, there was significant differences within the treatments of ground and wastewater irrigation treatments (like, large and small particles sized wood derived biochar applied at lower rate of 5 t $^{-}ha^{-1}$). In contrast of the first year, there was not any increase in the yield biomass in the second year under groundwater irrigation. Interestingly, yield biomass was non-significantly increased under groundwater as compared to wastewater irrigation in the second year (*Table 3*).

In the first year, under groundwater irrigation, aboveground biomass was significantly increased in response of control, manure biochar at lower application rate of 5 t ha^{-1}, large-particle-sized wood biochar at higher rate of 15 t ha^{-1} and small-particle-sized wood biochar at lower rate of 5 t/h than small-particle-sized wood biochar at higher rate of 10 t ha^{-1}; however, aboveground biomass was also increased in response of large-particle-sized wood biochar at 15 t ha^{-1} and small-particle-sized wood biochar at lower rate of 5 t ha^{-1} than manure biochar at higher rate of 10, 15 t ha^{-1}, large particle sized wood biochar at higher rate of 10 t ha^{-1}. In the second year, there was no significant difference of organic amendments than control; however, aboveground biomass was significantly decreased in response of manure biochar at higher application rate of 10 t ha^{-1} than small-particle-sized wood biochar at higher application rate of 10 t ha^{-1} (*Fig. 2*).

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Table 3. *Yield biomass/per plot of pea plant during first and second year of the experiment under groundwater and wastewater irrigation treatments*

	2016		2017	
	GW	**WW**	**GW**	**WW**
Control	337.67 ± 63.08ab	254.33 ± 77.41ab	96.33 ± 4.6 b*	44 ± 10.07abc
BM5	299.33 ± 73.71abcd	220.3 ± 56.62ab	238.33 ± 67.18ab*	44 ± 07.78ab
BM10	270.00 ± 27.01bc	296.8 ± 71.50ab	150 ± 21.92a*	46.67 ± 16.22abc
BM15	321.67 ± 75.67abcd	110.46 ± 36.18b	157.67 ± 24.06a*	40.33 ± 05.04bc
BW5	433.33 ± 85.39ab*	256.46 ± 24.92a	258.33 ± 107.71ab*	53.33 ± 08.69ab
BW10	322.67 ± 62.54abc	289.6 ± 12.88a	212 ± 91.17ab*	40.67 ± 06.27b
BW15	414.33 ± 58.08a	208.8 ± 79.23ab	213.67 ± 70.01a*	28.67 ± 01.51c
(5) BW.65	465.67 ± 25.23a*	254.67 ± 54.05ab	237.67 ± 56.69a*	40 ± 04.49bc
(10)BW.65	214.33 ± 04.27c	208.93 ± 84.48ab	262 ± 33.14a*	42.33 ± 05.52bc
(15) BW.65	173.67 ± 08.11d	390.36 ± 76.09a	142.33 ± 55.39ab	73 ± 11.08a

Values are mean ± SE, bars with * represent significant difference between years for a given treatment ($P < 0.05$), while bars with different letters represent significant difference between treatments of a given year ($P < 0.05$)

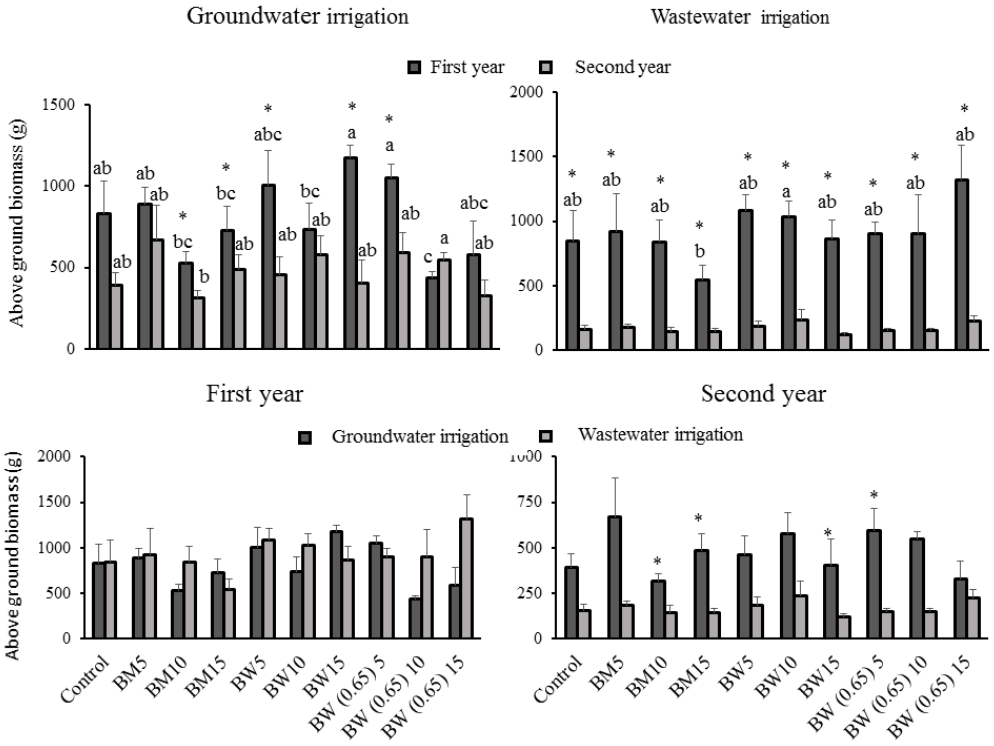

Figure 2. *Aboveground biomass (n = 3) illustrate difference between treatments within a year and between-year difference of a given treatment, values are mean ± SE, bars with * representing significant difference between years for a given treatment ($P < 0.05$), while bars with different letters represent significant difference between treatments of a given year ($P < 0.05$), * represent difference between irrigation types (groundwater and wastewater)*

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In the first year, aboveground biomass was significantly decreased in response of manure derived biochar at higher application rates of 15 t ha^{-1} than the large-particle-sized wood derived biochar amended at higher rate of 10 t ha^{-1} under wastewater irrigation. In the second year, biochar amendments did not influence aboveground biomass under wastewater irrigation ($P < 0.05$; *Fig. 2*).

Under both irrigations, there was a significant difference between the first year and the second year. Under groundwater irrigation, in the first year aboveground biomass was significantly higher in response of manure (higher application rate of 10, 15 t ha^{-1}) and wood biochar (large-particle-sized at higher application rate of 15 t ha^{-1} and small-particle-sized at lower application rate of 5 t ha^{-1}) than in the second year. Under wastewater irrigation, in the first year, aboveground biomass was higher in response of all organic amendments than in the second year. While, in the first year, there was no difference in aboveground biomass in between both irrigation treatments. While, in the second year, aboveground biomass was significantly higher under groundwater as compared to wastewater irrigation, that increased in aboveground biomass in response of biochar amendments (manure and wood) was the same as observed in between the first year and second year, under groundwater irrigation ($P < 0.05$; *Fig. 2*).

In the first year, under groundwater irrigation, harvest index was significantly decreased in response of manure derived at lower application rate of 5 t ha^{-1} than manure biochar at a higher rate of 10, 15 t ha^{-1} and large-particle-sized wood biochar at a higher rate of 10, 15 t ha^{-1}. In the second year, biochar did not influence the harvest index under groundwater irrigation; manure biochar at rate of 10 t ha^{-1} has significantly increased harvest index than at rate of 15 t ha^{-1}. While under wastewater irrigation, no biochar influence was observed for both years (*Fig. 3*). Harvest index increased in the first year as compared to the second year in control than in all other organic amendments; in contrast, increase was observed in response of large-particle-sized wood biochar in the second year as compared to first year under groundwater irrigation. While, there was no significant difference in between the first and the second year under wastewater irrigation. In the first year, there was a significant difference in between all treatments, except for manure biochar at a lower rate of 5 t ha^{-1} and small and large-particle-sized wood biochar at a higher rate of 15 t ha^{-1} (*Fig. 3*).

Ph, EC and organic matter in soil

Under both irrigation treatments, biochar amendments did not influence the soil pH, as well as did not have significant difference in between both irrigation treatments. Under groundwater irrigation, there was no significant influence of biochar on EC; however significantly the highest increase was observed in response of manure biochar at/higher rate of 10 t ha^{-1} than large-particle-sized wood biochar at lower rate of 5 t ha^{-1}. Under wastewater irrigation, EC was significantly increased in response of small-particle-sized wood biochar at lower rate of 5 t ha^{-1} than large-particle-sized wood biochar at higher rate of 10 t ha^{-1}. EC was significantly different in between groundwater and wastewater, this influence was followed in response of manure biochar at higher rate of 15 t ha^{-1}, large-particle-sized wood biochar at lower (5 t ha^{-1}) and higher rate (15 t ha^{-1}) and small-particle-sized at higher rate of 10 t ha^{-1}. EC was higher in wastewater than in groundwater irrigation (*Table 4*). Interactions between irrigation type and biochar types were significant for soil EC ($P < 0.05$; *Table 4*).

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Table 4. *Mean ± SD of pH, EC (µs), organic matter (%), mineral N (%), soluble mineral P, total C and total N in soil under groundwater (GW) and wastewater (WW) irrigation*

Treatments	pH		EC		Organic matter		N%		C%		C:N	
	GW	WW	GW	WW	GW	WW	GW	WW	GW	WW	GW	WW
Control	9.26±0.17	8.90±0.04	495.00±142.1ab	459.00±35.83ab	14.20±1.92b*	26.25±1.19c	0.11	0.18	2.68	3.14	24.59	17.52
BM5	9.63±0.10	8.76±0.09	411.33±57.15ab	652.00±55.89ab	16.92±1.31b*	25.68±0.26c	0.13	0.17	2.9	2.89	22.16	16.76
BM10	9.50±0.04	8.83±0.05	551.00±71.00a	552.33±176.35ab	16.32±1.82b*	21.59±1.63c	0.11	0.14	2.93	2.84	27.12	20.93
BM15	9.70±0.07	8.93±0.02	312.33±32.33b*	478.33±39.21ab	29.21±12.97ab	25.13±2.01bc	0.12	0.16	2.96	3.05	23.86	18.87
BW5	9.50±0.09	8.67±0.15	284.67±30.11b*	541.67±27.85ab	21.32±2.51ab*	38.47±2.35a	0.15	0.2	3.24	3.32	21.07	16.76
BW10	9.23±0.30	8.93±0.02	420.67±123.0ab	456.67±64.29b	22.83±2.13ab*	39.34±1.13a	0.12	0.17	3.29	3.45	28.29	19.81
BW15	9.46±0.19	8.83±0.02	392.00±41.68ab*	593.00±55.89ab	29.54±2.73a*	51.74±5.63a	0.12	0.12	3	3.11	25.73	25.59
BW(0.65)5	9.36±0.19	8.86±0.05	397.67±72.52ab	512.67±11.93ab	18.98±1.99a*	28.72±2.14c	0.1	0.18	2.9	3.41	28.03	18.92
BW(0.65)10	9.30±0.14	8.96±0.02	391.00±78.84ab*	680.00±70.76a	20.49±4.54ab	39.21±4.92ab	0.17	0.21	4.78	3.41	28.95	16.15
BW(0.65)15	9.43±0.19	8.86±0.02	540.00±82.67a	571.00±54.26ab	27.56±1.08a*	34.99±4.28ab	0.15	0.21	3.6	4.33	24.03	20.85

Values are mean ± SE and bars with different letters represent significant differences in between treatments of a given year and * represents significant differences in between the irrigation treatments (P < 0.05)

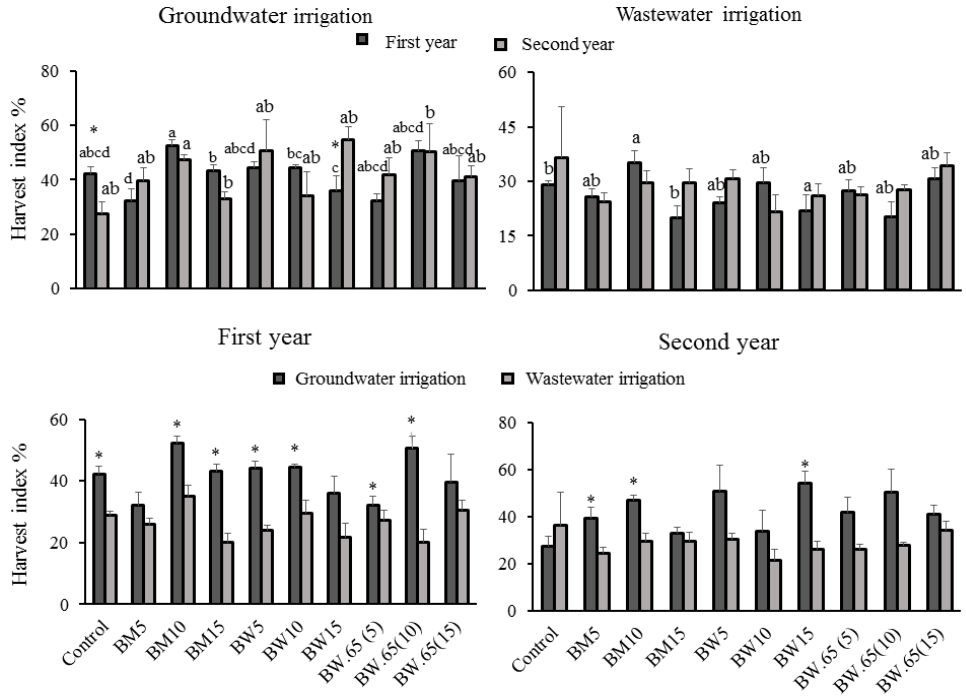

Figure 3. *Harvest index (n = 3) illustrates difference between treatments within year and between-year difference of a given treatment, values are mean ± SE, bars with * representing significant difference between years for a given treatment (P < 0.05), while bars with different letters represent significant difference between treatments of a given year (P < 0.05), * represents difference between irrigation types (groundwater and wastewater) (P < 0.05)*

Under groundwater irrigation, biochar did not have influence on soil organic matter; however, the highest increase was observed in response of large-particle-sized wood biochar at higher rate of 15 t ha^{-1} than the control and manure biochar at lower and higher rate of 5 t ha^{-1}, 10 t ha^{-1}, respectively. Under wastewater irrigation, significantly the highest increase was in response of large-particle-sized wood biochar at higher rate of 15 t ha^{-1} than the lowest in response of small-particle-sized wood biochar at lower rate of 5 t ha^{-1}. Significant differences

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between groundwater and wastewater irrigation were in response of control, manure biochar at lower (5 t ha^{-1}) and higher rate (10 t ha^{-1}) and including all treatments of wood biochar except small-particle-sized wood biochar at higher rate of 10 t ha^{-1}. As compared to groundwater, wastewater has maximum increased in organic matter (*Table 4*).

Finally, wastewater irrigation with biochar amendments has a more positive influence on EC and organic matter than soil pH. C and N concentrations were higher in response of wastewater irrigation than the groundwater irrigation but in contrast, C: N ratio was higher in response of groundwater irrigation than the wastewater irrigation. These results are based on pooled soil samples; although, C and N concentrations were higher in response of wood derived biochar than the manure derived biochar under both irrigations than the control ($P < 0.05$; *Table 4*).

Mineral nitrogen (N) and soluble phosphorus (P)

Under groundwater irrigation, soluble phosphorus (P) was significantly higher in response of manure derived biochar at higher rate of 15 t ha^{-1}, lower rate of 5 t ha^{-1} and large sized wood derived biochar at higher rate of 15 t ha^{-1} than control respectively; amongst all the treatments manure derived at higher of 15 t ha^{-1} had profound influence. While, under wastewater irrigation biochar did not influence P in soil; there was significant difference between both irrigation treatments like, control, wood derived biochar at all its application rates (5,10 and 15 t ha^{-1}) and small particle sized wood derived biochar at 5 and 10 t ha^{-1}. In contrast of P, nitrogen (N) was significantly higher under wastewater irrigation (*Fig. 4*). However under groundwater irrigation P was significantly higher in response of manure derived biochar at higher rate of 15 than control and under wastewater irrigation N was decreased in response of manure derived biochar at higher rate of 15 t ha^{-1} than control, manure derived biochar at lower rate of 5 t ha^{-1}, large sized particle of wood derived biochar at the application rate of 10, 15 t ha^{-1} and small sized wood derived biochar amendments at lower and higher rate of 5, 10 t ha^{-}, respectively. There was significant difference between both irrigation treatments except manure derived biochar at lower rate of 5 t ha^{-1} and small sized wood derived biochar at lower rate of 5 t/ha and higher rate of 15 t ha^{-1}.

Macro and microaggregates stability

Under groundwater irrigation, for 2000 µm aggregates stability was significantly increased in response of small-particle-sized wood biochar at higher rate than manure biochar at lower (5 t ha^{-1}) and higher rate (15 t ha^{-1}), large-particle-sized wood biochar at higher rate of 10 ha^{-1}. For 2000-650 µm aggregates stability was significantly decreased in response of large-particle-sized wood biochar at higher rate of 10 t ha^{-1} than control. For 650-85 µm aggregates stability was significantly decreased in response of large-particle-sized wood biochar at higher rate of 10 t/h than manure biochar at lower rate of 5 t ha^{-1} and there was no significant influence of biochar for 85-10.6 µm aggregates stability. Under wastewater irrigation, for 2000 µm aggregates stability was significantly increased in response of manure biochar at lower rate of 5 t ha^{-1} than manure biochar at higher rate of 10, 15 t ha^{-1} and small-particle-sized wood biochar at lower rate of 5 t ha^{-1}. For 650-85 µm aggregates, stability was significantly decreased in response of manure biochar at lower rate of 5 t ha^{-1} than control, manure biochar at higher rate of 10, 15 t ha^{-1}. However, large-particle-sized wood biochar at higher rate of 10 t ha^{-1} and for 85-10.6 µm aggregates stability significantly increased in response of manure biochar at higher rate of 10 t ha^{-1} than manure biochar at higher rate, small-large-particle-sized wood biochar at higher rate of 15 and 10 t ha^{-1}, respectively (*Fig. 5*).

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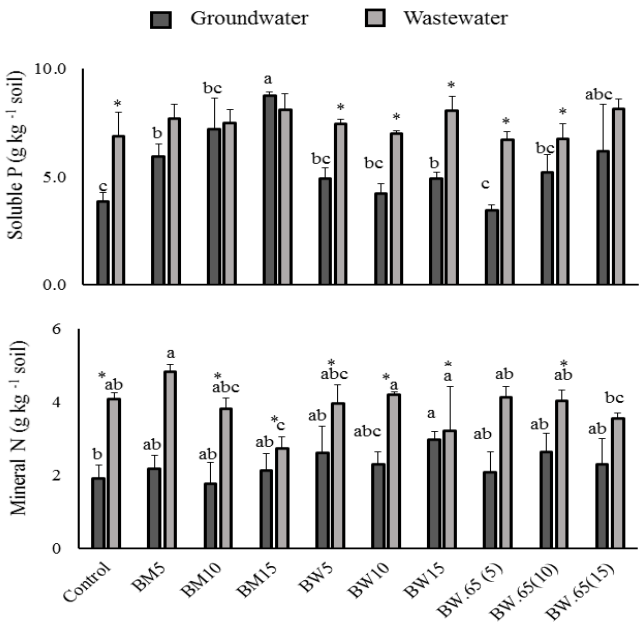

Figure 4. *Soluble phosphorus and Mineral nitrogen in soil (n = 3) under-ground and waste water irrigation treatments. Values are mean ± SE, Bars with different letters represent significant difference between organic amendment treatments of a given irrigation treatment while * represents differences between irrigation treatments (groundwater vs wastewater)*

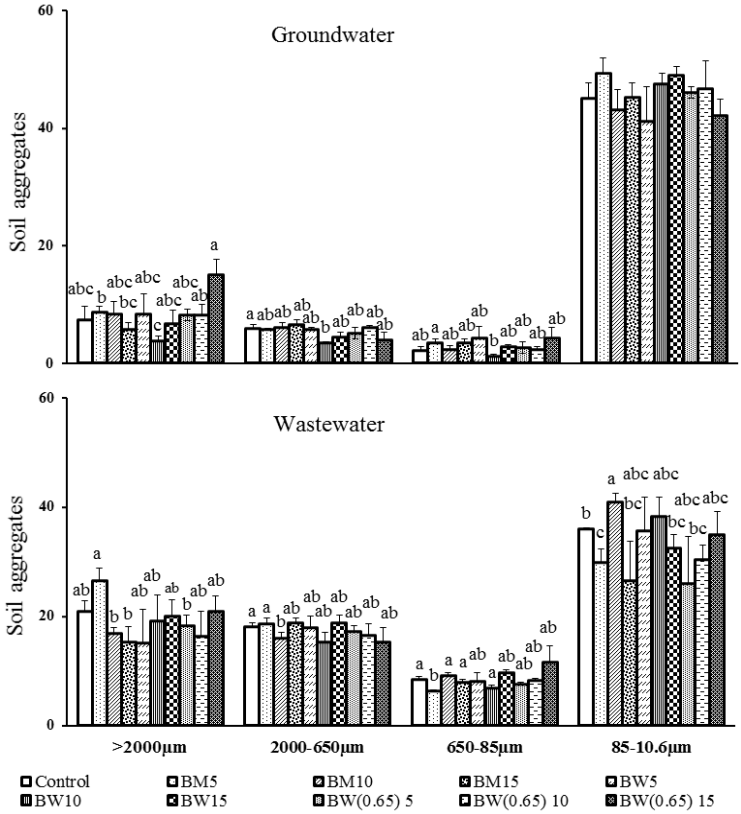

Figure 5. *Soil aggregates of various sizes of soils under groundwater and wastewater irrigation. Values are mean ± SE and bars with different letters represent significant difference between treatments of a given year ($P < 0.05$)*

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However, macroaggregates stability (2000 µm) was significantly different in between the groundwater and wastewater irrigation except in response of large-particle-sized wood biochar at lower rate of 5 t ha^{-1}, small-particle-sized wood biochar at higher rate of 10 and 15 t ha^{-1}; however, wastewater has higher aggregates stability for macroaggregates than the groundwater (*Fig. 6*).

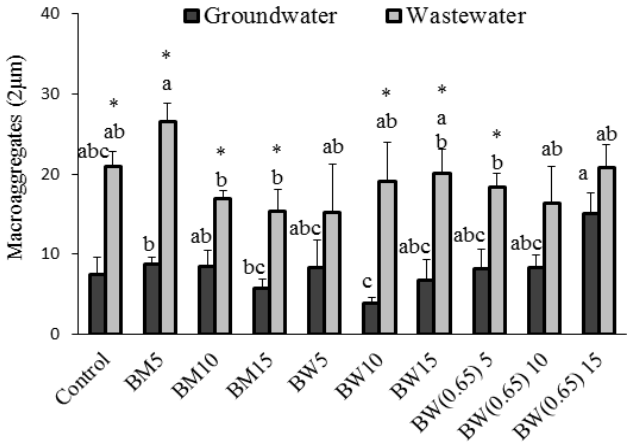

Figure 6. *Macroaggregates in soil (n = 3) under-ground and waste water irrigation treatments. Values are mean ± SE, Bars with * represent the difference within irrigation treatments (P < 0.05)*

Bacterial diversity

Two phylogenetic trees describe the bacterial diversity under groundwater and wastewater irrigation (*Fig. 7*). These results are based on pooled soil samples; however, number of bacterial genera are two-fold higher in the soil samples from groundwater irrigation treatment as compared to the soil samples from wastewater irrigation treatment. Under groundwater, phylogenetic tree indicates the presence of three bacterial genera i.e., *Vibrio, Tepidamorphus gemmatus and Brevundimonas diminuta*. Under wastewater irrigation, phylogenetic tree indicates the presence of three species that belong to the genera *Vibrio* i.e., *V. parahaemolyticus*, *V. alginolyticus* and *V. campbelli* (*Fig. 8*).

Discussion

Harvest index of yield

In the first year, under groundwater irrigation, yield biomass and aboveground biomass significantly decreased due to higher surface area of small-particle-sized wood biochar that has the highest adsorption capacity of nutrients at higher application rate (10 t ha^{-1}), this might have reduced the nutrients availability and in contrast, lower application rate have significantly increased the aboveground biomass (Manzoor et al., 2019). However, the influence of small-particle-sized wood derived biochar at higher application rate was inconsistent for the first and second year. In the first year, nutrients availability decreased by adhering the nutrients with biochar surface area, however in the second year, continuous biochar amendments have increased the free nutrients availability for the crop more than the first year. While in the second year, aboveground

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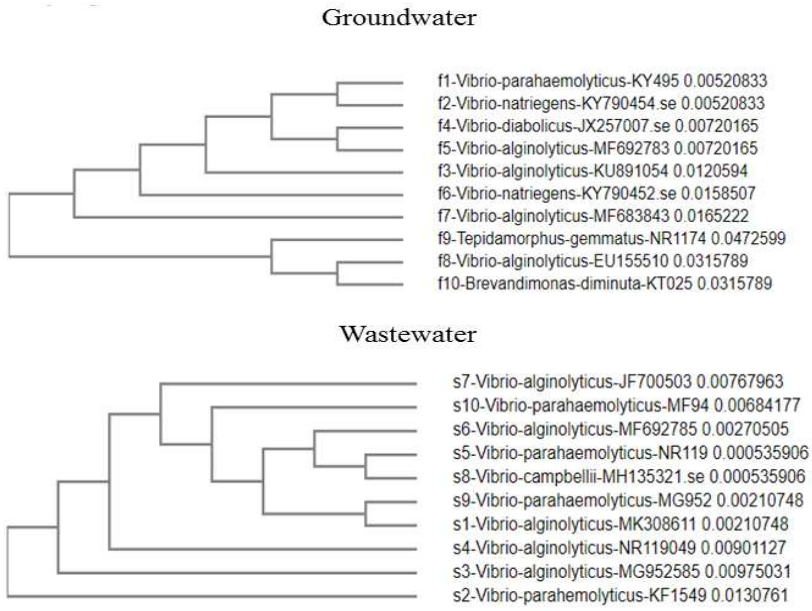
biomass was decreased in response of manure biochar at higher application rate of 10 t ha^{-1}. Manure biochar is the rich source of nutrients than the other biochar types, for this, continuous amendments of biochar built up the osmotic pressure on roots reduced the aboveground biomass (Manzoor et al., 2019). In response of second year, decrease in yield was not in favor of previous empirical reports of our finding that biochar amendments increase the crop production (Gul and Whalen, 2016; Rawat et al., 2019).



S=sewage, F=fresh (1: control, 2: BM5,3: BM 10,4: BM15, 5: BW5, 6: BW 1 7: BW15, 8: BW (0.65) 5, 9: BW (0.65) 10, 10: BW (0.65) 15)

Figure 7. *Phylogenetic tree of the microbial structure present in soil under groundwater and wastewater irrigation*

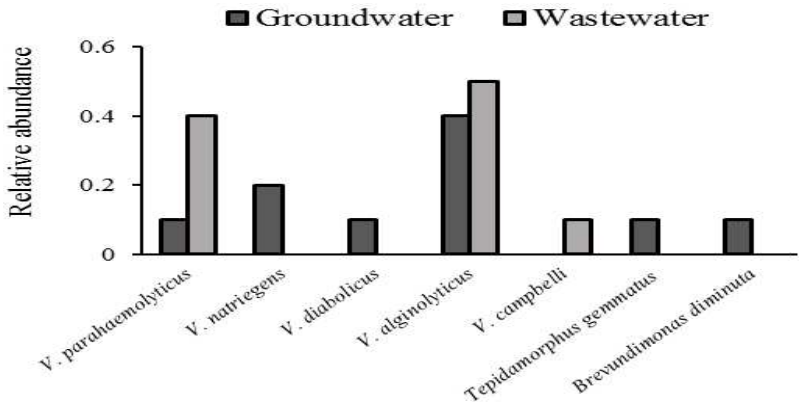


Figure 8. *Relative abundance of microorganisms underground and wastewater irrigation in response of biochar amendments*

In the first year, under wastewater irrigation, aboveground biomass was significantly decreased in response of higher application rate than large-particle-sized wood derived biochar. Interestingly, our findings are in favor of Manzoor et al. (2019) report for in

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response of organic amendments (decrease in manure derived at high application rate of 15 t ha^{-1}, increase in large particle-sized wood derived biochar at high application rate of 10 t ha^{-1}) under wastewater irrigation; however large-particle-sized have less adsorption capacity than the small particle sized, this increase in nutrients availability have positive influence on aboveground biomass. Despite the wastewater, is a rich source of nutrients the yield reduced in response of biochar amendments.

Possible explanation of decrease in harvest index, under groundwater irrigation is that manure and wood biochar at higher rate adhere the trace elements with biochar surface (Haider et al., 2019); while under wastewater irrigation, this results in profound negative influence on crop yield. Harvest index was significantly higher as compared to wastewater, under groundwater irrigation; however, profound influence of biochar might be suppressed in response of non-pyrogenic organic amendments with biochar amendments of manure at application rate of 5 t ha^{-1} (Bonanomi et al., 2017). Interestingly, Kammann et al. (2016) reported that influence of biochar and non-pyrogenic organic matter amendments have very few studies and merits further evaluation. In contrast, in the second year, as compared to wastewater, under groundwater irrigation harvest index was not significantly different in between the treatments except for manure derived biochar at higher rate of 5, 10 t ha^{-1}, large-particle-sized wood derived biochar at higher rate of 15 t ha^{-}. Overburden of biochar have negative influence on crop production; continuous amendments of biochar did not have positive influence in the second year than first year on aboveground biomass and harvest index. Our findings are in agreement of previous literature that groundwater irrigation have more positive influence on the following crops; pea plant and maize (Mensah and Frimpong (2018); Manzoor et al., 2019) respectively. Although wastewater is reported to improve soil quality, very few studies reported biochar responses under wastewater irrigation. This merits further long-term experiments to understand the influence of biochar under wastewater irrigation and impact on crops yield.

Ph, EC and organic matter in soil

In case of pH, our findings are not in agreement with Dume et al. (2016); Hameeda et al. (2019) reported that biochar have positive influence and are in agreement of other empirical reports that organic amendments did not have influence (Abrishamkesh et al., 2015) on soil; however, have negative influence (Gul et al., 2015) on pH in alkaline soil (*Table 2*). Rather than alkaline soil, biochar amendments reported for positive influence on soil pH under acidic soil. While, an increase in EC is related to the nutrients availability in soil under wastewater irrigation treatment; for this, EC was increased in response of biochar amendments at higher rate by increasing available nutrients adsorption. As a result, biochar amendments have a more positive influence for an increase in EC under wastewater irrigation than ground water. Increase in total dissolved solids in wastewater increase EC. An increase in pH and EC based on physical and chemical properties of biochar type; like, Chintala et al. (2013) reported that corn biochar (stover) have higher increase in pH than switchgrass biochar at all biochar amendments. EC and organic matter was increased in response of wood derived biochar at higher rate of (15 t ha^{-1}) than the manure biochar. Higher rate of biochar amendments increases the release of free cation and anions in the soil, which results in an increase in EC (Reeve et al., 2016); biochar have positive influence on EC and organic matter in response of wood biochar at higher rate on EC and organic matter in soil.

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In results of pooled soil samples, higher C under wastewater irrigation favors the indirect increase of organic matter and promote macroaggregates stability in biochar amended soil. Increase in organic matter favors the formation of macroaggregates in response of higher microbial diversity in soil. Our findings are against of Beidermen and Harpole (2012) findings that C: N ratio did not have pronounced influence crop productivity: interestingly, more extensive research is required to evaluate the biochar influence on C: N ratio. Finally, wastewater irrigation with biochar amendments have favored the increase in soil EC, organic matter and C% and N %.

Mineral nitrogen (N) and soluble phosphorus (P)

Possible increase in P in response of manure derived biochar at higher rate of 15 t/ha than control might be that manure is richer source of nutrients than wood derived biochar, so this favors the increase in P content under groundwater irrigation; as compared to ground water, wastewater irrigation has higher content of P at higher rates of all biochar amendments. Because, wood derived biochar at higher rate had positive influence on P in response of rich organic matter irrigation treatment (wastewater).

Under wastewater irrigation, N was significantly higher in response of manure derived biochar at lower rate of 5 t ha^{-1} than the higher rate of 15 t ha^{-1}. Rich source of nutrients in response of manure under wastewater irrigation has balanced more at lower rate than at the higher rate. While, second reason might be in response of available P and N content in biochar amended treatments and air-dried cow manure. For P, manure biochar and dried cow manure amendments were higher in P than the wood derived biochar. However, manure biochar and dried cow manure had higher N than the wood derived biochar. So, manure derived biochar response was profound for N and P availability in soil under both irrigation treatments than the wood derived biochar.

Finally, current findings are inconsistent with previous work that organic amendments increase mineral N and soluble P content in soil (Clough et al., 2013; Ameloot et al., 2015) but wastewater has high content of nutrients in response to biochar amendments and more increased N and P availability in soil than groundwater. This merits further research to understand biochar influence under wastewater irrigation with different soil types. Biochar amendments, reported for positive influence on the soluble phosphorus and nitrogen in soil (Nelson et al., 2017; Singh et al., 2018).

Macro and microaggregates stability

Soil organic matter improves the soil aggregates stability. In our findings, soil organic matter was higher in wastewater than in groundwater as a result aggregates stability was high under wastewater irrigation. Biochar has positive influence on soil organic matter and water saturation, this interaction favors the soil aggregates stability (Ouyang et al., 2013). In our findings, amendments of biochar has significant influence for macroaggregates (>200 μm) under wastewater irrigation (*Fig. 5*). Therefore, it appears that biochar has potential to improve the soil aggregates stability (Olmo et al., 2014; Gul et al., 2015). These findings merit further to evaluate association between biochar types at different rate and soil types (Herath et al., 2013; Nelison et al., 2014).

Bacterial diversity

Despite of significantly higher water-stable soil macroaggregates, with significantly higher concentration of mineral N and soluble mineral P, wastewater-irrigated soil had

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lower bacterial diversity as compared to groundwater irrigation. The electrical conductivity of wastewater-irrigated soil was in general higher than that of groundwater-irrigated soil, high concentration of nutrients and high electrical conductivity might have had a negative influence on microbial diversity. More extensive research is required to evaluate microbial abundance and diversity as influenced by wastewater-irrigation-induced high concentration of nutrients in soil and to link microbial properties to crop yield.

Conclusion

Contrary to present hypotheses, wastewater irrigation did not improve harvest index of pea as compared to groundwater irrigation and amendment of biochars in general did not cause a positive influence on pea growth and soil quality. Although, soil under two years of wastewater irrigation had significantly stronger macroaggregates, higher concentration of mineral N but soluble P was higher under groundwater irrigation, groundwater irrigated soil had 2-fold higher number of bacterial genera than the soil under wastewater irrigation. Lower bacterial diversity in wastewater-irrigated soil can be a reason of lower harvest index of plants as compared to groundwater irrigation. Further research is required to evaluate the influence of wastewater-irrigation-induced high level of nutrients and electrical conductivity on soil microbial abundance and diversity and to link microbial properties to crop yield (Tóthmérész, 1995).

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APPENDIX

Table A1. *Treatments with their replicates, mean and skewness values of harvest index (Data to Fig. 3 in the article)*

Treatments	GW				WW			
	2016		2017		2016		2017	
	HI	R+MSL	HI	R+MSL	HI	R+MSL	HI	R+MSL
Control	42.05		36.80		29.72		9.66	
	47.51	42.23	27.42	36.53	31.21	28.95	68.00	36.53
	37.13	SKEW=0.06	17.99	SKEW=-0.003	25.91	SKEW=-0.475	31.94	SKEW=0.279
BM5	31.45		50.88		21.39		18.03	
	41.68	32.17	35.41	24.30	26.04	25.83	27.48	24.30
	23.37	SKEW=0.14	32.41	SKEW=0.635	30.05	SKEW=-0.091	27.39	SKEW=-0.706
BM10	56.76		50.35		36.70		28.57	
	53.13	52.29	42.86	29.68	41.46	35.06	37.07	29.68
	46.97	SKEW=-0.30	48.31	SKEW=-0.492	27.03	SKEW=-0.388	23.40	SKEW=0.287
BM15	44.68		29.62		13.16		28.00	
	46.78	43.10	39.14	29.55	26.55	19.98	22.44	29.55
	37.85	SKEW=-0.55	30.42	SKEW=0.688	20.23	SKEW=-0.068	38.21	SKEW=0.342
BW5	43.32		39.46		22.80		24.91	
	49.30	44.28	78.48	30.64	28.19	23.96	31.52	30.64
	40.22	SKEW=0.36	34.14	SKEW=0.668	20.90	SKEW=0.510	35.48	SKEW=-0.295
BW10	41.86		14.34		31.83		12.73	
	44.44	44.37	35.67	21.64	36.96	29.62	20.13	21.64
	46.79	SKEW=-0.06	52.04	SKEW=-0.159	20.06	SKEW=-0.438	32.08	SKEW=0.278
BW15	44.56		46.09		32.76		25.83	
	40.97	35.98	66.35	26.05	18.53	21.83	18.99	26.05
	22.41	SKEW=-0.64	50.99	SKEW=0.539	14.20	SKEW=0.552	33.33	SKEW=0.055
BW.65 (5)	38.85		26.80		33.42		25.93	
	50.36	32.17	46.23	26.40	28.52	27.26	22.22	26.40
	45.65	SKEW=-0.22	52.41	SKEW=-0.541	19.83	SKEW=-0.326	31.06	SKEW=0.195
BW.65(10)	57.63		50.17		24.67		28.18	
	52.49	50.47	29.35	27.77	10.12	20.16	25.00	27.77
	41.29	SKEW=-0.42	71.69	SKEW=0.020	25.69	SKEW=-0.696	30.13	SKEW=-0.282
BW.65(15)	50.82		49.29		23.42		29.25	
	16.82	39.43	32.22	34.30	32.90	30.66	30.49	34.30
	50.66	SKEW=-0.71	41.14	SKEW=-0.055	35.67	SKEW=-0.562	43.18	SKEW=0.686

Table A2. *Treatments with their replicates, mean and skewness values of available phosphorus and nitrogen in soil (Data to Fig. 4 in the article)*

Treatments	Phosphorus				Nitrogen			
	GW		WW		GW		WW	
	Phosphorus	R+MSL	Phosphorus	R+MSL	Nitrogen	R+MSL	Nitrogen	R+MSL
Control	4.31		4.31		1.02		4.51	
	2.91	3.87	8.62	6.89	2.33	1.92	3.95	4.08
	4.39	SKEW=-0.7	7.75	SKEW=-0.591	2.41	SKEW=-0.69	3.78	SKEW=0.54
BM5	6.18		6.18		2.59		5.18	
	4.65	5.94	8.07	7.70	2.68	2.19	4.94	4.83
	6.98	SKEW=-0.365	8.86	SKEW=-0.453	1.31	SKEW=-0.697	4.36	SKEW=-0.45
BM10	7.56		7.71		3.10		3.60	
	4.08	7.22	5.99	7.47	0.77	1.79	3.33	3.82
	10.00	SKEW=-0.209	8.71	SKEW=-0.314	1.49	SKEW=0.42	4.51	SKEW=0.56

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BM15	9.00		7.00		2.75		**3.42**	
	8.92	8.74	9.88	8.12	2.62	2.14	2.65	2.74
	8.29	SKEW=-0.675	7.46	SKEW=0.636	1.06	SKEW=-0.693	2.16	SKEW=0.26
BW5	5.28		7.64		4.41		4.72	
	5.75	4.91	7.77	7.45	1.81	2.62	2.75	3.97
	3.71	SKEW=-0.558	6.95	SKEW=-0.636	1.63	SKEW=0.696	4.43	SKEW=-0.65
BW10	4.25		7.14		2.22		4.36	
	5.17	4.22	7.18	6.99	3.07	2.3	4.19	4.22
	3.24	SKEW=-0.061	6.64	SKEW=-0.691	1.61	SKEW=0.199	4.11	SKEW=0.45
BW15	4.21		6.60		3.42		4.67	
	5.35	4.92	9.44	8.06	3.01	2.99	4.74	3.22
	5.19	SKEW=-0.652	8.12	SKEW=-0.087	2.55	SKEW=-0.055	0.26	SKEW=-0.71
BW.65 (5)	3.24		7.40		2.15		4.18	
	3.05	3.45	5.90	6.71	3.26	2.08	3.46	4.13
	4.06	SKEW=0.606	6.83	SKEW=-0.280	0.83	SKEW=-0.107	4.74	SKEW=-0.16
BW.65(10)	4.44		7.42		3.75		4.52	
	4.06	5.22	7.80	6.76	2.56	2.63	4.25	4.03
	7.15	SKEW=0.665	5.07	SKEW=-0.655	1.59	SKEW=0.123	3.32	SKEW=-0.57
BW.65(15)	2.59		7.68		1.22		3.94	
	4.79	6.22	7.45	8.12	4.01	2.30	3.36	3.55
	11.28	SKEW=0.523	9.23	SKEW=0.664	1.68	SKEW=0.630	3.36	SKEW=0.71

Table A3. *Treatments with their replicates, mean and skewness values of pH and electrical conductivity of soil (Data to Table 4 in the article)*

Treatments	**EC**				**pH**			
	GW		**WW**		**GW**		**WW**	
	EC	**R+MSL**	**EC**	**R+MSL**	**pH**	**R+MSL**	**pH**	**R+MSL**
Control	812		384		9		8.8	
	461	495.00	457	459	9.1	9.27	8.9	8.90
	212	SKEW=0.20	536	SKEW=0.048	9.7	SKEW=0.65	9	SKEW=0.00
BM5	551		447		9.9		8.7	
	333	411.33	601	652.00	9.5	9.63	9	8.76
	350	SKEW=0.69	908	SKEW=0.380	9.5	SKEW=0.71	8.6	SKEW=0.53
BM10	719		322		9.4		8.9	
	506	551.00	351	552.33	9.5	9.50	8.9	8.83
	428	SKEW=0.50	984	SKEW=0.702	9.6	SKEW=0.00	8.7	SKEW=-0.71
BM15	391		438		9.7		8.9	
	265	312.33	574	478.33	9.8	9.67	9	8.93
	281	SKEW=0.66	423	SKEW=0.681	9.5	SKEW=-0.38	8.9	SKEW=0.71
BW5	310		605		9.7		8.3	
	212	284.67	488	541.67	9.3	9.50	8.8	8.67
	332	SKEW=-0.61	532	SKEW=0.292	9.5	SKEW=0.00	8.9	SKEW=-0.63
BW10	267		300		9.7		8.9	
	722	420.67	521	456.67	9.5	9.23	8.9	8.93
	273	SKEW=0.71	549	SKEW=-0.674	8.5	SKEW=-0.63	9	SKEW=0.71
BW15	475		624		9.8		8.8	
	299	392.00	693	593.00	9	9.47	8.8	8.83
	402	SKEW=-0.21	462	SKEW=-0.447	9.6	SKEW=-0.53	8.9	SKEW=0.71
BW.65 (5)	575		490		9.7		8.8	

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	300	397.67	508	512.67	9.5	9.37	9	8.87
	318	SKEW=0.70	540	SKEW=0.327	8.9	SKEW=-0.53	8.8	SKEW=0.71
BW.65 (10)	583		603		9.3		9	
	277	391.00	853	680.00	9.6	9.30	9	8.97
	313	SKEW=-0.67	584	SKEW=0.694	9	SKEW=0.00	8.9	SKEW=-0.71
BW.65 (15)	739		538		9		8.8	
	408	540.00	476	571.00	9.5	9.43	8.9	8.87
	473	SKEW=0.60	699	SKEW=0.483	9.8	SKEW=-0.29	8.9	SKEW=-0.71

Table A4. *Treatments with their replicates, mean and skewness values of organic matter of soil (Data to Table 4 in the article)*

Treatments	GW		WW	
	OM	R+MSL	OM	R+MSL
Control	14.22		27.30	
	10.12	14.21	28.10	26.25
	18.28	SKEW=-0.01	23.36	SKEW=-0.63
BM5	18.81		25.35	
	13.71	16.92	25.36	25.68
	18.25	SKEW=-0.67	26.33	SKEW=0.71
BM10	17.08		19.79	
	12.14	16.33	19.42	21.60
	19.77	SKEW=-0.34	25.60	SKEW=0.70
BM15	14.53		21.46	
	12.15	29.22	29.82	25.13
	60.98	SKEW=0.70	24.11	SKEW=0.41
BW5	15.76		43.28	
	26.40	21.32	33.33	38.47
	21.81	SKEW=-0.17	38.81	SKEW=-0.12
BW10	19.27		39.09	
	27.92	22.83	41.88	39.34671
	21.31	SKEW=0.55	37.08	SKEW=0.20
BW15	24.38		54.09	
	35.81	29.55	62.34	51.74
	28.46	SKEW=0.33	38.80	SKEW=-0.35
BW.65 (5)	20.39		33.81	
	14.24	18.99	25.07	28.73
	22.34	SKEW=-0.54	27.30	SKEW=0.52
BW.65 (10)	29.93		49.56	
	20.86	20.49	39.43	39.22
	10.68	SKEW=-0.07	28.66	SKEW=-0.04
BW.65 (15)	25.35		45.48	
	29.95	27.56	29.95	34.99
	27.38	SKEW=0.14	29.55	SKEW=0.71

Table A5. *Treatments with their replicates, mean and skewness values of soil aggregates under groundwater irrigation in soil (Data to Fig. 5 in the article)*

Treatments	GW							
	2 mm	R+MSL	1.5 mm	R+MSL	1 mm	R+MSL	0.5 mm	R+MSL
Control	2.736009		4.888195		2.429318		50.65841	
	7.285806	7.43	5.63797	5.95	3.543336	2.15	39.63722	45.12
	12.28112	SKEW=0.06	7.310892	SKEW=0.43	0.478925	SKEW=-0.32	45.05026	SKEW=0.02
BM5	9.985176		6.28561		1.973618		45.9584	

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	9.748876	8.79	5.502547	5.78	3.52692	3.47	46.37159	49.38
	6.637308	SKEW=-0.69	5.543312	SKEW=0.70	4.91096	SKEW=-0.07	55.79639	SKEW=0.70
	12.45582		4.973254		0.867607		36.45588	
BM10	3.919806	8.42	5.015299	6.03	3.068777	2.34	50.85363	43.09
	8.870346	SKEW=-0.19	8.110636	SKEW=0.71	3.080003	SKEW=-0.71	41.96749	SKEW=0.28
	5.742632		5.102102		1.627448		39.38699	
BM15	3.240332	5.77	8.272209	6.59	4.493055	3.44	48.31318	45.30
	8.324085	SKEW=0.02	6.396105	SKEW=0.22	4.198041	SKEW=-0.68	48.20249	SKEW=-0.70
	4.60829		5.81425		1.08485		49.24993	
BW5	16.7014	8.33	6.419233	5.75	8.969978	4.25	26.98852	41.26
	3.666492	SKEW=0.69	5.02482	SKEW=-0.16	2.695575	SKEW=0.59	47.55044	SKEW=-0.69
	5.043806		3.125664		0.886441		43.67078	
BW10	4.386047	3.82	3.865082	3.53	0.651206	1.17	51.15278	47.60
	2.025192	SKEW=-0.57	3.589129	SKEW=-0.30	1.981292	SKEW=0.62	47.9755	SKEW=-0.18
	4.750178		2.172818		0.906237		43.12011	
BW15	12.93283	6.77	7.090777	4.52	4.675428	2.85	48.52959	48.98
	2.633543	SKEW=0.59	4.288641	SKEW=0.17	2.975353	SKEW=-0.12	55.29207	SKEW=0.14
	7.340262		3.271642		2.293869		47.86555	
BW.65 (5)	3.840444	8.26	6.791311	5.19	3.438216	2.63	42.26027	46.13
	13.60005	SKEW=0.33	5.504442	SKEW=-0.32	2.154307	SKEW=0.68	48.24992	SKEW=-0.70
	7.444499		6.498663		1.561937		35.22507	
BW.65(10)	5.253505	8.29	6.565133	6.13	2.188311	2.36	51.64258	46.77
	12.17171	SKEW=0.41	5.338416	SKEW=-0.70	3.329189	SKEW=0.34	53.42874	SKEW=-0.68
	10.50179		6.983791		4.382236		36.30166	
BW.65(15)	21.34858	15.02	2.463665	4.06	0.48168	4.29	41.37479	42.10
	13.19686	SKEW=0.53	2.722287	SKEW=0.70	8.008052	SKEW=-0.04	48.61392	SKEW=0.21

Figure A6. *Treatments with their replicates, mean and skewness values of soil aggregates under wastewater irrigation in soil (Data to Fig. 5 in the article)*

Treatments	**Wastewater irrigation**							
	2 mm	**R+MSL**	**1.5 mm**	**R+MSL**	**1 mm**	**R+MSL**	**0.5 mm**	**R+MSL**
	17.09		19.05		8.06		35.59	
Control	20.74	21.00	19.07	18.16	9.63	8.47	36.74	35.96
	25.16	SKEW=0.12	16.35	SKEW=-0.71	7.73	SKEW=0.62	35.56	SKEW=0.71
	22.52		19.98		6.18		33.21	
BM5	31.92	26.61	16.54	18.74	6.88	6.33	23.76	29.82
	25.38	SKEW=0.44	19.69	SKEW=-0.69	5.94	SKEW=0.52	32.48	SKEW=-0.69
	15.58		18.24		8.00		40.12	
BM10	15.88	16.99	15.94	16.11	10.28	9.15	44.60	40.98
	19.52	SKEW=0.69	14.14	SKEW=0.15	9.18	SKEW=-0.04	38.22	SKEW=0.45
	8.47		20.59		9.07		9.41	
BM15	19.64	15.34	16.98	18.80	6.80	7.93	38.12	26.61
	17.90	SKEW=-0.64	18.84	SKEW=-0.04	7.92	SKEW=0.01	32.30	SKEW=-0.59
	30.13		22.73		7.16		22.47	
BW5	7.50	15.20	13.56	17.93	5.76	8.20	48.70	35.69
	7.98	SKEW=0.71	17.50	SKEW=0.17	11.68	SKEW=0.55	35.90	SKEW=-0.03
	30.28		18.51		5.43		30.14	
BW10	10.18	19.15	11.62	15.37	6.88	6.82	44.76	38.35
	16.98	SKEW=0.37	15.98	SKEW=-0.31	8.16	SKEW=-0.08	40.16	SKEW=-0.42
	27.27		22.11		9.33		26.47	
BW15	17.98	20.11	17.38	18.83	8.98	9.78	34.92	32.52
	15.08	SKEW=0.55	17.00	SKEW=0.69	11.02	SKEW=0.63	36.18	SKEW=-0.66
	14.50		18.92		6.41		6.41	
BW.65 (5)	18.48	18.37	14.82	17.25	8.26	7.52	42.78	26.04

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	22.12	SKEW=-0.05	18.02	SKEW=-0.57	7.90	SKEW=-0.60	28.92	SKEW=-0.28
BW.65(10)	25.65		21.74		7.71		27.27	
	6.50	16.42	14.96	16.56	8.96	8.33	36.64	30.50
	17.12	SKEW=-0.13	12.98	SKEW=0.56	8.32	SKEW=0.03	27.60	SKEW=0.70
BW.65(15)	25.02		10.23		18.41		29.38	
	14.04	20.88	14.72	15.38	10.50	11.60	45.26	34.90
	23.58	SKEW=-0.66	21.20	SKEW=0.22	5.90	SKEW=0.31	30.06	SKEW=0.70

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SOIL ORGANISM DIVERSITY AND FUNCTIONS IN PLASTIC SHED AND OPEN FIELD SOILS UNDER DIFFERENT CULTIVATION METHODS

TAO, J.[1,3*] – CHEN, J.[2] – DU, S. Y.[1,3] – CHEN, X.[1,3] – LI, S. Y.[1,3] – ZHOU, J.[1,3]

[1]*Key Laboratory of Environmental Changes and Natural Disaster, Ministry of Education, Beijing Normal University, Beijing 100875, China*

[2]*Key Laboratory for Ecology and Pollution Control of Coastal Wetlands (Environmental Protection Department of Jiangsu Province), School of Environmental Science and Engineering, Yancheng Institute of Technology, Yancheng 224051, China*

[3]*Faculty of Geographical Science, Beijing Normal University, Beijing 100875, China*

**Corresponding author*
e-mail: juntao@bnu.edu.cn; phone: +86-10-588-00336

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Abstract. The aim of this study was to investigate the influence of four different cropping management practices in plastic shed soils and open-field soils under organic cultivation or conventional cultivation on soil biodiversity and functions. Soils under plastic sheds and open-field were sampled in Beijing, China, in areas where organic cultivation had been conducted for 5 years and conventional cultivation had also been employed. The results showed that plastic shed production resulted in lower soil bacterial richness and diversity than open-field but higher soil mesofaunal Shannon diversity and Pielou's species evenness indexes. Plastic shed production decreased soil urease and phosphate activities compared to open-field under conventional cultivation. Organic cultivation resulted in higher soil urease, phosphatase and catalase activities than conventional cultivation except for soil urease activity in open soil. Plastic shed soils exhibited significantly higher soil electric conductivity (EC) and NO_3-N contents than open-field soils only under conventional cultivation. By contrast, organic cultivation decreased soil EC and nitrate contents compared to conventional cultivation in plastic shed soils. Organic cultivation resulted in higher soil bacterial OTU, Shannon diversity, Chao1 and ACE indexes than conventional cultivation, but significant differences were observed in the open-field soils.
Keywords: *soil bacterial, soil mesofaunal, enzyme activity, cultivation practices, plastic shed production*

Introduction

Rapid economic development and increasing living standards in China have encouraged the rapid development of plastic shed production. Consequently, the land area used for plastic shed production in China has rapidly increased in recent years. The total area of vegetable crops planted under plastic shed production in China is estimated to reach 3.70 million ha (Luo et al., 2020), and China has become the country that produces the most vegetable crops via plastic shed production worldwide (Duffy, 2017). Moreover, most of the newly developed plastic shed production land has been converted from croplands (Min et al., 2011). However, increasing concerns have been raised regarding soil degradation after plastic shed production. Plastic shed production systems are usually characterized by controlled environmental conditions and can strongly alter soil environmental conditions such as soil temperature and moisture. The higher evapotranspiration observed in plastic shed soils compared with open-field soils can also influence the distribution of the soil solution in the soil profile (Ge et al., 2010). For

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example, soil salinity is significantly higher in plastic shed its than in open-field its (Ju et al., 2007), which potentially affects soil biodiversity and functions in plastic shed its (Paoletti, 1999; Jiang et al., 2015). Soil microorganisms and fauna are an integral part of the soil ecosystem due to their high biodiversity (Van Straalen, 1998; Nannipieri et al., 2003; Andre et al., 2002). They play vital roles in maintaining soil functions such as nutrient cycling, organic matter decomposition, bioremediation and plant growth (Berg, 2009) that are critical for soil sustainability and health (Bhat, 2013; Goldford et al., 2018). Although those affecting these organisms are a relevant concern in plastic shed production, they are still poorly studied, especially regarding the effects on soil biodiversity and function.

In addition, to obtain a higher vegetable crop yield, large amounts of water, chemical fertilization and insecticides are applied in plastic shed production. These conventional agricultural practices may not only increase salt accumulation (Liu et al., 2005), soil acidification, nutrient imbalances and environmental pollution but also alter soil biodiversity and functions (Coolon et al., 2013; Bender et al., 2016). For instance, insecticide application may directly promote changes in population biodiversity and dynamics by killing components of the soil microbial and faunal community (Giller et al., 1997). As a consequence of the decline in biodiversity, increasing concern has arisen about the sustainability of farming practices (Hole et al., 2005). Organic cultivation is considered an important tool for combatting the negative effects of conventional methods in which the inputs are substituted to meet regulations (Geiger et al., 2010; Goldberger, 2011). Many researchers have reported that organic cultivation practices have positive effects on soil microbial populations, diversity and activities (Clark et al., 1998; Girvan et al., 2004; Ponce et al., 2011). A study by Mäder et al. (2002) showed that organically cultivated soils exhibit greater faunal diversity than conventionally cultivated soils. Two well-known meta-analyses conducted by Hole et al. (2005) and Bengtsson et al. (2005) demonstrated the benefits of organic cultivation on soil fauna communities. Soil enzyme activities in soils under organic cultivation were shown to be higher than those under conventional cultivation. Although the majority of research has shown increased soil organism diversity in soils from organic cultivation systems compared to those from conventional cultivation, some studies have obtained different results. A study of Shannon et al. (2002) showed that the differences in the microbial communities of soils under organic and conventional cultivation were subtle rather than dramatic. Some studies have found that organic field soils exhibit lower arthropod diversity than conventional field soils (Shah et al., 2003; Ponce et al., 2011). The inconsistent results between studies suggest that the benefits of organic cultivation for soil biodiversity may vary according to factors such as management systems, climate and crop type (Hole et al., 2005). For example, organic cultivation in plastic shed soils exhibited fewer examples of specific studies than in open-field soils. Soil biodiversity, including that of both microorganisms and fauna, has been less well studied under organic cultivation in plastic shed fields (Madzaric et al., 2018), and there are a lack of studies concerning soil enzyme activity.

In this study, we characterized soil bacterial and mesofaunal diversity and measured the activities of soil extracellular enzymes and soil properties in plastic shed and open-field soils under organic and conventional cultivation. We hypothesized that (1) plastic shed production would decreased the diversity of soil organisms and enzyme activities compared with open-field under conventional cultivation, but the negative effects may be alleviated by organic cultivation, and (2) organic cultivation increases soil

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biodiversity and enzyme activities compared to conventional cultivation, but plastic shed production may reduce these positive effects. Furthermore, this investigation could help farmers to improve their practices and enable stakeholders to develop future strategies for soil organism diversity and functioning and sustainable agriculture by saving inputs and preventing environmental damage.

Methods and material

Soil sampling

The study was conducted at Horticultural Farms located at two sites in Shunyi, Beijing, China, in March 2016. The climate in the region is a warm temperate subhumid climate, with 80% of annual precipitation (610 mm) falling from June to August. The mean annual air temperature is 11.5 °C, and the total annual sunshine (hours) is 2750 h.

The soils were sampled when the final harvest of the crops was performed to avoid the effects of direct fertilization during the next growing season. The fields managed under an organic farming approach were located in Beiwu County (40° 04′ N, 116° 49′ E). Crops have been grown under greenhouse conditions at these sites, typically in plastic sheds and open fields. All of these fields were located near each other within a continuous field area of approximately 11 ha. They were conventionally cultivated for several years before being converted to an organic system in 2010. On the organic horticultural farm, no chemical fertilizers or pesticides were used. The fields managed under the conventional farming approach were located in Lisui County (40° 05′ N, 116° 45′ E). These fields have been conventionally cultivated for more than 6 years. Crops in these areas have been grown under greenhouse conditions, typically in plastic sheds and in open-field soils. Further details of the major crops that were grown and fertilizer applied to the soils in the various fields are provided in *Table 1*.

Table 1. *Characteristics of the land management practices investigated in this study*

Treatments	Major crop	Fertilizer	Other farming practices
Plastic shed field + organic cultivation	Rotation: *Solanum lycopersicum, Capsicum annuum, Solanum integrifolium*	Organic fertilizer included: Total N 1.94 g/kg, Total P 0.58 g/kg, Total K 0.84 g/kg. Organic matter 182.45 g/kg; Application amount: 10000-12000 kg/ha	Soil disinfestation: irrigated and covered with plastic film lasted for a period of four weeks in July every year (Huang et al., 2019); Pollination method: bee-pollinated (drone density: (1200 individual/ha); Pest control: stick insect net in yellow; Weed control: manual weeding
Open field + organic cultivation	Corn	Organic fertilizer was same as above; Application amount: 5000-6000 kg/ha	Soil disinfestation: no; Pollination method: Natural pollination; Pest control: no; Weed control: manual weeding
Plastic shed field + conventional cultivation	**Rotation:** *Solanum lycopersicum, Capsicum annuum, Solanum integrifolium*	Chemical fertilizer was composed of diammonium phosphate and compound fertilizer (6:1), and included: total nutrients ≥ 680 g/kg, N 180 g/kg, P_2O_5 460 g/kg, K_2O 70 g/kg; Application amount of basic fertilizer: 450 kg/ha; Application amount of supplement fertilizer: 360 kg/ha	Soil disinfestation: no Pollination method: hand pollination; Pest control: Chemical insecticide (neonicotinoids, pyrethroid, benzoylurea and carbamate); Weed control: herbicide (organophosphorus and ether-derivative)
Open field + conventional cultivation	Corn	Chemical fertilizer was same as above; Application amount of basic fertilizer: 200 kg/ha; Application amount of supplement fertilizer: 180 kg/ha	Soil disinfestation: no Pollination method: hand pollination; Pest control: chemical insecticide (neonicotinoids, pyrethroid, benzoylurea and carbamate); Weed control: herbicide (organophosphorus and ether-derivative)

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Three 10-cm-deep soil cores (5 cm diameter) were taken from the three subplots and mixed to form a composite soil sample. After removing visible plant roots and stones, the composite samples were passed through a 2-mm sieve and divided into two halves. The first half was air-dried and subsequently stored at 4 °C for the analyses of soil physicochemical properties. The second half of the soil samples was packed in polyethylene bags and immediately stored at −20 °C until DNA extraction. Next-generation high-throughput sequencing was applied to determine the composition and diversity of soil bacterial communities.

Bacterial community

Soil genomic DNA was extracted from 0.5 g of dried soil per sample with the Fast DNA Spin Kit (MP, Biomedicals, USA) following the manufacturer's protocol. The extracted DNA was diluted to 10 ng/μL, checked using 1% agarose gel electrophoresis, and stored at −20 °C until PCR analysis. Soil bacterial communities were evaluated by amplifying the V4 region of the 16S rRNA gene using the primer set 515F (5′-GTGCCAGCMGCCGCGG TAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT -3′).

All PCR amplifications were performed in a 30-μL reaction volume containing 15 μL Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 μM forward/reverse primers, and 10 ng template DNA (Xiong et al., 2012). The PCR conditions were as follows: 98 °C for 1 min; 30 cycles of 10 s at 98 °C, 30 s at 50 °C and 30 s at 72 °C, and finally 5 min at 72 °C (Xue et al., 2017). Amplicons (200–400 bp) were confirmed on 2% EtBr agarose gels and purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Following quantitation, equal concentrations of the purified amplicons were combined in a single tube. Sequencing libraries were generated with an NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocol, and index codes were added. Library quality was assessed on a Qubit 2.0 Fluorimeter (Thermo Fisher Scientific, Carlsbad, CA, USA) and an Agilent Bioanalyzer 2100. The pooled amplicons were subjected to paired-end sequencing on the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) (Caporaso et al., 2012).

The paired-end reads were merged with FLASH, a fast and accurate tool designed specifically for overlapping reads. Sequence reads were assigned according to sample-specific barcodes. The sequences were analyzed in QIIME (Quantitative Insights into Microbial Ecology) with in-house Perl scripts for calculating alpha- (within-sample) and beta- (between-sample) diversity. The reads were passed through QIIME quality filters before pick_de_novo_otus.py was used to select operational taxonomic units (OTUs) with an OTU table. Sequences showing 97% nucleotide similarity were assigned to the same OTUs. A representative sequence for each OTU was screened and used to assign the taxonomic composition in the Greengenes (bacterial 16S rRNA) databases.

Mesofaunal community

Modified Tullgren funnel extractors were used for collecting the soil mesofauna. The extracted samples were collected from one soil core (10 ×10 cm), and the depth of the soil core was 15 cm. These samples were taken back to the laboratory and extracted for 48 h at 28 °C. A total of 24 soil mesofauna samples were collected (4 treatments × 3 duplications × 3 plots). All of the soil fauna samples were preserved in 75% alcohol.

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The soil fauna were counted under an OLYMPUS SZX16 stereoscopic microscope (Olympus Co., Tokyo, Japan) and were identified to the family level or to the suborder if identification to the family level was not possible (Yin, 1998; Zheng and Gui, 1999). Soil fauna community diversity was quantified using the Shannon-Wiener index (*H'*), Pielou evenness index (*J*) and Menhinick richness index (*d*) (Huang et al., 2006).

Soil properties and enzyme activity

The soil analysis followed the 'Analysis of soil characteristics' guidelines (Lu, 2000). The organic matter content was analyzed in ground soil by using the Walkley and Black dichromate oxidation method. Soil pH was measured in a 1:5 soil:water (distilled water) slurry using a glass electrode. Available P was extracted with 0.5 mol/L $NaHCO_3$ by the Olsen method. Available K was extracted with 1 mol/L NH_4OAc and was determined in all pot soil samples. Soil electrical conductivity determined with a conductivity meter following extraction using a 1:5 soil:water suspension. Soil mineral N was extracted from 40 g equivalent dry soil with 100 ml of a 1 M KCl solution. NO_3^- was determined with an AA3 continuous flow analyzer. Microbial biomass carbon and microbial biomass nitrogen were determined using the chloroform fumigation extraction methods of Vance et al. (1987) and Potthoff et al. (2003).

Soil enzyme activities were analyzed and assayed as described by Guan (1986). Dedydrogenase (DED) activity (mg TPF g^{-1}) was determined by the reduction of triphenyl tetrazolium chloride (1%) to triphenyl formazan. Protease (PRO) activity (mg tyrosine g^{-1}) was measured by the determination of the amino acid release after the incubation of samples with sodium caseinate (2%). Phosphatase (PHO) activity (mg phenol g^{-1}) was estimated by the determination of phenol release after the incubation of samples with phenyl disodium phosphate (0.5%). Urease (URE) activity (mg NH_4^+ g^{-1}) was measured by the determination of NH_4^+ released in the hydrolysis reaction after the incubation of samples with urea (1%). Catalase (CAT) activity was measured by back-titrating residual H_2O_2 with $KMnO_4$.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to determine the effects of plastic shed and organic cultivation on soil electrical conductivity, NO_3^--N, soil organic matter, available P, available K, microbial biomass carbon and microbial biomass nitrogen, urease, protease, phosphatase, dehydrogenase and catalase activities, and the soil bacterial OTU, Shannon and Chao1, soil mesofauna Shannon, Pielou and Menhinick indexes. Duncan's test was used to examine the significant differences in the mean values between different treatments at a probability level of 0.05 for detecting significant differences. Redundancy analysis (RDA) and the linear canonical community ordination method were used to visualize the relationships between the response variable values (soil bacterial and mesofaunal diversity), the environmental parameters and the samples with CANOCO 5.0 software (Microcomputer Power Inc., Ithaca, NY). For the RDA in this study, soil bacterial and mesofaunal diversities were used as the explained variables. Seven environmental factors (SOM, NO_3-N, EC, AP, AK, MBC and MBN) were used as the explanatory variables. Additionally, redundancy analysis (RDA) and the linear canonical community ordination method were used to visualize the relationships between the response variable values (soil enzyme activities), the environmental parameters and the samples with CANOCO 5.0 software

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(Microcomputer Power Inc., Ithaca, NY). For the RDA in this study, soil enzyme activities were used as the explained variables. Seven environmental factors (SOM, NO_3-N, EC, AP, AK, MBC and MBN) and seven soil bacterial and mesofaunal diversity indexes (Bac-OUT, Bac-Shannon, Bac-Chao1, Bac-ACE, Fau-Shannon, Fau-Pielou and Fau-Menhinick) were used as explanatory variables.

Results

Diversity of the soil bacterial and mesofaunal communities

The soil bacterial Shannon diversity associated with plastic shed production was lower than that associated with open-field under both organic and conventional cultivation. Plastic shed production decreased the bacterial OTU, Chao1 and ACE evenness indexes compared to open-field only under organic cultivation (*Fig. 1*). Organic cultivation increased bacterial Shannon diversity compared to conventional cultivation in both plastic shed soil and open-field soils. Organic cultivation increased the bacterial OTU, Chao1 and ACE evenness indexes compared to conventional cultivation only in the open-field soils (*Table 2; Fig. 1*). Plastic shed production resulted in higher soil mesofaunal Shannon diversity than open-field under both organic and conventional cultivation. Plastic shed production resulted in a higher soil mesofaunal Pielou index than open-field under both organic and conventional cultivation, but a significant difference was observed only for organic cultivation. Organic cultivation did not affect soil mesofaunal diversity in either plastic shed or open-field soils (*Table 2; Fig. 2*).

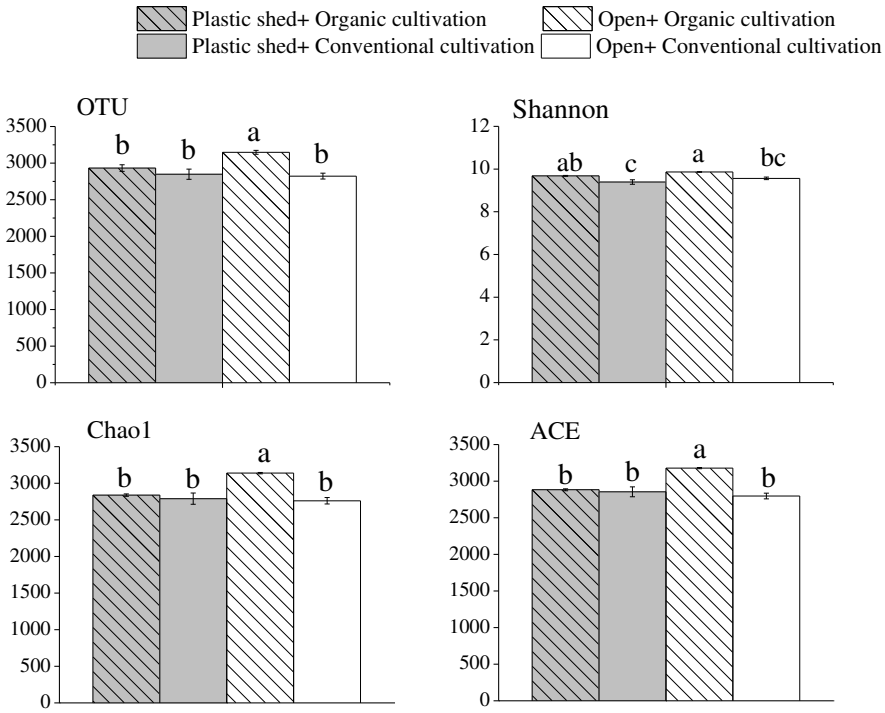

Figure 1. *The OTU, Shannon, Chao1 and ACE index of soil bacteria in the following treatments: plastic shed and open-field soils under organic and conventional cultivation. Vertical lines indicate standard deviation of the mean. Values with different letters differ significantly at $p < 0.05$ across different treatments*

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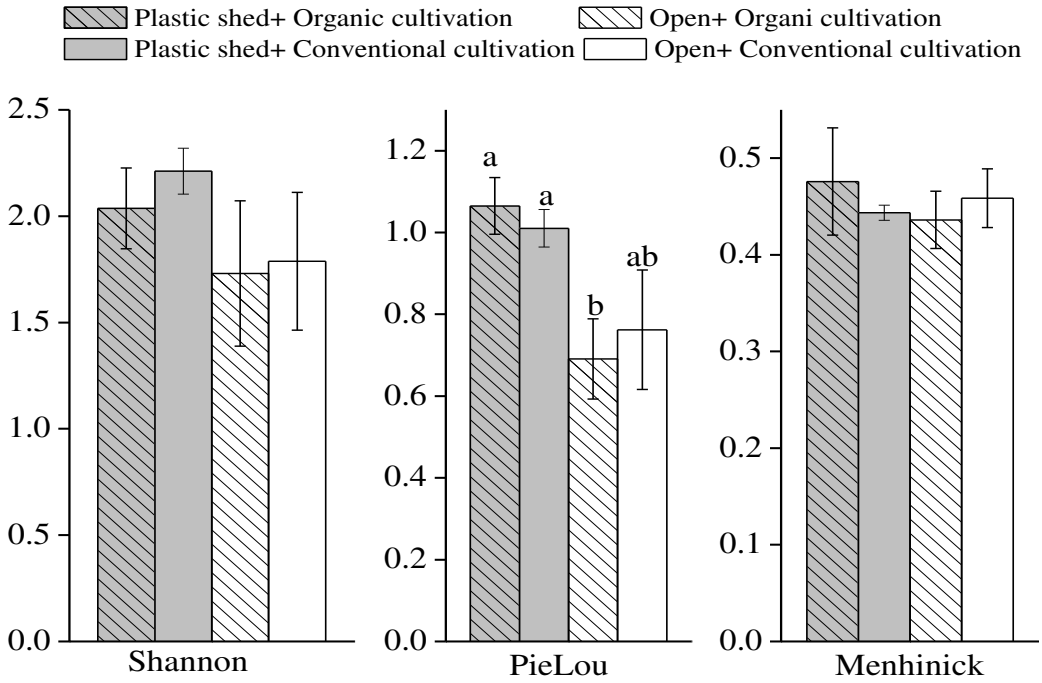

Figure 2. *The Shannon, PieLou and Menhinick index of soil mesofauna in the following treatments: plastic shed and open-field soils under organic and conventional cultivation. Vertical lines indicate standard deviation of the mean. Values with different letters differ significantly at $p < 0.05$ across different treatments*

Table 2. *F values and error from two-way ANOVA on the effects of plastic shed and organic cultivation and their interactions on the diversity of soil bacterial and mesofaunal community in the all treatments*

Treatment	*df*	The diversity of soil bacterial community				The diversity of soil mesofaunal community		
		OTU	Shannon	Chao1	ACE	Shannon	Pielou	Menhinick
Plastic shed (PS)	1	3.85	8.23*	8.93*	8.95*	1.98	10.11*	0.15
Organic cultivation (ORG)	1	18.30**	22.32**	21.72**	26.23**	0.20	0.01	0.01
PS×ORG	1	6.35*	0.01	13.03**	19.43**	0.05	0.41	0.60
Error	8							

* Significant at the 0.05 probability level
** Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Soil enzyme activities

Plastic shed production decreased soil urease, protease and phosphatase activities compared to open-field only under conventional cultivation (*Table 3*). Under organic cultivation, plastic shed production resulted in higher urease, protease and phosphatase activities than open-field, but the only significant difference was found for protease activity (*Table 3*). Compared with open-field, plastic shed production increased soil dehydrogenase activity under conventional cultivation and decreased soil dehydrogenase activity under organic cultivation. Compared to conventional cultivation, organic cultivation decreased soil urease and protease activities in open-field soils but increased their activities in plastic shed soils. Organic cultivation

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increased soil phosphatase, dehydrogenase and catalase activities compared to conventional cultivation in both plastic shed and open-field soils (*Table 3*).

Soil properties

Plastic shed production significantly increased soil EC and NO_3^--N concentrations compared to open-field only under conventional cultivation. Organic cultivation significantly decreased soil EC compared to conventional cultivation in both plastic shed and open-field soils (*Table 4; Fig. 3*). Organic cultivation significantly decreased soil NO_3^--N compared to conventional cultivation only in association with plastic shed production. Plastic shed production significantly increased SOM, AP and AK concentrations compared to open-field under both organic and conventional cultivation. Organic cultivation significantly increased soil SOM and AK concentrations compared to conventional cultivation only in plastic shed soils (*Table 4; Fig. 3*). Plastic shed production increased soil MBC and MBN compared to open-air vegetation under both organic and conventional cultivation (*Table 5*). Organic cultivation produced significant soil MBN compared to conventional cultivation only in open-air vegetation, while there was no difference in soil MBN between organic and conventional cultivation under plastic shed production (*Table 5*).

Table 3. *The mean value (±SE) of soil urease, protease, phosphatase, dehydrogenase and catalase activity in the following treatments: plastic shed and open-field soils under organic and conventional cultivation*

	Urease activity (mg/g)	**Protease activity (mg/g)**	**Phosphatase activity (mg/g)**	**Dehydrogenase activity (mg/g)**	**Catalase activity (ml/g)**
Plastic shed + organic cultivation	87.57 ± 11.7 ab	116.2 ± 7.03 a	113.8 ± 8.03 a	79.03 ± 6.41 b	189.5 ± 20.4 ab
Plastic shed + conventional cultivation	79.76 ± 29.6 b	12.24 ± 1.18 d	20.45 ± 3.53 c	54.21 ± 4.95 c	111.6 ± 20.9 b
Open-field + organic cultivation	55.86 ± 15.1b	61.42 ± 1.97 c	108.2 ± 4.64 a	97.46 ± 6.96 a	230.0 ± 33.6 a
Open-field + conventional cultivation	119.1 ± 4.14 a	80.33 ± 5.94 b	58.77 ± 9.20 b	16.87 ± 3.07 d	113.3 ± 39.1 b
Analysis of variance					

	df	F	df	F	df	F	df	F	df	F
Plastic shed (PS)	1	0.05	1	1.94	1	5.83*	1	2.89	1	0.50
Organic cultivation (ORG)	1	2.44	1	80.4***	1	111.3***	1	89.88***	1	10.77*
PS×ORG	1	4.01	1	167.6***	1	10.53*	1	25.15**	1	0.43

Values with different letters within a column show means with treatment-specific significant differences ($p < 0.05$; Duncan test). The lower part of the table shows F-values from the analysis of variance; degrees of freedom *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 4. *F values and error from two-way ANOVA on the effects of plastic shed and organic cultivation and their interactions on soil electrical conductivity (EC), pH, NO3-N, SOM, available P and available K in plastic shed and open-field soils under organic and conventional cultivation*

Treatment	***df***	**EC**	**pH**	**NO_3-N**	**SOM**	**AP**	**AK**
Plastic shed (PS)	1	15.14**	0.87	93.85***	584.1***	91.76***	203.8***
Organic cultivation (ORG)	1	301.03***	0.67	72.30***	158.8***	1.81	11.60**
PS×ORG	1	35.95***	0.09	62.50***	177.4***	0.09	37.47***
Error	8						

*Significant at the 0.05 probability level
**Significant at the 0.01 probability level
***Significant at the 0.001 probability level

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Table 5. *The mean value (±SE) of soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in the following treatments: plastic shed and open-field soils under organic and conventional cultivation*

	MBC	**MBN**
Plastic shed + organic cultivation	165.57 ± 21.68 a	36.82 ± 2.85 a
Plastic shed + conventional cultivation	137.98 ± 18.06 a	30.68 ± 2.38 a
Open-field + organic cultivation	66.44 ± 2.54 b	22.24 ± 1.69 b
Open-field + conventional cultivation	63.18 ± 8.14 b	13.37 ± 1.49 c

Analysis of variance

	df	F	df	F
PS	1	34.79***	1	53.73***
ORG	1	1.09	1	11.90**
PS×ORG	1	0.68	1	0.39

Values with different letters within a column show means with treatment-specific significant differences ($p < 0.05$; Duncan test). The lower part of the table shows F-values from the analysis of variance; degrees of freedom *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

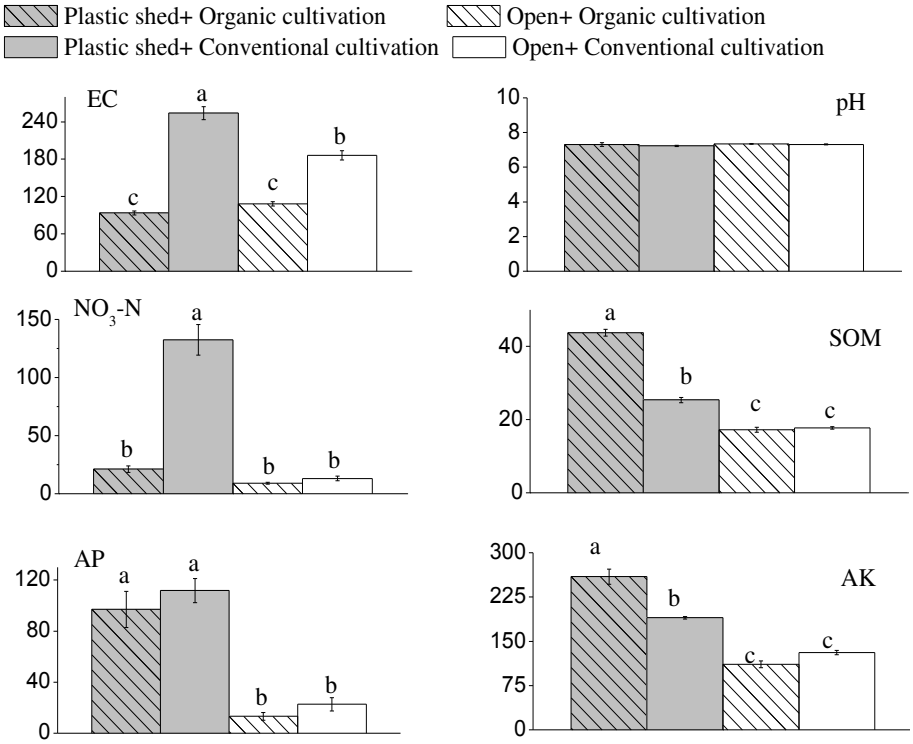

Figure 3. *Soil electric conductivity (EC), pH, NO3-N, organic matter (SOM), available P (AP) and available K (AK) in the following treatments: plastic shed and open-field soils under organic and conventional cultivation. Vertical lines indicate standard deviation of the mean. Values with different letters differ significantly at $p < 0.05$ across different treatments*

Correlations among soil organism diversity, soil enzyme activities and soil properties

The first ordination RDA axis explained 52.55% of the variation in the soil bacterial and mesofaunal data, and the second axis explained 17.43% (*Fig. 4*). The RDA

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suggested that the soil available P (which explained 26.8% of the variance, $P = 0.022$) was the most important parameter contributing to the diversity of the soil bacteria and mesofauna, followed by soil EC (which explained 24.1% of the variance, $P = 0.028$), and NO_3-N (which explained 19.0% of the variance, $P = 0.07$) (*Fig. 4*). The first ordination RDA axis explained 57.00%, and the second axis explained 31.20% of the variation in the soil enzyme data (*Fig. 5*). The RDA suggested that the soil EC (which explained 55.0% of the variance, $P = 0.002$) was the most important parameter contributing to soil enzyme activity, and thereafter, the most important parameters were the soil NO_3-N (which explained 47.7% of the variance, $P = 0.01$), Bac-Shannon (which explained 40.1% of the variance, $P = 0.004$), Bac-Chao1 (which explained 25.4% of the variance, $P = 0.042$), Bac-ACE (which explained 24.9% of the variance, $P = 0.046$), and Bac-OTU (which explained 21.8% of the variance, $P = 0.072$) (*Fig. 5*).

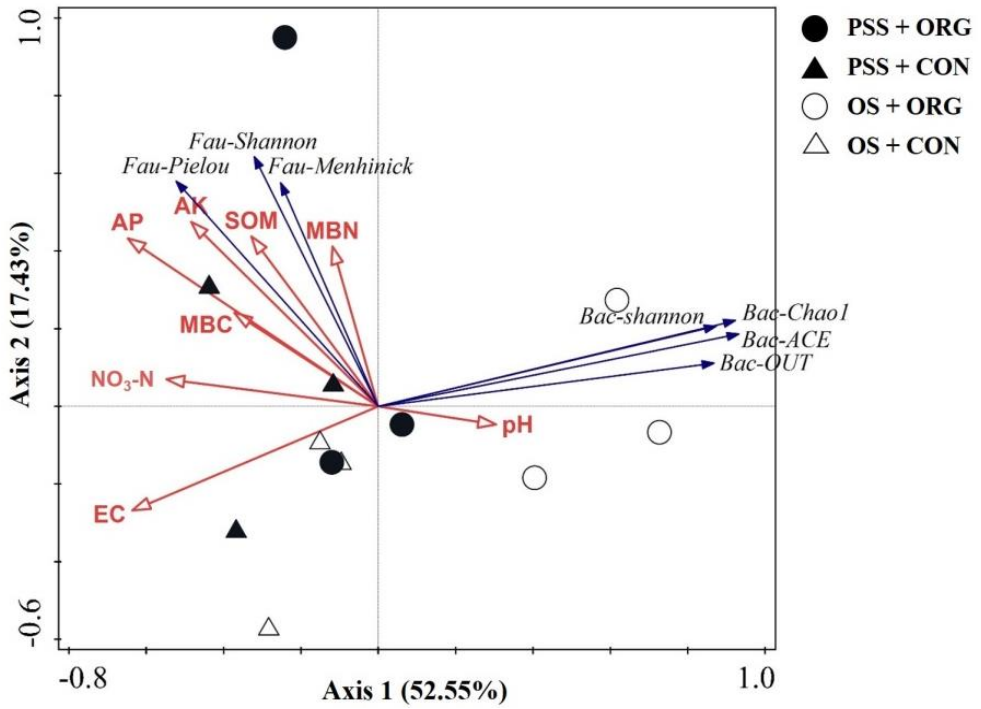

Figure 4. *Redundancy analysis between soil bacterial and mesofaunal diversity and soil environmental parameters. Bac-Shannon bacterial Shannon diversity index; Bac-Chao1, bacterial Chao1 index; Bac-ACE, bacterial ACE index; Fau-Shannon, mesofaunal Shannon diversity index; Fau-Pielou, mesofaunal Pielou index; Fau-Menhinick, mesofaunal Menhinick index; SOM, soil organic matter; NO3-N, nitrate; EC, electric conductivity; AP, available phosphorus; AK, available potassium; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; Bac-OUT, bacterial OUT; PSS + ORG, plastic shed soils + organic cultivation; PSS + CON, plastic shed soils + conventional cultivation; OS + ORG, open-field soils + organic cultivation; OS + CON, open-field soils + conventional cultivation*

Discussion

The soil bacterial diversity and richness index in plastic shed soils were lower than those in open-field soils. This may be due to changes in soil environmental factors that play important roles in shaping the bacterial community composition (Horner-Devine et al., 2004). Plastic shed production not only directly changed soil temperature and

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moisture but also indirectly altered soil nutrients, which contributed to the changes in the soil bacterial community. In the present study, we observed that soil bacterial diversity was significantly negatively correlated with soil available P, EC and NO_3-N content. Our results partly agree with the findings of Ma et al. (2018) and Chen et al. (2019), who found that NO_3-N and electrical conductivity were the most important soil properties controlling the variation in bacterial community structure. The results of previous studies have shown that high soil nutrient levels in plastic shed soils could decrease soil bacterial diversity (Ramirez et al., 2010; Sun et al., 2015; Chen et al., 2019). Although the present study did not measure environmental conditions, several studies have suggested that the soil temperature and moisture content in plastic shed soils are higher than those in open-field soils (Chen et al., 2008). Soil bacteria that are adapted to the high temperatures and moisture levels in plastic shed soils will be the dominant population, which may result in a decrease in bacterial diversity. Organic cultivation increased bacterial Shannon diversity compared to conventional cultivation in both plastic shed and open-field soils. This result is consistent with the findings of many previous studies showing that organic cultivation usually results in much higher soil biodiversity compared to conventional cultivation. The increase in bacterial diversity also increases the resilience of soils, leading to improved soil health (van Bruggen and Semenov, 2000).

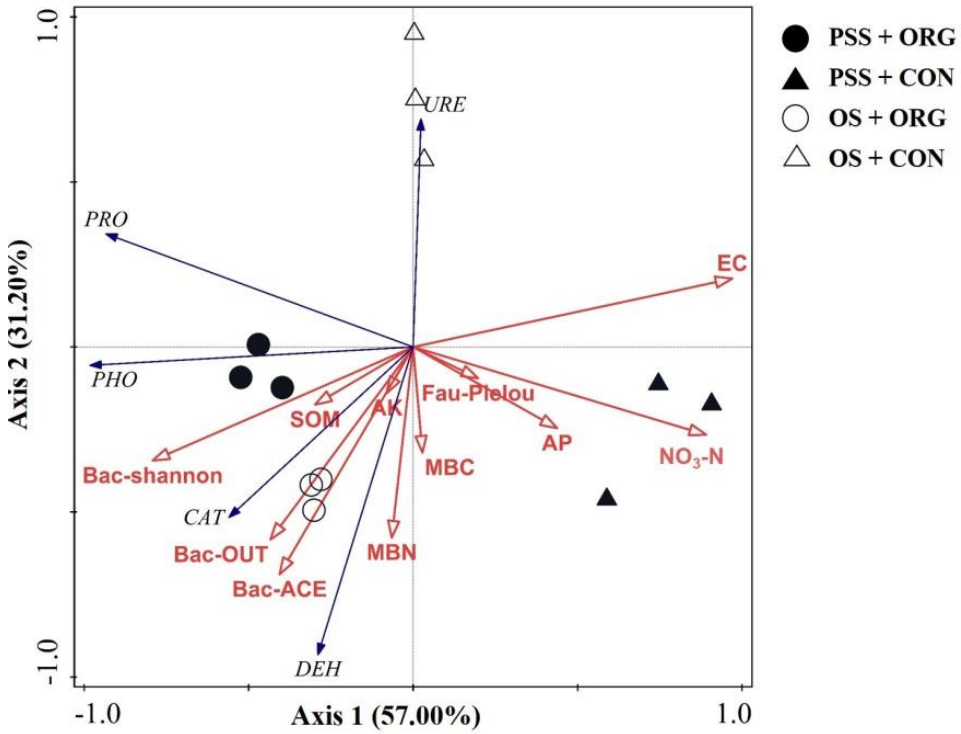

Figure 5. *Redundancy analysis between soil enzyme activities and soil environmental parameters. DEH, dehydrogenase; URE, urease; PRO, protease; PHO, phosphatase; CAT, catalase; SOM, soil organic matter; NO3-N, nitrate; EC, electric conductivity; AP, available phosphorus; AK, available potassium; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; Bac-OUT, bacterial OUT; Bac-Shannon bacterial Shannon diversity index; Bac-Chao1, bacterial Chao1 index; Bac-ACE, bacterial ACE index; Fau-Shannon, mesofaunal Shannon diversity index; Fau-Pielou, mesofaunal Pielou index; Fau-Menhinick, mesofaunal Menhinick index. PSS + ORG, plastic shed soils + organic cultivation; PSS + CON, plastic shed soils + conventional cultivation; OS + ORG, open-field soils + organic cultivation; OS + CON, open-field soils + conventional cultivation*

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Plastic shed production resulted in higher soil mesofaunal diversity and evenness indexes compared to open-field under both organic and conventional cultivation. This result is consistent with the findings of previous studies (Shah et al., 2003; Li and Gu, 2009; Ponce et al., 2011) in which the authors speculated that this change may be due to the improvement of soil nutrients. In the present study, we observed that the Shannon, Pielou and Menhinick indexes of the soil mesofauna were positively correlated with soil available P, soil microbial biomass carbon, microbial biomass nitrogen and nitrate contents. However, other researchers have shown that plastic shed production leads to lower Shannon diversity and Pielou evenness of the soil fauna community compared to open-field (Dong et al., 2008; Wang, 2009). This may occur because the soil macrofauna is more severely affected by plastic shed production than the meso- and microfauna (Postma-Blaauw et al., 2010; Ponge et al., 2013). The nonsignificant differences in mesofaunal diversity and evenness indexes between organic and conventional cultivation were not consistent with the findings of many studies (Cotes et al., 2010; Jiang et al., 2015) that have shown increased diversity of the soil fauna (arthropods) under organic cultivation. Such nondiscriminating results could be attributed to the heterogeneity of organic practices applied within soil systems as well as climate parameters and the different responses of species to management disturbance (Hole et al., 2005; Bengtsson et al., 2005; Gkisakis et al., 2016)

In soils, enzymes play an essential role in mediating biochemical transformations and nutrient cycling and can thus be used as a sensitive index to monitor changes in soil microbial activity and functioning. Under conventional cultivation, plastic shed production decreased soil urease, protease and phosphatase activities compared to open-field. These results suggest a decrease in soil function related to N and P transformation (Sinsabaugh et al., 2008). However, under organic cultivation, we observed that plastic shed production resulted in higher soil urease, protease and phosphatase activities compared to open-field. These results indicated that organic cultivation could alleviate the adverse effect of plastic shed production on soil enzyme activities related to soil N and P. In the present study, we observed higher soil urease, protease, phosphatase, dehydrogenase and catalase activities under organic cultivation than under conventional cultivation in plastic shed soils. These results indicated that the soils of organic cultivation systems exhibit higher overall microbial activity and a higher capacity to cleave proteins and organic phosphorus. In the present study, soil enzyme activities showed no significant correlation with most of the examined soil properties. These results differ from those of a previous study showing that soil properties such as available P and N exhibit close relationships with soil enzyme activities (Ling et al., 2014). However, the present study indicates that soil enzyme activities are significantly positively correlated with bacterial the Shannon diversity, Chao1 and ACE indexes. The results were in accordance with the findings of Chen et al. (2019) showing that soil enzyme activities were correlated with the microbial diversity index. The findings of Carrara et al. (2018) also showed that extracellular enzyme activities were significantly correlated with the bacterial community composition. This may be explained by the results of a study suggesting that a change in microbial diversity impacts soil functions such as enzyme activities (Colombo et al., 2016). This also supports the conclusion that the microbial community composition is more important than soil nutrient properties in influencing soil functioning (Nannipieri et al., 2012; Stark et al., 2014). In addition, the present study revealed that soil enzyme activities were significantly negatively correlated with soil electrical conductivity and NO_3-N. These results indicated that the

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accumulation of salt in the soil impaired soil enzyme activities (Tejada et al., 2006; Tripathi et al., 2007).

Under conventional cultivation, plastic shed production significantly increased soil EC compared to open-field. These results indicated that under conventional cultivation, plastic shed production causes soil salinization. This agrees with the findings of a previous study (Ju et al., 2007). These results may be due to the following two reasons. First, under conventional cultivation, heavy application rates of chemical fertilizers in vegetable production cause excessively high nutrient accumulation in soil. Soil nitrate is one major factor leading to soil salinization, which occurs easily in plastic shed soils due to the heavy use of fertilizers (Shi et al., 2009). In the present study, plastic shed production significantly increased the soil NO_3-N concentration compared to open-field only under conventional cultivation. The accumulated NO_3-N content reflects the impact of chemical fertilizers on soil EC. Second, plastic shed production usually causes higher evapotranspiration compared with open-air fields (Han et al., 2009), which induces the upward movement of soil water and the soil solution from subsoil, resulting in the accumulation of soil salt ions in topsoil (Ge et al., 2010). However, under organic cultivation, the soil EC values were not different between the plastic shed and open-field soils. These results indicated that organic cultivation can significantly alleviate soil salinization in plastic shed soil. Organic cultivation can reduce soil nitrate accumulation, which may improve denitrification processes (Huang et al., 2019). In the present study, significantly lower soil EC and nitrate contents were observed under organic cultivation than under conventional cultivation. Together, the available evidence suggests that the observed effects of organic cultivation management are attributable to its roles in alleviating soil salinization (or nitrate accumulation), which likely benefit from the alteration of soil N-cycling processes. The soil organic matter, available K and available P contents observed in association with plastic shed production were significantly higher than those associated with open-field under both organic and conventional cultivation. These results indicate that the nutrient contents of the plastic shed soils were maintained at high levels to achieve sustained soil chemical fertility (Chen et al., 2019; Xie and Tan, 2001; Yang et al., 2011). The soil organic matter and available K contents under organic cultivation were significantly higher than those under conventional cultivation associated with plastic shed production. These results occurred because N fertilizer in organic shed production systems is generally replaced with organic fertilizer, which includes carbon and K in different forms.

Plastic shed production increased soil microbial biomass carbon and microbial biomass nitrogen compared to open-field under both organic and conventional cultivation. These findings are consistent with the results of several previous studies (Yu, 2007) and may be due to the higher soil nutrient contents associated with plastic shed production compared with open-field (Mele and Crowley, 2008; Zhong et al., 2010). In the present study, we observed that soil microbial biomass carbon and microbial biomass nitrogen were correlated with soil nutrients such as soil organic matter, NO_3^--N, available P and available K. Organic cultivation significantly increased soil microbial biomass carbon and microbial biomass nitrogen compared to conventional cultivation in both plastic shed and open-field soils, with the exception of soil microbial biomass nitrogen associated with plastic shed production. These results are in agreement with other studies (Liu et al., 2007; van Diepeningen et al., 2006). Under organic cultivation, more organic carbon there is applied to fields to maintain the organic matter content in soils, which may simultaneously increase the microbial biomass.

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Conclusion

This research provides evidence that plastic shed soils exhibit lower soil bacterial richness and diversity than open-field soils but present higher soil mesofaunal Shannon diversity and Pielou's species evenness indexes. Plastic shed production significantly decreased soil urease and phosphate activities compared with those in open soil only under conventional cultivation. Organic cultivation mostly resulted in higher bacterial abundance and diversity, soil microbial biomass and enzyme activities compared with conventional cultivation, but significant differences in some parameters were observed only in the open-field soils. Changes in soil enzyme activities occurred and were tightly linked to bacterial diversity and soil electrical conductivity, rather than to soil nutrient properties. Plastic shed production increased soil nutrient properties but caused soil salinization (mainly because of soil nitrate accumulation) under conventional cultivation, which may cause groundwater pollution. Organic cultivation decreased the soil EC and nitrate contents and increased soil organic matter and available K contents compared with conventional cultivation in plastic shed soils. Together, these results indicated that organic cultivation could be used to minimize the negative impacts of plastic shed production, particularly to decrease soil EC and nitrate contents and enhance soil functions, and the showed that the positive impacts of organic cultivation on soil microbial diversity and functions were reduced by plastic shed production. Further studies are still needed to determine the long-term effects of plastic shed production systems on soil nutrients, biodiversity and functions under different management practices.

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EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI ON MAIZE (*ZEA MAYS* L.) UNDER ZINC DEFICIENT AND TOXIC FIELD CONDITIONS

SABOOR, A.* – ALI, M. A.*

Department of Soil Science, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan
(phone: +92-334-682-8007)

**Corresponding authors*
e-mail: abdulsabooruaf36d@gmail.com; arif1056@bzu.edu.pk

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Abstract. Arbuscular mycorrhizal fungi (AMF) have potential to cope with nutrient stress environment when the soil is zinc (Zn) deficient or toxic. Current study was conducted under field conditions to investigate the potential of mycorrhizal inoculation on maize to mitigate Zn nutrient stress condition. The treatments were organized according to randomized complete block design under factorial arrangements with three replicates. During this study soil nutrient status and maize nutrient uptake were observed. Soil analysis and determination of different parameters such as chlorophyll contents, total soluble protein including yield parameters was conducted to compare the change in nutrient status with mycorrhizal inoculation. Results showed that mycorrhizal inoculation (M+) reversed the stress effect of Zn stress and promoted maize growth. Inoculation increased Zn uptake by 98% in Zn deficient conditions while reduced the uptake of Zn by 39% in Zn toxic conditions. Plants height increased by up to 16% with fungal inoculation as compared to non-inoculated maize plants. The increased cob length ultimately resulted in higher grain yield with an increase of 15% and 8% under medium Zn toxicity and severe toxicity respectively with mycorrhizal inoculation. Moreover, inoculated maize also showed significant improvement in maize plant root colonization, chlorophyll contents and total soluble protein.
Keywords: *alkaline soil, growth attributes, physiological growth, yield traits, nutrients*

Introduction

Maize (*Zea mays* L.) is one of the most abundantly cultivated cereal and fodder crop globally. It is an important cereal crop as it provides essential foodstuff to many people all around the world (Khan et al., 2012). The composition of maize grains comprises of starch, protein, fiber, oil, sugar and ash as described in the literature (Chaudhary, 1993). Limited availability of zinc (Zn) in alkaline calcareous soil is one of the major constraints that hamper maize crop productivity (Joy et al., 2017). Deficiency of Zn reduces the plant growth due to less auxin production (Brown et al., 1993). According to an estimation of WHO, about 31% of the world's population have deficiency of Zn (World Health Organization, 2005). As Zn is an essential micronutrient for animals and plants, it plays important roles in various enzymatic activities involved in protein synthesis, lipid metabolism, carbohydrate and nucleic acid synthesis (Hussain et al., 2011; Ali et al., 2013; Broadley et al., 2007).

Due to its structural, catalytic and co-catalytic function, Zn plays a significant role in development, reproduction and signaling (Broadley et al., 2007; Cavagnaro, 2008; Roohani et al., 2013) of plants. Zinc takes part in about 300 enzymatic functions, and it is important for plant functioning and growth (Christie et al., 2004; Hacisalihoglu and Kochian, 2000). The studies showed that alkaline calcareous soils of arid and semi-arid regions comprising of 30% of the soils in the world have deficiency of Zn (Kochian,

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2000) because of low mobility of Zn (Cakmak et al., 1999; Broadley et al., 2007; Alloway, 2009). In soils with limited Zn mobility, it becomes very important to understand the mechanisms of Zn acquisition by plants from the soil (Impa and Johnson-Beebout, 2012). Soil Zn with less than 1 mg/kg concentration in soil is considered as deficient, 1-60 mg/kg is considered as optimum, 60-120 mg/kg is toxic and above 120 mg/kg, it is highly toxic (Alloway, 2009). Requirement of Zn for optimum growth of plants varies among different plant species and crop variety. Maize optimum Zn requirement for its proper growth is 4.7 mg/kg, and for maximizing the maize yield, it should be more than 7 mg/kg (Liu et al., 2017). Contrarily microbial strains confer positive influence by enhancing root colonization which assist in essential nutrient uptake and mitigates any kind of soil nutrient or abiotic stresses (Shahzad et al., 2017). Arbuscular Mycorrhizal Fungi (AMF) develop a symbiotic association with most of the terrestrial plants and increase uptake of mineral nutrients (Wahid et al., 2016). The AMF are reported to increase Zn uptake in plants (Rue et al., 1975; Chen et al., 2003; Kafkas and Ortas, 2009; Ortas, 2012). Through this mechanism, AMF can help to cope up Zn deficiency in animals and humans (Cavagnaro, 2008).

Meanwhile, concentration of heavy metals in soils, including Zn is increasing at a faster rate from the past few decades (Zarcinas et al., 2004). At high level of Zn in soil, stress is induced in plants, which causes stunted root and shoot growth, death of leaf tips, curling of young leaves, leaf chlorosis, reduced photosynthetic rate, etc. (Rout and Das, 2003; Shi et al., 2015). Environmental Zn pollution is usually caused by anthropogenic activities like mining, electroplating, smelting and improper waste disposal (Bacon and Dinev, 2005; Bi et al., 2006). High or toxic amount of Zn availability in soil can have negative effect on plant growth, germination of seeds (Wang et al., 2009), development of roots (Lingua et al., 2008), loss of membrane structure (Stoyanova and Doncheva, 2002) ultimately, leading to the death of cells (Chang et al., 2005).

Under such conditions of high Zn levels, AMF can play a significant role in improving crop growth and development (He and Nara, 2007; Cavagnaro, 2008). Arbuscular mycorrhizal fungi are symbiotic fungi occurring worldwide, they belong to phylum Glomeromycota (Schubler et al., 2001) and form symbiotic association with the majority of terrestrial plants. The prominent function of AMF is its efficiency in absorbing less mobile nutrients, such as P and Zn (Bolan, 1991; Burkert and Robson, 1994; Marschner and Dell, 1994; Jansa et al., 2003). The role of AMF in absorbing P from the soil is well studied; however, their role for uptake of micronutrients is not well established under field relevant conditions. The study aimed to investigate the effects of arbuscular mycorrhizal fungi on the growth, yield, chlorophyll contents and total soluble protein of maize under toxic or deficit zinc concentrations in a field experiment.

Materials and methods

The research experiment of the proposed study was conducted in Multan, Punjab, Pakistan at latitude 29°55N and longitude 71°31E (site selected due to Zn deficiency). Experiment was carried out under field condition at optimized selected toxic and deficient levels of Zn with and without AMF inoculation. The study comprised of the following treatments ($Zn_{0.45}$ zinc deficiency 0.45 mg kg^{-1}, Zn_{60} medium toxicity 60 mg kg^{-1} and Zn_{120} zinc toxicity 120 mg kg^{-1}) with and without mycorrhizal inoculations. Field soil Zn levels were maintained by $ZnSO_4.7H_2O$ salt addition in the surface soil layer. The treatments were arranged according to randomized complete block design (RCBD) under factorial

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arrangements with three repeats. The net plot size was 6 m^2 with 65 cm row to row and 15.5 cm plant to plant distance. The hybrid maize cultivar (YH-1898) was sown on 15th July. Mix consortia of mycorrhizal inoculum having glomus species (inoculum purchased from Bustan urban Gardening Essential, Toronto, Canada having 158 propagule/gram) were used as seed priming. In AMF controlled pots (M-), Topsin M (Thiophanate Methyl 70% WP) was applied at 50 mg/kg soil, for rendering AMF root colonization. Standard agronomic practices of irrigation with tube well water and recommended dose of nitrogen (N) in three split doses, optimum level of half dose of (P) recommended and potassium (K) fertilizer were applied N:P:K@ 92:29:37 kg $acre^{-1}$, respectively. Other agronomic practices like weed and pest control were also applied. At the time of maturity, full plot was harvested, and root and leaf samples were collected randomly from whole plot and the growth, and physiological parameters were recorded.

Mycorrhizal colonization

Roots were harvested for AMF root colonization assessment by gridline intersect method (Giovannetti and Mosse, 1980), cleared in 10% KOH solution and tryphan blue stain was used for staining (Phillips and Hayman, 1970).

Physiological parameters

Chlorophyll a, b was determined by following the procedure of Arnon (1949). The intensity of green color extract of fresh plant leaves in acetone was measured by a spectrophotometer at 645 and 663 nm wavelength and chlorophyll a, b was calculated by the formula proposed by Arnon (1949).

$$\text{Chlorophyll '}a\text{' (mg g}^{-1}) = 100 \times [(0.0127 \times A663 - A645 \times 0.00269)] / 0.5$$

$$\text{Chlorophyll '}b\text{' (mg g}^{-1}) = 100 \times [(0.0229 \times A645 - 0.00468 \times A663)] / 0.5$$

Nutrient concentration

All of the fresh plant leaves and roots were removed and rinsed with water and oven dried at 72 °C for 24 h. Plant leaves and roots fresh weight and oven dried weight was recorded by an analytical/precision electrical balance. Plant Zn was determined by using standard procedure of atomic absorption spectrophotometer by Lindsay and Norvell (1978) method. Phosphorus in plants was analyzed by following the protocol of malachite green method (Ohno and Zibiliski, 1991). Soil Zn was determined by extractable DTPA-Zn as prescribed by Lindsay and Norvell (1978) and available P was extracted by sodium bicarbonate solution (Olsen and Sommers, 1982) method and further quantified by malachite green method (Ohno and Zibiliski, 1991). Soil N analysis was performed by Kjeldahl method (Bremner, 1960) and K was determined by extracting K from soil in ammonium acetate solution by flame photometer instrument (Shuman and Duncan, 1990).

Total soluble protein (mg g^{-1})

Total soluble protein was measured by using the procedure of Bradford (1976). The plant material (200 µL) was extracted from leaves. After this extracted material was added

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into 780 µL deionized water and 20 µL of coomassie blue dye, and absorbance of this prepared mixture was read at 595 nm in a spectrophotometer.

Statistical analysis

The data collected was analyzed statistically using analysis of variance (ANOVA) with arrangement of two factorial randomized complete block design (Steel et al., 1997). Mean values were compared for significance by conducting least significance difference test ($P \leq 0.05$). Principal component analysis (PCA) and correlation matrix was performed by using XLSTAT-2014.

Results

Data regarding mycorrhizal colonization was measured from the roots of both inoculated and un-inoculated maize plants. Significant ($P \leq 0.05$) proportion increase in mycorrhizal colonization was observed under all zinc deficient and toxic soil conditions (*Fig. 1*).

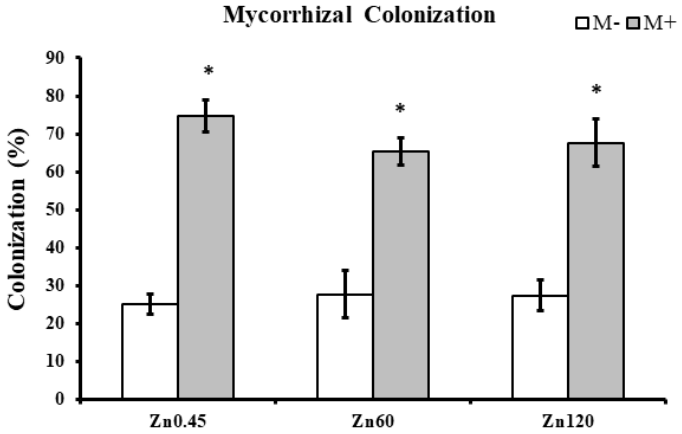

Figure 1. *Effect of arbuscular mycorrhizal fungi inoculation on mycorrhizal colonization by maize under zinc deficient and toxic soil conditions $Zn_{0.45}$ zinc deficiency (0.45 mg Kg^{-1}), Zn_{60} medium zinc toxicity (60 mg Kg^{-1}), Zn_{120} severe zinc toxicity (120 mg Kg^{-1}). Inoculated with AMF (M + grey), un-inoculated (M- white). Vertical bars represent standard error and asterisk (*) shows significant difference ($P \leq 0.05$) among treatments*

Soil nutrient status of the Zn deficient and toxic soil under investigation in the current study was noted. It was observed that the mycorrhizal inoculation enhanced the nutrient status of the soil irrespective of macro nutrients (N and P) as well as micronutrient (Zn) (*Fig. 2*). However, the behavior for Zn contents was different with the subjected Zn environment. Soil Zn content was reported to be increased with the inoculated maize plants under Zn deficiency (0.45 mg Kg^{-1}), however contrasting results were observed under both medium and severe toxic Zn growing medium. Maize inoculated plants showed ameliorative effect against Zn toxicity. Highly significant ($P \leq 0.05$) twofold decrease (11.80 mg Kg^{-1}) was noted with inoculation in soil Zn contents under severe Zn toxicity (*Fig. 2c*).

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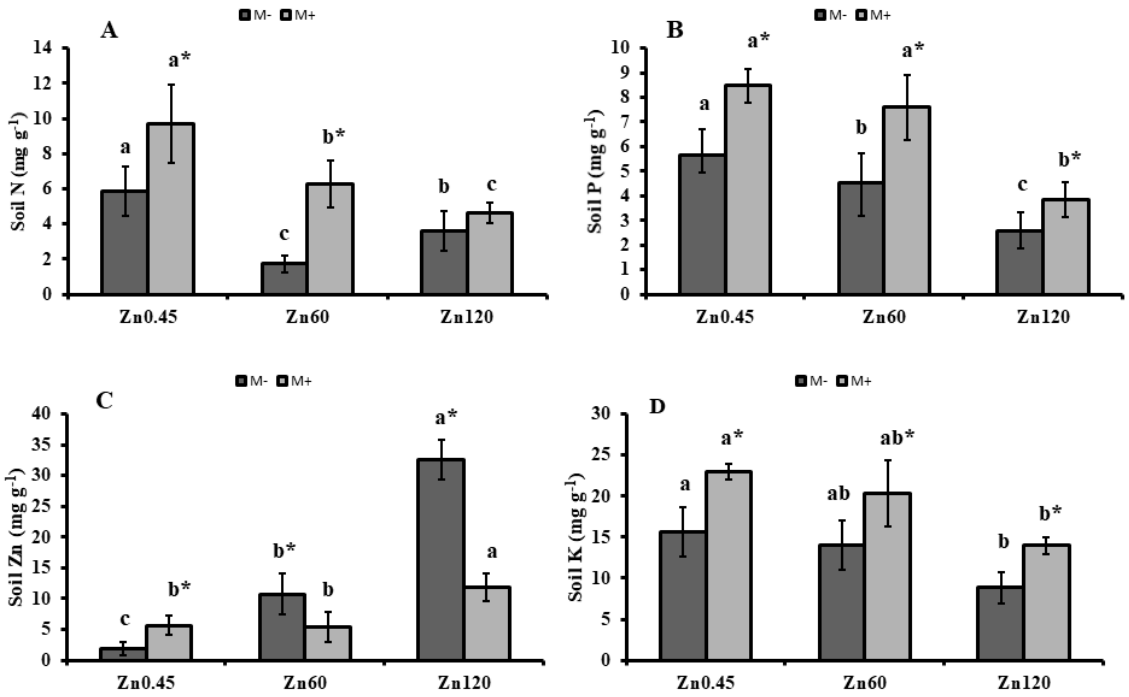

Figure 2. *Influence of arbuscular mycorrhizal inoculation maize plants on soil nutrient status under zinc deficient and toxic soil environments. (a) soil nitrogen contents (b) soil phosphorus contents (c) soil zinc contents (d) soil potassium contents. $Zn_{0.45}$ zinc deficiency (0.45 mg Kg^{-1}), Zn_{60} medium zinc toxicity (60 mg Kg^{-1}), Zn_{120} severe zinc toxicity (120 mg Kg^{-1}). among treatments. Inoculated with AMF (M + grey), un-inoculated (M- white). Vertical bars represent standard error and asterisk (*) shows significant difference ($P \leq 0.05$) among treatments. Alphabets sharing same letter shows non-significant ($P \geq 0.05$) difference whereas, different letter shows significant ($P \leq 0.05$) difference among different treatments*

Plant root is the only organ that is in direct contact with the soil environmental adversities and it is also the base of plant for nutrient uptake to ensure sturdy plant growth. Maize nutrient uptake and its accumulation in roots showed contrary results with inoculated and un-inoculated maize plants. Zinc contents in the root of inoculated maize plant were significantly ($P \leq 0.05$) higher while in the upper portion (shoots) it was lower. However un-inoculated maize roots showed lower Zn contents with a decrease of 96% (0.04 mg g^{-1}) and P content decrease was of 90% (1.90 mg g^{-1}) under severe Zn toxicity (120 mg Kg^{-1}), whereas it created significant nutrient toxic conditions with higher uptake of Zn in shoots of maize plants (*Table 1*).

Table 1. *Effect of arbuscular mycorrhizal fungi inoculation on nutrient accumulation in roots and its uptake by maize under zinc deficient and toxic soil conditions*

	Zn in root (mg g^{-1})		Zn in shoot (mg g^{-1})		P in root (mg g^{-1})		P in Shoot (mg g^{-1})	
	M-	M+	M-	M+	M-	M+	M-	M+
$Zn_{0.45}$	0.04±0.00b	0.10±0.00a	5.21±0.01a	3.18±0.02c	1.90±0.01e	3.32±0.01b	4.14±0.01a	1.51±0.01e
Zn_{60}	0.04±0.01b	0.09±0.01a	4.59±0.01b	2.94±0.03d	2.37±0.02c	5.47±0.01a	3.27±0.01b	1.77±0.01d
Zn_{120}	0.02±0.00c	0.04±0.00b	1.40±0.03f	2.78±0.03e	0.12±0.01f	1.97±0.02d	0.32±0.01f	2.13±0.00c

Mean values ± standard error. Lettering represents significance; different letters shows significant difference ($P \leq 0.05$). $Zn_{0.45}$ zinc deficiency (0.45 mg Kg^{-1}), Zn_{60} medium zinc toxicity (60 mg Kg^{-1}), Zn_{120} severe zinc toxicity (120 mg Kg^{-1})

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Maize plants that were subjected to Zn deficient (0.45 mg kg^{-1}) and severe toxic (120 mg kg^{-1}) soil conditions exhibited lower chlorophyll a and b contents. Maize showed the same trend in decrease of chlorophyll contents in the case of both mycorrhizal inoculation and un-inoculation. Significant ($P \leq 0.05$) difference was noted in chl a and b contents of maize with AMF inoculated plants under both Zn deficient and toxic soil regimes (*Table 1*). Maize plants inoculated with AMF showed higher total soluble protein.

Significant differences were noted with all the inoculated plants under varying Zn levels. The $Zn_{0.45}$ with mycorrhizal inoculation depicted the highest TSP with an increase of 46% (26.49 mg g^{-1}) as compared with the same medium toxicity of Zn but having non-mycorrhizal inoculation (*Table 2*).

Table 2. *Effect of arbuscular mycorrhizal fungi inoculation on chlorophyll and total soluble protein of maize under zinc deficient and toxic soil conditions*

	Chl a (mg g^{-1})		**Chl b (mg g^{-1})**		**TSP (mg g^{-1})**	
	M-	**M+**	**M-**	**M+**	**M-**	**M+**
$Zn_{0.45}$	1.32±0.29c	1.71±0.49a	0.26±0.03e	0.82±0.01a	18.16±1.80b	26.49±1.67a
Zn_{60}	1.19±0.80d	1.62±0.26a	0.38±0.01d	0.59±0.02b	13.42±2.54c	18.95±2.48b
Zn_{120}	1.29±0.20cd	1.47±0.36b	0.36±0.02d	0.51±0.06c	11.36±1.01c	15.93±0.98b

Mean values ± standard error. Lettering represents significance; different letters shows significant difference ($P \leq 0.05$). $Zn_{0.45}$ zinc deficiency (0.45 mg Kg^{-1}), Zn_{60} medium zinc toxicity (60 mg Kg^{-1}), Zn_{120} severe zinc toxicity (120 mg Kg^{-1}). Chl a and b (chlorophyll a and b contents of maize), whereas TSP represents total soluble protein

Results showed that mycorrhizal inoculation improved maize growth attributes (plant height, stem girth, no of leaves and plant biomass) under zinc deficient and zinc toxic conditions as compared to maize plants grown in un-inoculated treatments. Significant ($P \leq 0.05$) increase of 16% was observed in plant height with fungal inoculation. The stem girth improved with an increase of 8% (8.45 cm) under zinc toxicity. The plants no of leaves were also decreased with zinc toxicity, on the other hand mycorrhizal inoculation resulted in higher number of leaves (*Fig. 3*).

Results regarding yield attributes of maize are presented in *Figure 4*. This data showed that mycorrhizal inoculation was effective in improving the yield attributes (cob length and weight, 1000 grain weight, harvest index, biological yield and grain yield) of maize in zinc toxic and deficient conditions. Increase in cob weight, 1000 grain weight, harvest index, biological yield and grain yield of maize was observed in zinc problematic conditions under inoculation compared to treatment without inoculation. The increase in cob length was 30% (17.03 cm) under slight zinc toxic soil conditions. The increased cob length resultantly conferred higher grain yield with an increase of 17% under $Zn_{0.45}$ zinc toxicity without inoculation to 6.06 Kg ha^{-1} and 8% under severe toxicity 3.93 Kg ha^{-1} to 4.26 Kg ha^{-1} with mycorrhizal inoculation.

Principal component analysis

The interrelationship among the variables under Zn deficient and toxic soil were evaluated by biplot principal component analysis (PCA) as shown in *Figure 5*. It showed that the first two components explained 90.42% variance (contributed by PC1 75.78%, and PC2 14.64%) under Zn deficient and toxic soil SS conditions. PCA biplot showed the grouping of the mycorrhizal and non-mycorrhizal treatments on their response to the

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tested morphological and physiological traits. The mycorrhizal inoculation was highly responsive to influence the tested variables. The Zn1M (mycorrhizal inoculation under Zn deficiency) and Zn2M (mycorrhizal inoculation under medium Zn toxicity), showed higher response for the variables, i.e., cob weight, grain yield, harvest index, plant height, number of leaves, stem girth, cob length, total soluble protein, potassium in soil, P accumulation in roots, Zn in root, nitrogen in soil. The Zn3M (mycorrhizal inoculation under high zinc toxicity) showed average response. The Zn1NM (non-mycorrhizal under zinc deficiency) showed higher accumulation of P in shoot. The Zn2NM and Zn3NM were non-responsive to influence the traits of maize.

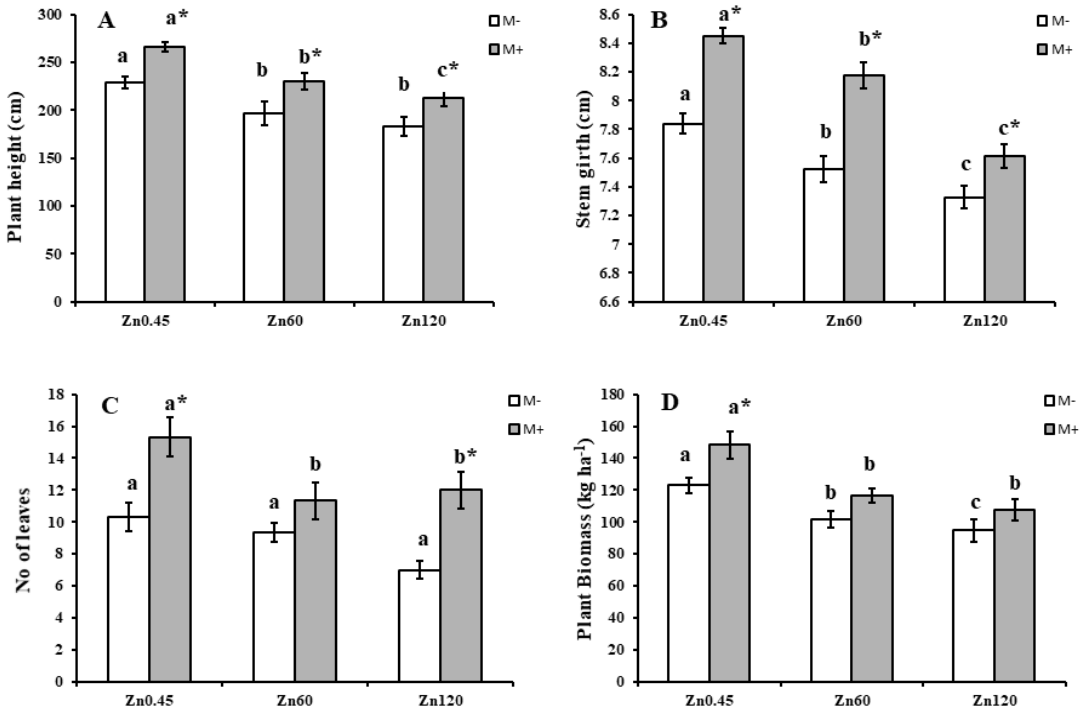

Figure 3. *Influence of arbuscular mycorrhizal inoculation on maize growth characteristics under zinc deficient and toxic soil environments. (a) plant height (cm) (b) stem girth (c) no of leaves (d) plant biomass. Zn0.45 zinc deficiency (0.45 mg Kg^{-1}), Zn60 medium zinc toxicity (60 mg Kg^{-1}), Zn120 severe zinc toxicity (120 mg Kg^{-1}). Inoculated with AMF (M + grey), un-inoculated (M- white). Vertical bars represent standard error and asterisk (*) shows significant difference ($P \leq 0.05$) among treatments. Alphabets sharing same letter shows non-significant ($P \geq 0.05$) difference whereas, different letters show significant ($P \leq 0.05$) difference among different treatments*

Correlation matrix

Results of Pearson correlation coefficient (r) among the morphological and physiological traits of maize under Zn deficient and toxic soil conditions were summarized in *Table 1*. Plant height had a strong positive correlation soil N, soil P, soil K and Zn in root, and negative correlation with Zn in soil. Similarly, grain and yield had positive correlation with soil N, soil P and total soluble protein, whereas both exhibited negative correlation with soil Zn. The soil P had a positive correlation with soil N and K, chlorophyll a, and total soluble protein, and a negative correlation with soil Zn, while strongly correlated with Zn in root (*Table 3*).

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Table 3. *Pearson's correlation between different traits under Zn deficient and toxic soil conditions in maize (n = 6)*

	PH	SG	PB	NL	CL	CW	GW	HI	BY	GY	SN	SP	SZ	SK	ZR	ZS	PR	PS	CHLA	CHLB
PH																				
SG	0.962**																			
PB	0.981**	0.923**																		
NL	0.926**	0.861*	0.885**																	
CL	0.947**	0.922**	0.904**	0.964**																
CW	0.906**	0.921**	0.843*	0.869*	0.836*															
GW	0.973**	0.877*	0.963**	0.957**	0.933**	0.859*														
HI	0.948**	0.840*	0.976**	0.850*	0.857*	0.762*	0.963**													
BY	0.984**	0.907**	0.992**	0.912**	0.925**	0.833*	0.984**	0.986**												
GY	0.925**	0.885**	0.963**	0.801*	0.791*	0.854*	0.891**	0.914**	0.927**											
SN	0.932**	0.901**	0.929**	0.823*	0.923**	0.713	0.890**	0.916**	0.940**	0.811*										
SP	0.916**	0.981**	0.877*	0.796*	0.842*	0.943**	0.812*	0.771*	0.842*	0.887**	0.808*									
SZ	-0.734*	-0.697	-0.668	-0.675	-0.577	-0.894**	-0.725	-0.649	-0.672	-0.718	-0.474	-0.749*								
SK	0.937**	0.981**	0.884**	0.879*	0.900**	0.971**	0.862*	0.784*	0.866*	0.872*	0.812*	0.985**	-.0770*							
ZR	0.866*	0.960**	0.805*	0.818*	0.885**	0.888**	0.760*	0.673	0.779*	0.775*	0.796*	0.957**	-0.601	0.967**						
ZS	0.254	0.17	0.274	0.16	-0.012	0.45	0.287	0.3	0.247	0.447	-0.043	0.284	-0.751*	0.262	0.038					
PR	0.61	0.763*	0.482	0.57	0.607	0.825*	0.477	0.338	0.461	0.495	0.456	0.824*	-0.686	0.829*	0.852*	0.19				
PS	0.128	0.026	0.125	0.065	-0.118	0.335	0.186	0.185	0.123	0.269	-0.158	0.127	-0.704	0.121	-0.113	0.970**	0.114			
CHLA	0.828*	0.871*	0.752*	0.831*	0.938**	0.716	0.766*	0.685	0.777*	0.6	0.882*	0.782*	-0.403	0.827*	0.886**	-0.273	0.667	-0.354		
CHLB	0.735*	0.780*	0.695	0.834*	0.889**	0.658	0.702	0.584	0.696	0.596	0.742*	0.71	-0.271	0.777*	0.861*	-0.306	0.572	-0.437	0.896**	
TSP	0.993**	0.960**	0.987**	0.929**	0.957**	0.880*	0.965**	0.943**	0.984**	0.932**	0.940**	0.910**	-0.668	0.931**	0.877*	0.191	0.58	0.049	0.839*	0.786*

** significant at $p \leq 0.01$; * significant at $p \leq 0.05$; PH: Plant height, SG: Stem girth, PB: Plant biomass, NL: No of leaves, CL: Cob length, CW: cob weight, GW: 1000 grain weight, HI: harvest index, BY: biological yield, GY: grain yield, ZR: Zn in root, ZS: Zn in shoot, PR: Phosphorus in root, PS: phosphorus in shoot, PS: phosphorus in soil, ZS: zinc in soil, SN: nitrogen in soil, SZ: zinc in soil, ZS: zinc in shoot, ZR: zinc in root, PR: phosphorus in root, SK: potassium in soil, TSP, total soluble protein, CHLA: chlorophyll a, CHLB: chlorophyll b

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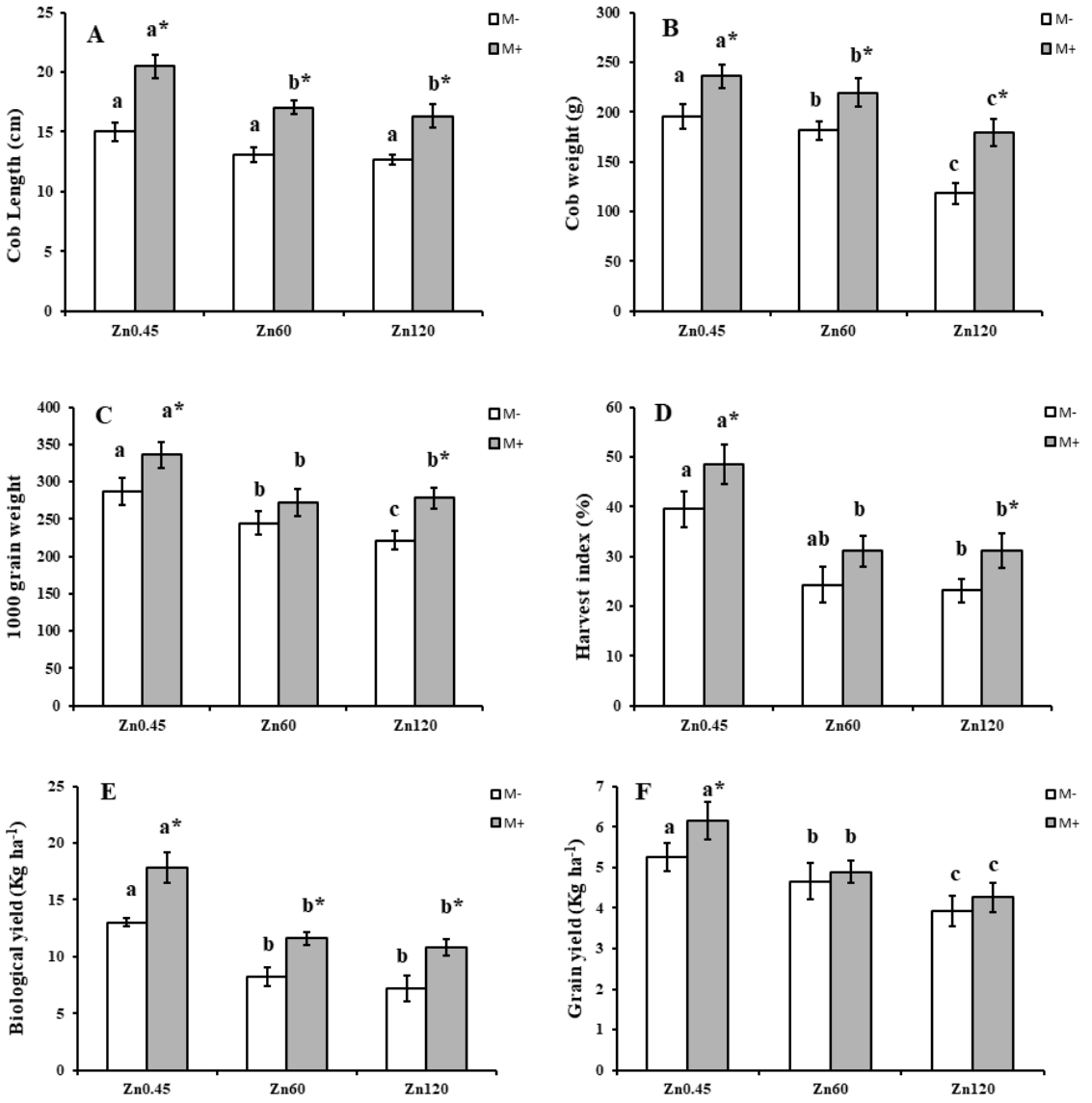

Figure 4. *Influence of arbuscular mycorrhizal inoculation maize yield attributes under zinc deficient and toxic soil conditions. (a) cob length (cm) (b) cob weight (c) 1000 grain weight (d) harvest index (e) biological yield (f) grain yield. $Zn_{0.45}$ zinc deficiency (0.45 mg Kg^{-1}), Zn_{60} medium zinc toxicity (60 mg Kg^{-1}), Zn_{120} severe zinc toxicity (120 mg Kg^{-1}). Inoculated with AMF (M + grey), un-inoculated (M- white). Vertical bars represent standard error and asterisk (*) shows significant difference ($P \leq 0.05$) among treatments. Alphabets sharing same letter shows non-significant ($P \geq 0.05$) difference whereas, different letters show significant ($P \leq 0.05$) difference among different treatments*

Discussion

Zinc deficiency is a common problem for many cereal crops. The deficiency of Zn in crops also provide deficient food among their consumers over the extended period. In this way, much of the world's human population cannot fulfil their daily Zn requirement. This can lead to serious issues regarding human health (Brown and Wuehler, 2000). To avoid nutritional deficiencies, it is important to understand, how plants uptake and utilize Zn from the soil. While the concern for increasing the

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concentration of Zn in staple crops is widely recognized (Brown and Wuehler, 2000; Burns et al., 2010). Microbial inoculants have received attention to fortify the micronutrients. If this is to change, we must develop a sound understanding how plants and AMF acquire Zn from soil. Current study evaluated the effect of mycorrhizal inoculation for plants under deficient and toxic field conditions under optimum P fertilizer application rates. In this study, mycorrhizal inoculation improved the zinc uptake in zinc deficient condition, while reduced the zinc uptake in zinc toxic concentration. That might be due to myccorhizae prevented zinc deficiency by promoting the plant zinc and phosphorons acquisition and under toxic soil Zn conditions immoblize Zn, AMF reduced the soil Zn availability as reported in present study (Chen et al., 2003; Kafkas and Ortas, 2009; Ortas, 2012). Protecting the plants against excessive zinc concentration could also be attributed to mycorrhizal application as mycorrhizae colonize the plant roots lower its uptake and tissue zinc concentration (Chen et al., 2003; Christie et al., 2004). Improvement in phosphorous uptake in stress conditions by mycorrhizal inoculation might be due to that mycorrhizal inoculation solubilized the unavailable phosphorous in stress conditions and promoted the uptake of P in plants (Javot et al., 2007). Improved P nutrition of mycorrhizal maize plants is due to the hyphal P uptake beyond the P depletion zone resulting in absorption of P from the soil solution, which otherwise cannot be replenished, as its mobility is poor in soil (Karandashov and Bucher, 2005). The results of this study are consistent with the earlier report of improved P uptake in mycorrhizal maize plants (Battini et al., 2017). Our results regarding phosphorus solubilization were further strengthened by the report of Wahid et al. (2016) in maize plants. Results reported in this study showed that mycorrhizal inoculation improved the Chlorophyll (a and b) contents under zinc stress conditions. This might be due to mycorrhizae increased the uptake of essential nutrients especially N. Increase in N content in plants promote the chlorophyll content that ultimately assist in improved photosynthetic rate. Results regarding improved chlorophyll contents in response to stress conditions are in agreement with earlier study reported by Sheng et al. (2008). It was also noticed that mycorrhizal inoculation reversed the toxic effect of zinc deficiency and toxicity on maize growth and yield, and improved maize growth such as higher plant height, stem girth, plant biomass, number of leaves and yield attributes which includes cob weight, cob length, 1000 grain weight, harvest index, biological yield and grain yield under zinc toxic and zinc deficient conditions. Improvement in maize growth and yield characteristics under zinc deficient conditions by mycorrhizal inoculation is due to the beneficial role of mycorrhizal inoculation which solubilized unavailable zinc, phosphorous and other essential nutrients and promoted their uptake in maize plants resulting in improved maize growth and yield in zinc deficient conditions (Smith and Read, 2010; Nadeem et al., 2014). The study conducted by Amanullah et al. (2011) also corroborated the results of this study with higher yield of maize with mycorrhizal inoculation in maize plants. While improvement in maize growth and yield attributes under zinc toxic conditions by fungal application might also be due to that inoculation reduced the uptake, accumulation, and translocation of zinc in plant tissues (Smith and Read, 2010). Moreover, it is also due to that mycorrhizal application protects the plants against excessive zinc concentration as mycorrhizae colonize the plant roots, lower uptake and tissue zinc concentration (Chen et al., 2003; Christie et al., 2004).

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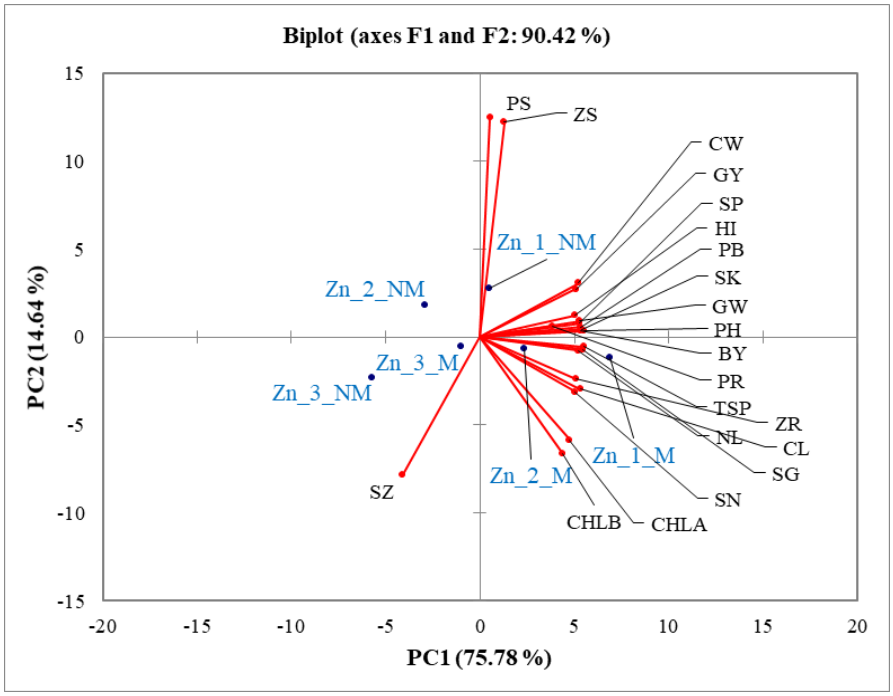

Figure 5. *PCA biplot for morpho-physiological variables of maize grown under zinc deficient and zinc toxic soil conditions with mycorrhizal (M) and non-mycorrhizal (NM) inoculations. PCA biplot is a combination of score plot of zinc treatments with M and NM (represented in blue text) and loading plot of variables (represented by red vectors; black text). Zn1: zinc deficiency (0.45 mg Kg^{-1}), Zn2: Zn60 medium zinc toxicity (60 mg Kg^{-1}), Zn3: Zn120 severe zinc toxicity (120 mg Kg^{-1}), PH: Plant height, SG: Stem girth, PB: Plant biomass, NL: No of leaves, CL: Cob length, CW: cob weight, GW: 1000 grain weight, HI: harvest index, BY: biological yield, GY: grain yield, ZR: Zn in root, ZS: Zn in shoot, PR: Phosphorus in root, PS: phosphorus in shoot, SP: phosphorus in soil, ZS: zinc in soil, SN: nitrogen in soil, SZ: zinc in soil, ZS: zinc in shoot, ZR: zinc in root, PR: phosphorus in root, SK: potassium in soil, TSP, total soluble protein, CHLA: chlorophyll a, CHLB: chlorophyll b*

Conclusion

It was concluded from the study that mycorrhizal inoculation reversed the stress effect of Zn deficiency and promoted maize growth, nutrient uptake, and yield. Inoculation of AMF imparted dual beneficial effect on maize as it increased Zn uptake in Zn deficient conditions while reduced the uptake of Zn in toxic conditions. Moreover, it can be suggested by observing the beneficial role of AMF, that it can be used to mitigate Zn deficiency and toxicity for healthy growth of maize. Further experiments should be conducted for determining the molecular mechanism behind this. Moreover, experiments should be performed on different crops, soils with varying texture, soil pH, nutrient status and moisture contents, as all of these factors strongly influenced Zn availability and translocation.

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APPENDIX

Analysis of variance table for biological yield

Source	DF	SS	MS	F	P
Myco	1	69.896	69.8956		
Zinc	2	144.904	72.4522	65.26	0.0000
Error	14	15.544	1.1103		
Total	17	230.344			

Grand mean 11.448 CV 9.20

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Analysis of variance table for cob length

Source	DF	SS	MS	F	P
Myco	1	85.238	85.2383		
Zinc	2	36.787	18.3933	9.95	0.0020
Error	14	25.877	1.8484		
Total	17	147.902			

Grand mean 15.768 CV 8.62

Analysis of variance table for cob weight

Source	DF	SS	MS	F	P
Myco	1	6063.6	6063.6		
Zinc	2	20506.3	10253.1	69.54	0.0000
Error	14	2064.3	147.5		
Total	17	28634.1			

Grand mean 193.34 CV 6.28

Analysis of variance table for chlorophyll a

Source	DF	SS	MS	F	P
Myco	1	0.49336	0.49336		
Zinc	2	0.05710	0.02855	4.48	0.0313
Error	14	0.08914	0.00637		
Total	17	0.63960			

Grand mean 1.4333 CV 5.57

Analysis of variance table for chlorophyll b

Source	DF	SS	MS	F	P
Myco	1	0.45442	0.45442		
Zinc	2	0.03484	0.01742	1.54	0.2478
Error	14	0.15798	0.01128		
Total	17	0.64724			

Grand mean 0.4944 CV 21.48

Analysis of variance table for grain weight

Source	DF	SS	MS	F	P
Myco	1	9038.4	9038.40		
Zinc	2	13254.1	6627.07	72.21	0.0000
Error	14	1284.8	91.77		
Total	17	23577.3			

Grand mean 273.25 CV 3.51

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Analysis of variance table for grain yield

Source	DF	SS	MS	F	P
Myco	1	0.92934	0.92934		
Zinc	2	7.40468	3.70234	153.47	0.0000
Error	14	0.33774	0.02412		
Total	17	8.67176			

Grand mean 4.8428 CV 3.21

Analysis of variance table for harvest index

Source	DF	SS	MS	F	P
Myco	1	216.94	216.944		
Zinc	2	911.59	455.796	15.52	0.0003
Error	14	411.13	29.366		
Total	17	1539.67			

Grand mean 32.402 CV 16.72

Analysis of variance table for number of leaves

Source	DF	SS	MS	F	P
Myco	1	72.000	72.0000		
Zinc	2	36.111	18.0556	6.71	0.0090
Error	14	37.667	2.6905		
Total	17	145.778			

Grand mean 10.889 CV 15.06

Analysis of variance table for plant biomass

Source	DF	SS	MS	F	P
Myco	1	562.24	562.242		
Zinc	2	1883.36	941.682	65.37	0.0000
Error	14	201.68	14.406		
Total	17	2647.28			

Grand mean 112.00 CV 3.39

Analysis of variance table for plant height

Source	DF	SS	MS	F	P
Myco	1	3618.5	3618.45		
Zinc	2	10581.7	5290.87	118.57	0.0000
Error	14	624.7	44.62		
Total	17	14824.9			

Grand mean 222.26 CV 3.01

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Analysis of variance table for phosphorus in roots

Source	DF	SS	MS	F	P
Myco	1	20.2248	20.2248		
Zinc	2	24.8323	12.4162	76.17	0.0000
Error	14	2.2819	0.1630		
Total	17	47.3390			

Grand mean 2.5256 CV 15.99

Analysis of variance table for phosphorus in shoot

Source	DF	SS	MS	F	P
Myco	1	2.6912	2.69120		
Zinc	2	8.6987	4.34937	3.80	0.0479
Error	14	16.0036	1.14312		
Total	17	27.3936			

Grand mean 2.1889 CV 48.85

Analysis of variance table for mycorrhizal root colonisation

Source	DF	SS	MS	F	P
Myco	1	8149.39	8149.39		
Zinc	2	35.11	17.56	1.20	0.3297
Error	14	204.44	14.60		
Total	17	8388.94			

Grand mean 47.944 CV 7.97

Analysis of variance table for shoot girth

Source	DF	SS	MS	F	P
Myco	1	1.20125	1.20125		
Zinc	2	1.37301	0.68651	74.60	0.0000
Error	14	0.12883	0.00920		
Total	17	2.70309			

Grand mean 7.8206 CV 1.23

Analysis of variance table for soil potassium

Source	DF	SS	MS	F	P
Myco	1	177.033	177.033		
Zinc	2	200.406	100.203	16.71	0.0002
Error	14	83.952	5.997		
Total	17	461.391			

Grand mean 15.975 CV 15.33

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Analysis of variance table for soil nitrogen

Source	DF	SS	MS	F	P
Myco	1	44.305	44.3054		
Zinc	2	55.922	27.9612	12.51	0.0008
Error	14	31.297	2.2355		
Total	17	131.525			

Grand mean 5.3033 CV 28.19

Analysis of variance table for soil phosphorus

Source	DF	SS	MS	F	P
Myco	1	25.2998	25.2998		
Zinc	2	47.6024	23.8012	22.95	0.0000
Error	14	14.5174	1.0370		
Total	17	87.4196			

Grand mean 5.4533 CV 18.67

Analysis of variance table for soil zinc

Source	DF	SS	MS	F	P
Myco	1	251.78	251.777		
Zinc	2	1115.27	557.636	16.60	0.0002
Error	14	470.35	33.596		
Total	17	1837.39			

Grand mean 11.357 CV 51.04

Analysis of variance table for total soluble protein

Source	DF	SS	MS	F	P
Myco	1	209.169	209.169		
Zinc	2	198.507	99.253	51.94	0.0000
Error	14	26.754	1.911		
Total	17	434.430			

Grand mean 17.714 CV 7.80

Analysis of variance table for zinc in root

Source	DF	SS	MS	F	P
Myco	1	0.00720	0.00720		
Zinc	2	0.00668	0.00334	18.21	0.0001
Error	14	0.00257	0.00018		
Total	17	0.01644			

Grand mean 0.0556 CV 24.37

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Analysis of variance table for zinc shoot

Source	DF	SS	MS	F	P
Myco	1	2.6758	2.67576		
Zinc	2	14.8597	7.42987	9.92	0.0021
Error	14	10.4899	0.74928		
Total	17	28.0254			

Grand mean 3.3511 CV 25.83

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MORPHO-PHYSIOLOGICAL AND MOLECULAR RESPONSES OF COWPEA (*VIGNA SINENSIS* L.) TO NICKEL TOXICITY

AL-MUWAYHI, M. A.

Department of Physics and Chemistry, Faculty of Science, Shaqra University, P.O. Box 33, Shaqra 11961, Kingdom of Saudi Arabia
(e-mail: malmuwayhi@su.edu.sa; phone: +966-11-6224-4118)

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Abstract. One of the environmental risks that threatens us is the toxicity of nickel (Ni^{2+}). Cowpea (*Vigna sinensis* L.) plants require it in trace amounts, while high concentrations of it influence their biochemical and physiological processes. In the current study, the effect of various concentrations of nickel (100, 200, and 300 mg Ni^{2+} / kg soil) has been investigated to identify their effects on *Vigna sinensis*. The results revealed the negative effects of nickel on most of the studied physiological and morphological traits compared with those of the control. The concentrations of mineral elements and chlorophyll decreased significantly. Reducing, non-reducing and total sugar content were affected negatively. Furthermore, the activity of studied enzymes (APX, GPX, CAT, SOD) decreased significantly. Moreover, the negative effects of nickel concentrations on the root, stem, leaves, seeds, flowers and fruits were obvious. On a molecular level, the ISSR markers could not detect any polymorphism, indicating no toxic effect of nickel on the DNA at the studied concentrations. It is obvious that the nickel has a toxic effect on *Vigna sinensis* at physiological and morphological levels. The conduction of more investigations on the effects of nickel on a molecular level using more molecular markers is highly recommended.
Keywords: *enzyme, lipid, reducing sugar, protein, DNA, ISSR*

Introduction

Cowpea (*Vigna sinensis* L.) is a very important leguminous plant throughout the world especially in rural areas, as it is a good source of protein and energy (Granito et al., 2005). There are many environmental hazards surrounding living organisms that pose a major risk to their life, safety and health, including heavy environmental hazards (Panda and Panda, 2002). The toxicity of heavy metals is one of the current major environmental health problems and is potentially dangerous due to their bioaccumulation through the food chain and in plant products that are used for human consumption. Therefore, soil and plant pollution with heavy metals has become an increasing problem (Kaveriammal and Subramani, 2013), as the important biological processes in the plant are greatly affected by the increase in the percentage of heavy metals such as the metabolism process and, consequently, the plant traits and yield (Sinha and Gupta, 2005). Increased levels of heavy metals in the plant may lead to food poisoning (Macnair, 1993). Nickel is considered one of the important and abundant elements in the earth's crust. It is one of the basic mineral nutrients found in natural soil with minimal concentrations (Hussain et al., 2013). It is one of the important heavy metals in the environment, which gains wide recognition and is also essential for plants (Harasim and Filipek, 2015). It is also one of the essential nutrients needed by the plant and there are many studies suggesting its importance and that it has beneficial effects for the plant at low concentration (Sabir et al., 2011; Rathor et al., 2014). Despite the primary role of the nickel in the plant, toxicologists considered that the toxicity of nickel poses an environmental threat to biological systems when these are overexposed to it. For instance: the study of Asagba et al. (2019) conducted on *Vigna unguiculate* L. indicated that there are significant

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differences when treating the soil with a concentration of 100 ppm of nickel with adverse effects on plant height, plant growth rate and leaf Ca^{2+} ATPase activity. The main reasons for the high levels of nickel pollution in the environment are industrial and agricultural activities. To understand the role and importance of nickel for plants, it is necessary to understand the functional properties and toxic effects of nickel. The amount of nickel the plant needs for natural growth and development is very small, however it is considered an important component of the different enzymes in plants and a regulator of specific enzyme activities that are involved in maintaining the appropriate cellular oxidation state and many of the biochemical and physiological responses such as photosynthesis, plant pigments, cell membranes and other growth processes. High concentrations of nickel affect the morphological traits of the plant and prevent root growth (Hussain et al., 2013). There are many sources of nickel in the environment that may cause a problem if the nickel element is present in high concentrations because it is toxic to plants and other organisms (Harasim and Filipek, 2015). A study of Jagetiya and Bhatt (2007) conducted on cowpea (*Vigna unguiculata* L.) indicated that high concentrations (1000 µm) of nickel led to a decrease in fresh mass, dry matter production and chlorophyll contents of seedlings. The treatment of cowpea (*Vigna unguiculate* L.) with high concentrations (1.8 mM and 2.4 Mm) of nickel led to a significant impact on the performance of seedlings growth and the effect on physiological and biochemical characteristics. Moreover, the concentration of 1.2 mM affected negatively the number of leaves, length of seedlings, fresh weight, and dry weight of seedlings. Furthermore, concentrations of 0.6 mM, 1.2 mM, 1.8 mM and 2.4 mM showed less influence on leaf area, leaf weight ratio, root fresh and dry weight, while having a significant effect on the relative water content and chlorophyll A, B, and total chlorophyll, carotenoids and phenols (Mujeeb et al., 2019). Rathor et al. (2014) reported that high concentrations of nickel negatively affected maize plant. They showed that the dry matter decreased at the concentration of 50 mg/kg. The results of many studies confirmed that the high concentrations of nickel become toxic to most plant species, as it affects the processes of photosynthesis, respiration, mineral nutrition, water relations, transport of mineral, and induction of oxidative stress (Llamas and Sanz, 2008; Llamas et al., 2008). The increase in the rates of accumulation of heavy metals also leads to a significant change in the genes of the organism and thus affects the genetic diversity (Panda and Panda, 2002). The toxicity of nickel caused clastogenicity and the aneugenicity in onion (*Alium cepa*) (Dovgalyuk et al., 2001). Scientists have been able to link and produce genetic maps using molecular markers such as AFLP, RAPD, RFLP, SSR. The development of genetic maps using molecular markers is one of the most important steps in the genome studies of plants (Ma et al., 2001; Nguyen et al., 2001). Al-Qurainy (2009) in his study on the molecular detection of Al and Ni toxicity on the Phaseolus vulgaris plant, concluded that the toxicity of heavy metals in plants is due to the stimulation of oxidative stress associated with oxidation of proteins and membranous fats and changes in the DNA damage response. The genetic toxicity induced by these minerals was evaluated using RAPD and this technique was useful in assessing the genetic toxicity. Moreover, a genetic mutation at a concentration of 150 mg Ni / kg of soil was detected. The genetic toxicity of nickel was also assessed with RAPD molecular marker in a study on Barbados nut (*Jatropha curcas*) plants (Sarkar et al., 2010). RAPD analysis produced only 5 polymorphic bands (3.225%) out of a total of 155 bands from 18 selected primers. Only three primers OPK-19, OPP-2, OPN-08 produced polymorphic bands. Gjorgieva et al. (2013) analyzed the biological effects induced by bioaccumulation of metals in common bean (*Phaseolus vulgaris* L.). Their results

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suggested that mineral nutrient imbalance is involved in changes of antioxidant levels and DNA damages of the seedlings, which may help to understand the mechanism of metal toxicity in plants. DNA polymorphism detected by ISSR analysis offered a useful molecular marker for the identification of mutants in gamma radiation-treated plants and demonstrated that the DNA of the hybrid "Doi Tung 554" of siam tulip (*Curcuma alismatifolia*) showed a greater response in induced mutation compared with the other varieties (Taheri et al., 2013). Al-Qurainy (2010) utilized ISSR molecular markers to study the effect of toxicity of three heavy metals (Zn, Pb, and Cd) on DNA in the *Eruca sativa* L. plant, where 20 ISSR primers were used. The ranking of genotoxic potencies of the three heavy metals was in the descending order of $Cd^{2+} > Pb^{2+} > Zn^{2+}$. Among these heavy metals, high concentration of Cd (150 mg/l) and Pb (150 mg/l) generated mutations along with changed morphology of seedlings. Despite the importance of nickel-polluted soils throughout the world, little is known about the activity of nickel required to reduce plant growth and the effects that nickel toxicity has on the plant. The aim of this study is to identify the toxic effects of nickel on physiological, morphological and molecular traits using ISSR molecular markers in *Vigna sinensis* L.

Materials and methods

Plant material and experimentation

The seeds of "Buff" variety of *Vigna sinensis* were purchased from the local market and the experiment was conducted at the Department of Chemistry, Faculty of Science, Shaqra University, Kingdom of Saudi Arabia. Three seeds were planted in each plastic pot containing 1 kg of sterilized soil. The concentrations of Ni ($NiSO_4$) used for soil treatment were; 100 mg/kg soil, 200 mg/kg soil, 300 mg/kg soil, and the control. There were three replicates for all treatments. Plants were grown in a controlled air conditioned climate and light of green house (24°C and 13:11 hours dark-light cycle). Three plants from each replicate were sampled after 90 days of sowing (fruiting phenophase) and the targeted measurements were taken.

Physiological parameters

Phosphorous content was determined according to Murphy and Riley (1962). Potassium was determined using the Flame photometer according to Allen et al. (1989). Estimation of Ca and Mg were performed using the Atomic Absorption Spectroscopy instrument according to Stewart (Allen et al., 1974). Chlorophyll A, B and total were estimated using Metzner et al. (1965) method. Estimating non-reducing sugars was calculated by subtracting the value of reduced sugars from the value of total sugars. Determination of total sugars was done depending on Hedge et al. (1962). Reduced Sugars were estimated according to Li et al. (2013). Estimation of lipids was performed using the Soxhlet method. Total protein was estimated based on the nitrogen content of the sample using the Kjeldahl method (Jones Jr et al., 1991). Soluble protein was determined according to Bradford (1976). Superoxide dismutase (SOD) activity was estimated according to Giannopolitis and Ries (1977). Catalytic Activity (CAT) was estimated according to Aebi (1984). Guaiacol peroxidase (GPX) enzyme activity was estimated according to Egley et al. (1983). Ascorbate peroxidase (APX) enzyme activity was estimated according to Asada (1984).

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Genomic DNA isolation

Reagents and chemicals

The stock solution concentrations were: acetyl trimethyl ammonium bromide (CTAB) 3% (w/v), 1 M TrisCl (pH 8), 0.5 M EDTA (pH 8), 5 M NaCl, absolute ethanol (AR grade). The extraction buffer consisted of CTA B 3% (w/v), 100 mM Tris –Cl (pH 8), 25 mM EDTA (pH 8), and 2 M NaCl, respectively. The PVP and β-mercaptoethanol were freshly prepared and added in the extraction buffer.

DNA extraction

DNA was isolated from plants grown in green house using a modified CTAB method (Khan et al., 2007). In brief, the plant samples were ground into extraction buffer (100 mM tris buffer pH 8, 25 mM EDTA, 2 M NaCl, 3% CTAB, 3% PVP). The suspension was gently mixed and incubated at 65ºC for 20 min with occasional mixing. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 12,000 rpm for 5 min. The clear upper aqueous phase was then transferred to a new tube and 2/3 volume of ice-cooled isopropanol was added followed by incubation at -20ºC for 30 min. The nucleic acid was collected by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 80% ethanol. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE (10 mM tris buffer pH 8, 1 mM EDTA) at room temperature and stored at 4ºC until used. The RNA from crude DNA was eliminated by treating the sample with RNase A (10 mg/ml) for 30 min at 37ºC. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 and 280 nm. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

PCR amplification and ISSR reaction

PCR amplification was done with ISSR primers according to the protocol developed by Zietkiewicz et al. (1994). Ten ISSR primers synthesized from Sigma Company were used to amplify the genomic DNA extracted from untreated and treated plants. After screening of the primers, two of them were promised to produce mono/polymorphism (*Table 1*).

Table 1. *List of ISSR Primers used in the study*

Sequence	Code	Annealing temperature
5'-ACACACACACACACACC-3'	UBC-26	52.4ºC
5'-AGAGAGAGAGAGAGAGYC-3'	UBC-35	53.9ºC

The PCR reaction was carried out in 20 ml volume of master mixture purchased from Amerson Company (UK). In master mixture, 30 ng of template DNA and 30 ng of primer were added in each tube. Tubes were vortexed and briefly centrifuged after adding template DNA and primer in master mixture. The amplification was done on 96 well plates on a Primus PCR machine as per the program: First denaturation at 94°C for 3 min, segment denaturation at 94°C for 1 min, annealing at 45°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 3 min was performed for amplification.

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Statistical analysis

Completely Randomized Design was used (Stell et al., 1980). Analysis of variance was performed on the collected data using SAS 9.1, SAS Inc., North Carolina, USA (SAS, 2014). Thus, mean values were rated significant at $p < 0.05$. The least significant difference (LSD) test was used to discern differences among the mean values of the treatments and accessions.

Results

Effect of nickel on the content of mineral elements in the leaves

The results showed that there is an effect of different concentrations of nickel on phosphorous (P) content in the leaves of *Vigna sinensis* (*Table 2*). Generally, there were significant differences between all treatments compared to the control, however, there were no significant differences between the two concentrations (200 and 300 mg / Kg of soil). The results also showed that there is an effect of different concentrations of nickel on the potassium (K) content of the leaves of *Vigna sinensis*. The differences were significant at all concentrations of nickel (100, 200, 300 mg / kg of soil). The results also showed a negative effect of different concentrations of nickel on the Ca content of the leaves of the *Vigna sinensis*. There were significant differences between the concentration of 100 mg / kg of soil and the other concentrations (200, 300 mg / kg of soil), but no significant differences between the two concentrations (200 and 300 mg / kg of soil). The content of the leaves of Ca treated at concentration of 100 mg / kg of soil reached 16.6 ppm, and the lowest content was at 300 mg / kg of soil (13.3 ppm). The results also showed that there is an effect of the different concentrations of nickel on the content of magnesium (Mg) in the leaves of *Vigna sinensis*. There were significant differences at the all concentrations.

Table 2. *Effect of various concentrations of nickel on the mineral elements in the leaves of Vigna sinensis*

Treatments (mg/kg soil)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)
Control	2.2a± 0.6	103.4a± 8.1	21.2a± 13.3	37.1a± 3.4
100	1.8c± 0.4	94.3b± 8.4	16.6b± 15.2	22.3b± 7.2
200	1.6b± 0.9	76.7c± 11.9	14.2c± 7.8	19.0c± 6.5
300	1.6b± 0.8	58.7d± 13.6	13.3c± 10.3	15.3d± 11.6
LSD 0.05	0.3	5.2	2.5	2.4

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the content of plant pigments in the leaves

The results shown in *Table 3.* showed that there is an effect of different concentrations of nickel on the chlorophyll a in the leaves of the *Vigna sinensis*. There were significant differences between concentration 100 mg / kg of soil and concentrations 200 and 300 mg / kg of soil, whereas no significant differences can be observed at the last two concentrations. It was observed that the concentration of chlorophyll a decreased as the

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toxicity of the nickel increased, compared to the control. The concentration of chlorophyll a in the plants exposed to a concentration of 100 mg / kg of soil is higher than the other concentrations of nickel. There is also an effect of different concentrations of nickel on the content of chlorophyll b in the leaves of *Vigna sinensis*.

Table 3. *Effect of various concentrations of nickel on plant pigment concentration in Vigna sinensis L. leaves*

Treatments (mg/g)	Chlorophyll a (µg /g)	Chlorophyll b (µg /g)	Carotenoids (µg /kg soil)
Control	0.68a± 0.15	0.41a± 0.12	0.029a± 0.01
100	0.41b± 0.15	0.23b± 0.07	0.026a± 0.01
200	0.24c± 0.15	0.26b± 0.11	0.031a± 0.02
300	0.23c± 0.17	0.18c± 0.09	0.027a± 0.02
LSD 0.05	0.05	0.04	-

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the sugar content in the leaves

The results showed that there is an effect of the different concentrations of nickel on the content of reducing sugars in the leaves of the *Vigna sinensis* (*Table 4*). There were significant differences between the concentration of 100 mg / kg of soil and the other concentrations (200 and 300 mg / kg of soil). It was observed that there is decrement in the concentration of reducing sugars as the toxicity of the nickel increases compared to the control. In terms of non-reducing sugars, there were significant differences between the concentration of 100 mg / kg of soil and other two concentrations (200 and 300 mg / kg of soil). No significant differences were detected between the two concentrations of 200 and 300 mg / kg of soil. The results indicate that the content of non-reducing sugars in the plants exposed to concentration of 100 mg / kg of soil was the highest. Total sugars in the leaves of the *Vigna sinensis* L. also were affected negatively as the plants were exposed to different concentration of nickel. The content of total sugars at a concentration of 100 mg / kg of soil was higher than the other two concentrations (200 and 300 mg / kg of soil) with significant difference.

Table 4. *Effect of various concentrations of nickel on sugar concentration in Vigna sinensis leaves*

Treatments (mg/kg soil)	Reducing sugars (mg/g)	Non-reducing sugars (mg/g)	Total sugars (mg/g)
Control	49.2a± 15.1	115.4a± 17.5	164. 6a± 34.1
100	30.7b± 11.2	112.2b± 23.7	142.9b± 50.4
200	24.5c± 3.8	97.1c± 41.4	121. 6c± 50.1
300	23.4c± 3.1	99. 4c± 45.9	122.8c± 51.2
LSD 0.05	3. 5	7. 4	6.3

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

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Effect of nickel on lipids, total protein and soluble protein in the leaves

The results revealed that there is an effect of the different concentrations of nickel on the content of lipids in the leaves of *Vigna sinensis* (*Table 5*). There were significant differences between the concentration of 100 mg / kg of soil and the other concentrations, but these differences were not significant between the concentrations of 200, and 300 mg / kg of soil. Total protein was negatively affected as a result of exposing the plants to different concentrations of nickel. There were significant differences between all concentrations. The total protein in the plants treated with a concentration of 100 mg / kg of soil was higher than at other concentrations. The results indicated that there is an effect of different concentrations of nickel on the soluble protein in the leaves. There are significant differences between concentration of 100 mg / kg of soil and other concentrations, but not the two concentrations of 200 and 300 mg / kg of soil. The soluble protein in plants treated with concentration of 100 mg / kg of soil was higher than that at other concentrations (200, 300 mg / kg of soil).

Table 5. *Effect of various concentrations of nickel on lipids, total protein and soluble protein in Vigna sinensis leaf*

Treatments (mg/kg soil)	Lipids (%)	Total protein (%)	Soluble protein (mg/g)
Control	4.43a± 1.65	19.4a± 3.1	9.2a± 0.11
100	4.12a± 1.15	16.2b± 3.7	9.1b± 0.12
200	2.21b± 0.55	12.4c± 6.2	8.9c± 0.16
300	1.94b± 0.47	11.0d± 4.0	8.5d± 0.08
LSD 0.05	0.25	0.973	0.07

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the activity of enzymes in the leaves

The results showed that there is an effect of different concentrations of nickel on the activity of Ascorbate peroxidase (APX) in the leaves of the *Vigna sinensis* compared with the control (*Table 6*). However, no significant differences were detected between the three nickel concentrations (100, 200, and 300 mg / kg of soil). Guaiacol peroxidase (GPX) activity was decreased significantly as a result to exposing the plants to various nickel concentrations (100, 200, and 300 mg / kg of soil). Oppositely, no significant differences were observed between nickel concentrations. There is a negative effect of nickel treatment on the activity of Catalase activity (CAT). It was observed that the general trend is a decrement in CAT activity as the toxicity of the nickel increases compared to the control. CAT activity in response to 100 mg / kg of soil was higher (47.1 µmol / min / mg) than that at other concentrations (200 and 300 mg / kg of soil). Superoxide dismutase (SOD) activity also significantly decreased in response to nickel treatments (100, 200 and 300 mg / kg of soil). The general trend is a decrement in the SOD activity as the toxicity of the nickel increases compared to the control. The results showed that the percentage of SOD activity in plants treated with a concentration of 100 mg / kg of soil is higher (41.3 µmol / min / mg) than the concentrations of 200 and 300 mg / kg of soil (38.4 and 38.1 µmol / min / mg), respectively.

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Table 6. *Effect of various concentrations of nickel on the activity of enzymes in leaves of Vigna sinensis*

Treatments (mg/kg soil)	Ascorbate peroxidase(APX) (µmol/min/mg protein)	Guaiacol peroxidase (GPX) (µmol/min/mg protein)	Catalase (CAT) (µmol/min/mg protein)	Superoxide dismutase (SOD) (µmol/min/mg protein)
Control	2.6a± 0.08	2.15a± 0.04	55.2a± 0.11	47.2a± 2.34
100	1.3b± 0.28	2.08b± 0.34	47.1b± 2.12	41.3b± 0.98
200	1.3b± 0.06	1.92b± 0.08	45.9c± 1.16	38.4c± 4.28
300	1.2b± 0.05	1.91b± 0.06	39.5c± 4.18	38.1c± 4.51
LSD 0.05	0.13	0.973	0.10	1.28

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on plant height, leaf area, and number of leaves

The results revealed that there is no significant effect of the different concentration of nickel (100, 200, and 300 mg / kg of soil) on the plant height neither comparing with the control nor within the different concentrations. However, leaf area and number of leaves were significantly decreased as a result of exposing to the different concentrations of nickel (100, 200, and 300 mg Ni^{2+} / kg of soil) compared with the control. Moreover, high significant differences between the nickel concentrations were detected as well (*Table 7*).

Table 7. *Effect of various concentrations of nickel on plant height, leaf area, and number of leaves in Vigna sinensis*

Treatments (mg/kg soil)	Plant height (cm)	Leaf area (cm^2)	Number of leaves
Control	65.4a± 21.1	959.4a± 366	15.3a± 3.1
100	64.1a± 21.2	798.2b± 274	11.1b± 2. 6
200	64.2a± 21.3	756.3c± 267	10.4c± 2. 2
300	62.4a± 22.2	746.4d± 254	10.1d± 2.1
LSD 0.05	2.6	36.5	0.62

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the wet weight of plant parts

The wet weight of the plant and all its parts (stem, leaf and root) were affected negatively in response to the different concentration of nickel (100, 200, and 300 mg Ni^{2+} / kg of soil). The statistical analysis of the results revealed high significant differences between the nickel concentrations and the control, and within the concentrations as well (*Table 8*). The general trend indicates a decrement in the wet weight of the plant and all its part as increasing the toxicity of nickel. The average wet weight of the stem ranged from 142.4 g at a concentration of 100 mg / kg of soil to 51.4 g at a concentration of 300 mg / kg of soil. The average wet weight the root at a concentration of 100 mg / kg of soil was greater than at the concentrations of 200 and 300 mg / kg of soil. It reached 32.2, 24.1, 23.4 g, respectively. The average wet weight of the plant at a concentration of 300 mg / kg of soil was 106.6 g.

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Table 8. *Effect of various concentrations of nickel on stem wet weight, leaf wet weight, root wet weight, plant wet weight in Vigna sinensis*

Treatments (mg/kg soil)	Stem wet weight (g)	Leaf wet weight (g)	Root wet weight (g)	Plant wet weight (g)
Control	142.4a± 65.5	126.4a± 45.1	40.3a± 10.5	309.1a± 124.1
100	109.2b± 44.5	86.2b± 24.7	32.2b± 7.5	227.6b± 73.7
200	59.1c± 27.5	42.1c± 8.2	24.1c± 2.5	125.3c± 36.2
300	51.4d± 9.7	31.8d± 10.7	23.4d± 1.7	106.6d± 19.7
LSD 0.05	3.5	4.03	2.3	4.6

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the dry weight of plant parts

The dry weight of the plant and all its parts (stem, leaf and root) were affected negatively in response to the different concentrations of nickel (100, 200, and 300 mg Ni^{2+} / kg of soil). The statistical analysis of the results revealed high significant differences between the nickel concentrations and the control, and within the concentrations as well (*Table 9*). The general trend indicates a decrement in the dry weight of the plant and all its part as the toxicity of nickel increased. The average dry weight of the stem ranged from 22.2 g at a concentration of 100 mg / kg of soil to 13.4 g at a concentration of 300 mg / kg of soil). The dry weight of the leaf at a concentration of 100 mg / kg of soil was greater than at the other two concentrations (200 and 300 mg / kg of soil). It was 31.2, 17.1 and 14.4 g, respectively. The average dry weight of the root ranged from 15.2 g at a concentration of 100 mg / kg of soil to 12.4 g at a concentration of 300 mg / kg of soil. The average of plant dry weight at a concentration of 100 mg / kg of soil was greater than at the concentrations of 200 and 300 mg / kg of soil. It reached 68.6, 46.0 and 40.2 g, respectively.

Table 9. *Effect of various concentrations of nickel on stem dry weight, leaf dry weight, root dry weight and plant dry weight in Vigna sinensis*

Treatments (mg/kg soil)	Stem dry weight (g)	Leaf dry weight (g)	Root dry weight (g)	Plant dry weight (g)
Control	38.7a± 4.51	44.3a± 9.14	17.3a± 4.24	100.3a± 13.24
100	22.2b± 4.76	31.2b± 5.56	15.2b± 1.46	68.6b± 9.36
200	15.8c± 3.54	17.1c± 2.54	13.1c± 1.44	46.0c± 6.24
300	13.4d± 1.73	14.4d± 1.71	12.4d± 1.81	40.2d± 2.91
LSD 0.05	2.2	2.1	1.1	2.7

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the number of flowers and flower weight

The results showed that there is an effect of the concentrations of nickel on the number of flowers. There are significant differences between the concentration of 100 mg / kg of soil and the concentrations of 200 and 300 mg / kg of soil, whereas there is no significant difference between the concentrations of 200 and 300 mg / kg of soil. The average number

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of flowers ranged from 18.2 at a concentration of 100 mg / kg of soil and 14.1 at a concentration of 300 mg / kg of soil (*Table 10*). The toxicity of nickel had an effect on the wet weight of flowers at all concentrations compared to the control. The results showed that there were significant differences between the concentration of 100 mg / kg of soil and the concentrations of 200, 300 mg / kg of soil. The wet weight of flowers at concentrations of 100, 200, and 300 mg / kg of soil were 8.2, 7.2, and 7.0 g, respectively. The average dry weight of flower at a concentration of 100 mg / kg of soil was greater than at concentrations of 200 and 300 mg / kg of soil with significant differences. It reached 0.52 g at a concentration of 100 mg / kg of soil and 0.42, 0.35 g at concentrations of 200 and 300 mg / Kg of soil, respectively.

Table 10. *Effect of various concentrations of nickel on number of flowers, flower wet weight, and flower dry weight in Vigna sinensis*

Treatments (mg/kg soil)	Number of flowers	Flower wet weight (g)	Flower dry weight (g)
Control	42.3a± 3.5	23.2a± 1.4	1.1a± 0.11
100	18.2c± 1.3	8.2b± 0.6	0.52b± 0.01
200	15.1d± 1.1	7.2c± 0.3	0.42c± 0.03
300	14.1d± 0.1	7.0c± 0. 1	0.35d± 0.01
LSD 0.05	3.45	0.11	0.01

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the number of fruits and fruit wet and dry weight

Treatment of the plants with different concentrations of nickel (100, 200, and 300 mg Ni^{2+} / kg of soil) affected number of fruits, fruit wet weight, and fruit dry weight in *Vigna sinensis* negatively. The statistical analysis reflected significant analysis between all the treatments compared with the control and among the concentrations of nickel (*Table 11*). The average number of fruits ranged from 10 at a concentration of 100 mg / kg of soil to 6.5 at a concentration of 300 mg / kg of soil. Average fruit wet weight at a concentration of 100 mg / kg of soil was greater than at the other two concentrations (200 and 300 mg / kg of soil). It was 12.2, 12.0, and 10.5 g, respectively. The average fruit dry weight ranged from 1.7 g at a concentration of 100 mg / kg of soil to 1.2 g at a concentration of 300 mg / kg of soil.

Table 11. *Effect of various concentrations of nickel on number of fruits, fruit wet weight, and fruit dry weight in Vigna sinensis*

Treatments (mg/kg soil)	Number of fruits	Fruit wet weight (g)	Fruit dry weight (g)
Control	17.6a± 2.5	38.2a± 3.21	5.6a± 0.70
100	10.1b± 1.6	12.2b± 2.32	1.7b± 0.22
200	5.3c± 0.6	12.0c± 2.03	1.4c± 0.11
300	6.5d± 1.2	10.5d± 1.71	1.2d± 0.10
LSD 0.05	3.1	6.11	0.8

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

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Effect of nickel on the number and weight of the seeds

The results showed that there is an effect of different concentrations of nickel on the number of seeds in *Vigna sinensis*. As illustrated in *Table 12*, there were no significant differences between the three concentrations of nickel (100, 200, and 300 mg / kg of soil). The seed weight was also affected by nickel treatments. The general trend revealed a decrement in the average seed weight as the toxicity of nickel increases compared to the control.

Table 12. *Effect of various concentrations of nickel on number of seeds, and seed weight in Vigna sinensis*

Treatments (mg/kg soil)	Number of seeds	Seed weight (g)
Control	270.3a± 35.5	36.4a± 6.1
100	60.1b± 5.5	8.1b± 0.8
200	54.2b± 6.5	7.2c± 0.4
300	45.4b± 2.7	6.5d± 0.5
LSD 0.05	32.5	5.4

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

Effect of nickel on the flowering

There is an effect of different concentrations of nickel on the beginning of flowering. There were significant differences between all treatments (*Table 13*). The general trend showed a delay in the flowering date as the toxicity of nickel increased compared with the control. The average flowering initiation period ranged from 55 days at a concentration of 100 mg / kg of soil to 56.4 days at a concentration of 300 mg / kg of soil. The effect of nickel toxicity on the end-flowering period was also observed in all concentrations compared to the control. As presented in *Table 13*, there were significant differences between the concentration of nickel (100, 200, and 300 mg / kg of soil). These results showed an effect of different concentrations of nickel on the flowering duration compared to the control. There are significant differences between the concentrations of 100, 200, and 300 mg / kg of soil. It was obvious that the average flowering duration decreased as the toxicity of nickel increased. It ranged from 40 days at a concentration of 100 mg / kg of soil to 37.9 days at a concentration of 300 mg / kg of soil.

Table 13. *Effect of various concentrations of nickel on start flowering date, end flowering date, and flowering duration in Vigna sinensis*

Treatments (mg/kg soil)	Start date of flowering (day)	End date of flowering (day)	Flowering duration (day)
Control	50.2c± 0.0	101.4a± 0.0	51.2a± 0.0
100	55.2b± 0.0	95.2b± 1.2	40.0b± 1.2
200	54.1b± 1.0	95.4c± 1.0	41.3c± 1.7
300	56.4a± 0.6	94.3c± 1.0	37.9d± 1.05
LSD 0.05	1.1	1. 7	2. 2

Values are mean ± SD for three replicates in each treatment. Within the same column, means followed by the same letter do not differ significantly ($p < 0.05$)

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Effect of nickel on the fruiting

The results showed that there is negative effect of different concentrations of nickel on the beginning of fruiting. There are significant differences between the concentrations (*Table 14*). The average start of fruiting ranged from 76.2 days at a concentration of 100 mg / kg of soil to 80.2 days at a concentration of 300 mg / kg of soil. The effect of nickel toxicity on the end of fruiting date was observed in all concentrations compared to the control. Although there was a significant difference between all concentrations of nickel compared with the control, there were no significant differences between the concentrations. The results also showed an effect of different concentrations of nickel on the fruiting duration compared with the control. There were significant differences between the treatments. The average fruiting period ranged from 26.9 days at a concentration of 100 mg / Kg of soil and 21.2 days at a concentration of 300 mg / kg of soil.

Table 14. *Effect of various concentrations of nickel on start fruiting date, end fruiting date, and fruiting duration in Vigna sinensis*

Treatments (mg/kg soil)	Start date of fruiting (day)	End date of fruiting (day)	Fruiting duration (day)
Control	64.3c± 1.0	110.4a± 1.2	46.1a± 2.2
100	76.2b± 1.2	103.1b± 1.5	26.9b± 1.9
200	77.1b± 0.6	102.3b± 1.2	25.2c± 0.7
300	80.2a± 0.0	101.4b± 1.7	21.2c± 1.4
LSD 0.05	1.8	2. 3	3. 2

Values are mean ± SD for three replicates in each treatment ($p < 0.05$)

Effect of nickel on the DNA

Two ISSR responding primers which produced clear cut and reproducible bands (UBC 26 and UBC 35) were further used to amplify genomic DNA from all treated and untreated plants. The results revealed that all appeared fragments were monomorphic (*Figures 1 and 2*) and no differences were observed between the treatments. These results indicated that there is no genotoxic effect of nickel on the DNA of *Vigna sinensis* at the treatments of 100, 200, and 300 mg/kg soil.

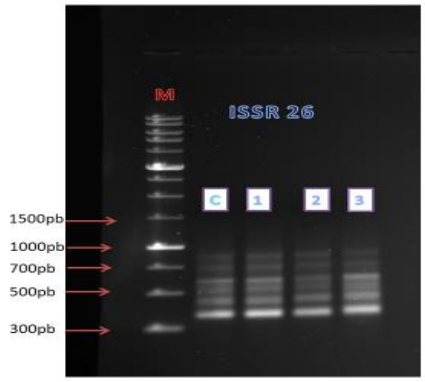

Figure 1. *ISSR fingerprints for Vigna sinensis treated with various concentrations of nickel with primer UBC 26. Lane M: 1 kb DNA ladder; Lane c: control; Lanes 1, 2, 3: plants treated with nickel at concentrations of 100, 200 and 300 mg/kg soil, respectively*

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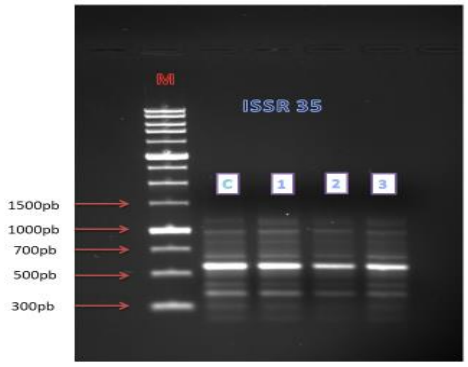

Figure 2. *ISSR fingerprints for Vigna sinensis treated with various concentrations of nickel with primer UBC 35. Lane M: 1 kb DNA ladder; Lane c: control; Lanes 1, 2, 3: plants treated with nickel at concentrations of 100, 200 and 300 mg/kg soil, respectively*

Discussion

Nickel (Ni^{2+}) is one of the essential nutrients for plants, but it is toxic to plants at high concentrations. Plant species differ greatly in their ability to mineral absorption and accumulation, and these differences often help in the explanation of the plant's tolerance to mineral toxins (Yang et al., 1996). In this study, the toxic effects of nickel on physiological, morphological and molecular traits of cowpea (*Vigna sinensis*). were investigated. Three concentrations of nickel were used (100, 200, and 300 mg / kg of soil). The results showed that the three nickel concentrations (100, 200, 300 mg / kg of soil) affected the physiological traits negatively. It was observed that the P, K, Mg, and Ca content of *Vigna sinensis* leaves decreased as the concentration of nickel increased compared with the control. These results are consistent with Harasim and Filipek (2015) who stated that nickel causes toxicity to the plant if it is present in high concentrations. However, it is inconsistent with the results of Piccini and Malavolta (1992) in their study on the bean plant (*Phaseolus vulgaris* L.). They found that when the concentration of nickel increased the levels of N, P, K, and Cu elements increased. Whereas there were no significant changes in the concentrations of Ca, Mg, Mn and Zn in the plant tissues. The results also revealed that there is an effect of the three nickel concentrations (100, 200, 300 mg / kg of soil) on the concentration of chlorophyll a & b, and carotenoids in the *Vigna sinensis* L. plant. It was obvious that the general trend is a decrement in the concentration of chlorophyll a, b and carotenoids as the concentration of the nickel increases compared with the control. This may be attributed to the fact that the exposure of the plant to the toxicity of nickel leads to a negative effect on the growth of chloroplasts, which leads to a noticeable effect in the process of chlorophyll synthesis as a result of reducing photosynthetic pigments. These results are in alignment with several studies. For instance: Piccini and Malavolta (1992), who found that the nickel toxicity had a significant impact on the reduction of chlorophyll in the bean leaf (*Phaseolus vulgaris* L.). Ewais (1997) who investigated the effect of heavy metals on the decrement of chlorophyll content in three herbaceous plants (*Cyperus difformis*, *Chenopodium ambrosioides*, and *Digitaria sanguinalis*). Kaveriammal and Subramani (2013) who studied the effect of nickel chloride ($NiCl_2$) on peanut (*Arachis hypogeaea* L.) and observed a negative effect of $NiCl_2$ on chlorophyll a & b, total chlorophyll, and carotenoids. Stanisavljevic et al. (2012) who investigated the effect of nickel on *Alyssum markgrafii* and found toxic effects of nickel on chlorophyll a & b, total chlorophyll, and

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carotenoids. Singh and Pandey (2011) who investigated the nickel toxicity on chlorophyll a & b in Pistia stratiotes. Hussain et al. (2013) who concluded that nickel inhibits the activity of plant pigments. The results indicated that the sugar content (reducing, non-reducing and total sugars) of *Vigna sinensis* leaves were affected negatively by the toxicity of nickel at all studied concentrations (100, 200, and 300 mg / kg of soil) compared with the control. The reason behind may be due to the accumulation of nickel in the leaves and to the small leaf area where the sugars are manufactured. These results are in parallel with what was indicated by Ashraf et al. (2011) in their experiments on sunflower (*Helianthus annuus* L.). They observed that treatment of the plant with various levels of nickel led to a disturbance in the metabolism of the chemical and, consequently, affected the availability of sugars that produce metabolic energy due to the toxicity of nickel. Our findings found that there is an effect of nickel various concentrations (100, 200, and 300 mg / kg soil) on lipid ratio, total protein and soluble protein in the leaves of *Vigna sinensis*. They indicated the effect of nickel toxicity on the percentage of fat as the concentration of toxicity of nickel increased compared with the control. The ratio of total protein and soluble protein in the leaves was affected by the toxicity of nickel at high concentrations (300 mg / kg of soil) compared to the control, and this may be due to the accumulation of nickel in the leaves and the decrease in the dry matter of the vegetative and root parts of the plant, which negatively affected the protein content. These findings are consistent with Stanisavljević et al. (2012) who indicated that high concentrations of nickel lead to oxidation of fats in *Alyssum markgrafii* plants. The results of our study are also in alignment with those of Kumar et al. (2011) in their study on barley, where they observed a significant decrease in the protein thiol content of leaves. Several researchers also reported that the toxicity of nickel in the leaves of plants affects the physiological characteristics and works to decrease the contents of protein and fats (Ewais, 1997; Ashraf et al., 2011; Kumar et al., 2011; Singh and Pandey, 2011; Stanisavljević et al., 2012; Kaveriammal and Subramani, 2013). The results showed that nickel concentrations (100, 200, 300 mg / kg of soil) negatively affected the antioxidant enzymes including Ascorbate peroxidase (APX), Catalase (CAT), Guaiacol peroxidase (GPX) Superoxide dismutase (SOD) in the leaves of *Vigna sinensis*. The activity ratio of all enzymes decreased at different treatments of nickel and the decrease was significant at a concentration of 300 mg / kg of soil followed by the two concentrations (200 and100 mg / kg of soil). The reason may be attributed to the fact that the nickel toxicity has the ability to bind with the enzymes, which leads to changes in their potency and performance. These results are in parallel with Shahzad et al. (2018) who indicated that nickel is a toxic pollutant in agricultural environments and excessive concentration of nickel disrupts the nature of biological enzymes. It also agrees with the results of Ashraf et al. (2011) who noticed that the toxicity of nickel leads to a disturbance in the chemical metabolism in sunflower (*Helianthus annuus* L.). which leads to a shortage of amino acids necessary for the production of proteins and enzymes needed for fetal growth due to inhibition of activities of α - Amylase and protease. However, our results are in contrast with the results of Kumar et al. (2011), who observed a significant increase in the activities of Guaiacol peroxidase (GPX), Ascorbate peroxidase (APX) and Superoxide dismutase (SOD) and Glutathione reductase (GR) Catalase (CAT) when treating Barley malt plant with two concentrations of nickel (200 & 400 μM). The results of Maheshwari and Dubey (2009) in their conducted-on rice seedlings (*Oryza sativa* L.) showed an increase in the activity of guaiacol peroxidases (GPX) and ascorbate peroxidase (APX). Whereas our results agree with them in that the toxicity of nickel significantly reduces Glutathione reductase

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(GR) enzyme in the treated seedlings. Our results also showed that there is an effect of nickel various concentrations (100, 200, 300 mg / kg of soil) on the morphological traits of *Vigna sinensis*. Stem length, leaf area, and number of leaves decreased in response to high toxic concentration of nickel (300 mg / Kg of soil) compared with the control. This may be due to the fact that the nickel toxicity affects the processes of cell division, and thus this affects vegetative growth and the dry and fresh weight of the plant. The nickel toxicity might lead to the disruption of metabolic processes such as photosynthesis, respiration, protein synthesis, enzyme activity and other processes that lead to a decline in plant growth and yield. These results are in alignment with many findings of previous researchers who noticed the symptoms of nickel toxicity that resulted in a decrease in the average plant height, leaf area, and necrosis of the leaves (Piccini and Malavolta, 1992; Yadav and Aery, 2001; Al-Qurainy, 2009; Maheshwari and Dubey, 2009; Kumar et al., 2011; Singh and Pandey, 2011; Hussain et al., 2013; Kaveriammal and Subramani, 2013). However, these results are inconsistent with Asagba et al. (2019) who noticed that there are no significant changes in the plant height and growth rate when the plants were exposed to low concentrations of nickel. The results of the current study indicated that there is an effect of nickel concentrations (100, 200, and 300 mg / kg of soil) on the decrement of root weight and total plant weight. This decrease was significant at concentration of 300 mg / kg of soil compared with the two concentrations (200 &100 mg / kg of soil). This indicates that high concentrations of nickel affect the processes of cell division and thus this affects the weight of fresh and dry plants and the roots. The results of our study are in agreement with many previous studies which indicated that the exposure of the plant to the toxicity of nickel in high concentrations resulted in affecting the plant weight, root growth and length, and reducing fresh and dry weight and the length of the root tip (Yadav and Aery, 2001; Al-Qurainy, 2009; Maheshwari and Dubey, 2009; Kumar et al., 2011; Hussain et al., 2013; Kaveriammal and Subramani, 2013; Rathor et al., 2014). However, the results are in contrast with the findings of Asagba et al. (2019) who reported no significant changes in fresh weight and growth rate when the plant was treated with low concentrations of nickel. Regarding the seeds, results indicated that there was a negative effect for nickel treatments on the number of seeds and seed weight. This effect was increased by increasing concentrations of nickel (100, 200, and 300 mg / kg of soil). These results are in alignment with the findings of Yadav and Aery (2001) who reported that the toxicity of high concentrations of nickel affects seed production. Whereas it does not agree with the findings of Shahzad et al. (2018) and Ahmad et al. (2009) who concluded that low concentrations of nickel led to improved and stimulated seed germination, seedling growth and improvement in fresh and dry weights of seeds. In the means of flowering and fruiting, our results revealed that there is a negative effect of different concentrations of nickel on flowers and fruits, as the toxicity of nickel increases at a concentration of 300 mg / kg of soil compared with the control. These results are consistent with Hussain et al. (2013) who reported that the toxicity of nickel affects the activity of many morphological characteristics. At the molecular level, our ISSR molecular marker did not reveal any polymorphism neither between treatments of nickel (100, 200, and 300 mg / kg of soil) on *Vigna sinensis* nor with the control and all bands seem monomorphic. These finding is in contrast with other studies that used RAPD and ISSR molecular markers and showed genotoxicity of heavy metals on plants (Al-Qurainy, 2009, 2010; Taheri et al., 2013).

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Conclusion

In the current study, the effect of various concentrations of nickel (100, 200, and 300 mg Ni^{2+} / kg soil) has been investigated to identify their effects on *Vigna sinensis*. The results revealed the negative effects of nickel on the concentrations of mineral elements, chlorophyll a, reducing, non-reducing and total sugar content. Furthermore, the activity of studied enzymes (APX, GPX, CAT, SOD) decreased significantly. Moreover, the negative effects of nickel concentrations on the root, stem, leaves, seeds, flowers and fruits were obvious. On a molecular level, the ISSR markers could not detect any polymorphism, indicating no toxic effect of nickel on the DNA at the studied concentrations. It is obvious that the nickel has a toxic effect on *Vigna sinensis* at the physiological and morphological levels. The conduction of investigations on the effects of nickel on a molecular level using more molecular markers is highly recommended.

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EFFECT OF EXOGENOUS GIBBERELLIC ACID AND PRESENCE OR ABSENCE OF TESTA ON THE EX VITRO SEEDLING GROWTH OF BAY LAUREL (*LAURUS NOBILIS* L.)

CAVUSOGLU, A.[1,2]

[1]*Department of Plant Protection, Faculty of Agriculture and Natural Sciences, Kocaeli University, 41285 Kocaeli, Turkey*
(e-mail: cavusoglu@kocaeli.edu.tr; phone: +90-262-351-3281)

[2]*Department of Horticulture, Graduate School of Natural and Applied Sciences, Kocaeli University, 41001 Kocaeli, Turkey*

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Abstract. Seed germination, seedling growth and viability are the initial, critical steps and they are affected by various exogenous and endogenous factors including seed testa and plant growth regulators. This study investigated the effects of testa (seeds with or without testa) and gibberellic acid (GA_3; 0, 100, 500, 1000, 2000, 3000 and 4000 ppm) on seed germination, seedling growth parameters and seedling viability rate in bay laurel (*Laurus nobilis* L.). It has been definitely demonstrated that the seed testa extends seedling formation process at the final germination by nearly two times via delaying effects on the first germination time. For seed-with testa, all GA_3 concentrations were found to be similarly successful (between 83.3 and 88.3%) compared to control (68.3%) but in seed-without testa, high GA_3 concentrations (55.0% in 2000 ppm, 56.7% in 3000 ppm and 55.0% in 4000 ppm) showed greater negative effect on final germination than lower doses (80% in 100 ppm, 81.7% in 500 and 1000 ppm) and even control (63.3%). GA_3 concentrations showed no differences in most seedling parameters excluding root number in seeds with testa and maximum root length in seeds without testa. Despite the delaying effect of testa, seedling and viability capacity were higher than those of seeds without testa in high GA_3 concentrations. On the other hand, in control and lower doses of GA_3 (100, 200 and 500 ppm) gave similar results of these two parameters. All data confirmed that *Laurus nobilis* L. seed testa have effects on seed germination delaying but found more resistant to seedling capacity (83.09%) and seedling viability (94.39%) in average after acclimatization in which seed-without testa gave lower results with 67.63%; 78.24% respectively in the *ex vitro* studies.
Keywords: *Lauraceae, seed coat, germination, gibberellins, abiotic stress, plant physiology*

Introduction

Seed germination and seedling success is a vitally important factor not only for human nutrition, but for animal feeding, pharmaceutical and other livelihood occupations as well, it is also an important factor in protecting plants from environmental damage and maintaining generations especially plants which are substantially collected from nature mostly without setting a plantation as *Laurus nobilis* L.

In some conditions seed plants may contain some types of dormancy. There is quite a bit of diversity in dormancy at physiological, morphological and anatomical levels. Physical dormancy is one of the types and is caused by one or more water-impermeable layers of palisade cells in the seed or fruit coat. Mechanical or chemical scarification will promote germination in seeds with non-deep physiological dormancy (Baskin et al., 2000; Baskin and Baskin, 2004). Testa is maternal tissue, it is inherited maternally and in typical angiosperm seeds the embryo is surrounded by two covering layers: the endosperm and the testa (seed coat) (Finch-Savage and Leubner-Metzger, 2006). At the same time some seeds in the plant kingdom are recalcitrant and do not maintain high viability in storage, undergo little or no maturation drying and remain desiccation

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sensitive both during development and after they are shed (Berjak and Pammenter, 2002). Recalcitrant seeds typically have high water content, and well-developed embryos (Bannister et al., 1996).

Although there are numerous studies about dormancy types, dormancy breaking and germination requirements for seed (Baskin and Baskin, 2004; Rowarth et al., 2007; Tang et al., 2019; Sharma et al., 2020; Koutouan-Kontchoi et al., 2020) and numerous studies about seed behavior as orthodox, recalcitrant and intermediate (Berjak and Pammenter, 2002; Jaganathan et al., 2019; Viana et al., 2020; Azarkovich, 2020; Bharuth et al., 2020), there are limited studies focused on seed coat effects on seed germination of woody plants (Sari et al., 2006; Li et al., 2012; Gendreau and Corbineau, 2009).

Gibberellins are a group of plant hormones stimulating growth and development in nearly all steps including stem and internode elongation, regulation of flowering, male and female fertility besides germination. Seed germination is a complex process, controlled by both physical and internal regulating factors. Gibberellins are required for breaking seed dormancy (Gupta and Chakrabarty, 2013). There are limited studies about breaking dormancy in seeds with the aim of external usage of gibberellic acid in woody plants (Rehman and Park, 2000; El-Dengawi, 2005; Çetinbaş and Koyuncu, 2006; Fang et al., 2006) than monocots.

The model plant *Laurus nobilis* L. of the Lauraceae family is an evergreen, perennial, dioecious plant native in Turkey, grown as high-value spice crop, ornamental, importance of ecological and economical aims. Active chemicals from different plant parts are mostly obtained from traditionally grown or naturally existing plant. The high economic value of the species in Lauraceae has caused these to be destroyed in the natural habitats over years according to personal long-term observations.

The evergreen plant has one-seeded drupa fruits, that are oval shaped, and become green to glossy black (Aytürk and Ünal, 2013). There has been an increasing interest to the plant because of its biologically active substances from the under threatened plant. The plant has antimicrobial (Fernández et al., 2019; Nafis et al., 2020), antioxidant (Rincón et al., 2019; Hussein et al., 2019), pharmaceutic (Duletíc-Laušević et al., 2019; Riabov et al., 2020; Chbili et al., 2020) and plant protective (Ebrahimi et al., 2013; Fidan et al., 2019) chemical composition. The odor is caused by volatile chemicals and mostly fixed or essential oils that are found in different ratio in different plant parts as flower, leaf, bark, berry, seed etc. (Kilic et al., 2004; Yılmaz and Deniz, 2018).

Because of importance of *Laurus nobilis* L. some studies about its seed dormancy and storage behavior (Takos, 2001; Takos and Efthimiou, 2003; Sari et al., 2006; Konstantinidou et al., 2008; Ertekin and Çorbacı, 2018) have been carried out. Takos and Efthimiou (2003) emphasized that laboratory tests are more successful than field sowing because of low temperature in autumn, in that time *L. nobilis* fruit is getting mature. In another study on *L. nobilis*, Takos (2001) it was shown that removing pericarp and cold stratification lead to a big success in germination compared to intact pericarp. Ertekin and Çorbacı (2018) used cold stratification or gibberellic acid and polystimulins in combination with cold stratification and they found out that all treatments are higher in germination criterion than control and higher in most of plant growth criterions. Sari et al. (2006) also found that the highest germination rate was observed when seeds were sowed after completely removing seed coat. The results of the one of before study showed that the laurel seeds are recalcitrant and seed coat extended the germination time so the seedlings have to be grown as soon as possible (Cavusoglu et al., 2014).

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The purpose of the study was to investigate the effects of presence or completely removed seed coat and different doses of exogenous gibberellic acid (GA_3) on duration of seedling growth and plant growth criterions of *Laurus nobilis* L. which has increasing medicinal, aromatic and environmental importance.

Materials and methods

A pot experiment was conducted between 2017-2018 and 2018-2019 (December to May) at the Faculty of Agriculture and Natural Sciences, Kocaeli University, Turkey. The bay laurel fruits were collected from only one full grown female plant (*Fig. 1a, b*) for both years in November 12, at an orchard near private property in Kocaeli city, located at 57 m a.s.l. with coordinates 40°.71.8312 N and 29°.99.1608 E. In the first year of the study, a hundred randomized fruits were measured on the day of collection (*Fig. 1c*) and in average one fruit was found 1.12 g in weight, 11.2 mm in diameter and 14.5 mm in length. All fruits were left in the refrigerator for 1 months at 8 °C prior to usage.

Figure 1. *Laurus nobilis L.; (a) initial female plant, (b) a fruity branch sample of the used plant, (c) a fruit cluster sample of the used plant, (d) fruit and used seed samples of the plant – seed with pericarp (fruit), seed-with testa, seed-without testa from left to right*

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At the treatment day narrow and skinny fruit pericarp of half of fruits were removed to obtain seed-with testa and on the other side pericarp and testa of the other half of seeds were removed to obtain seed-without testa (naked seed) (*Fig. 1d*). All this step performed manually with paper towel without the help of water etc. At this step a hundred seeds with testa and without testa were measured; one was found 0.72505 g to 0.682025 g in weight, 9.7 mm to 9.5 mm in diameter, and 11.9 mm to 11.3 mm in length respectively. GA_3 concentrations were prepared the same day as 100, 500, 1000, 2000, 3000 and 4000 ppm besides 0 (Control), and prepared seeds were left in the prepared GA_3 solutions for 24 h in glass beaker under laboratory conditions. Control seeds were left in the same amount of water for the same duration (*Fig. 2a*). The treated seeds were placed and sowed without washing 1 cm deep from surface in trays filled with 0.5 dm^3 base substrate peat: perlite (1:1, w:w) without added nutrients (*Fig. 2b*). The trays were watered to field capacity and placed in the room (temperature between 16 and 23 °C; relative humidity between 57 and 75%) of the laboratory for 6 weeks for the naked-seeds and 12 weeks for the seeds-with testa. Then rooted and shooted seedlings were acclimatized to the greenhouse (temperature between 25 and 39 °C), where they remained for 8 weeks (*Fig. 2c, d*). The trial lasted a total of 14 weeks for naked-seeds and 20 weeks for seeds-with testa in both years. This was because the last germination needed a longer time in seeds with testa.

Figure 2. *Seed germination and seedling steps of Laurus nobilis L. plant; (a) GA_3 treatment to seeds with testa and seeds without testa separately, (b) Initial germination as shoot reached at least 1 cm on substrate surface, (c) A sample of germination and seedling steps, (d) Acclimatized seedling of Laurus nobilis L. in glasshouse*

The seedling growth (at least 1 cm primer shoot on the surface of substrate) was recorded once a week and at the end of both tests (testa and GA_3 effects); root

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number, maximum root length, average root length, shoot length per seedling and seedling capacity were calculated for seeds with testa or seeds without testa separately at the end of the seedling growth under ex vitro laboratory condition because of taking different duration. End data of seedling and viability capacity after 8 weeks acclimatization were also calculated comparatively. All treatments were tested in two years with 3 replications and each replication consisted of 20 seeds. In total 840 seeds (420 seeds-with testa and 420 seeds-without testa) were used in each year. Results were analyzed by ANOVA and Duncan Multiple Range Tests ($p<0.05$) were done to determine differences among the data of the treatments in average of the two years.

Results and discussion

For the treatment with or without testa in bay laurel, the highly significant differences among them were recorded for first seedling, last seedling and average seedling week. All parameters show earliness in naked seed than in seeds with testa (*Tables 1* and *2; Figs. 3* and *4*). In average, seeds with testa began to turn seedling on 5.43rd week first and on 10.87th week last on the other hand seeds without testa began to turn seedling on 2.41st week first and on 4.89th last (*Table 3*).

Table 1. *Seedling capacity of seed with testa treated with different concentration of GA_3 of Laurus nobilis L. along 12 weeks*

GA_3 concentration	**1.week***	**2.week***	**3.week***	**4.week***	**5.week****	**6.week****
Control	0	0	0	0	1.7 ab	11.7
100 ppm	0	0	0	0	1.7 ab	13.3
500 ppm	0	0	0	1.7	1.7 ab	6.7
1000 ppm	0	0	0	0	5.0 ab	13.3
2000 ppm	0	0	0	0	5.0 ab	20.0
3000 ppm	0	0	0	0	6.7 a	16.7
4000 ppm	0	0	0	0	0.0 b	13.3
Average in week	**0**	**0**	**0**	**0.24**	**3.11**	**13.57**
	7.week**	**8.week****	**9.week****	**10.week****	**11.week****	**12.week****
Control	18.3 b	36.7 d	53.3 b	61.7 b	65.0 b	68.3 b
100 ppm	35.0 a	53.3 bc	66.7 ab	81.7 a	86.7 a	88.3 a
500 ppm	30.0 ab	51.7 cd	71.7 ab	76.7 ab	78.3 a	83.3 a
1000 ppm	43.3 a	68.3 ab	75.0 a	83.3 a	83.3 a	86.7 a
2000 ppm	40.0 a	58.3 abc	65.0 ab	73.3 ab	81.7 a	83.3 a
3000 ppm	38.3 a	71.7 a	78.3 a	80.0 ab	85.0 a	85.0 a
4000 ppm	45.0 a	70.0 a	80.0 a	85.0 a	86.7 a	86.7 a
Average in week	**40.80**	**58.57**	**70.00**	**77.39**	**80.96**	**83.09**

*N.S., No significant difference in GA_3 concentrations in seedling capacity of seeds-with testa
**Lower-case letters denote significant differences at the $p<0.05$ level in GA_3 concentrations in seedling capacity of seeds with testa

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Table 2. *Seedling capacity of seeds without testa treated with different concentration of GA_3 of Laurus nobilis L. along 6 weeks*

GA_3 concentration	1.week*	2.week**	3.week**	4.week**	5.week**	6.week**
Control	0	6.7 ab	40.0 b	56.7 ab	61.7 abcd	63.3 ab
100 ppm	0	10.0 a	46.7 ab	63.3 ab	73.3 abc	80.0 a
500 ppm	0	3.3 ab	61.7 ab	73.3 a	81.7 a	81.7 a
1000 ppm	0	3.3 ab	70.0 a	73.3 a	75.0 ab	81.7 a
2000 ppm	0	0.0 b	48.3 ab	48.3 b	51.7 d	55.0 b
3000 ppm	0	1.7 b	43.3 ab	55.0 ab	56.7 abc	56.7 b
4000 ppm	0	3.3 ab	50.0 ab	53.3 ab	53.3 cd	55.0 b
Average in week	**0**	**4.04**	**51.43**	**60.46**	**64.77**	**67.63**

*N.S., No significant difference in GA_3 concentrations in seedling capacity of seeds-without testa

**Lower-case letters denote significant differences at the $p<0.05$ level in GA_3 concentrations in seedling capacity of seeds-without testa

Table 3. *First week, last week and average week of seedling capacity of seeds with or without testa treated with different concentrations of GA_3 of Laurus nobilis L.*

GA_3 concentration	First seedling Week***		Last seedling Week***		Average seedling Week***	
	Seed with testa*	Seed without testa**	Seed with testa**	Seed without testa*	Seed with testa**	Seed without testa*
Control	5.7 A	2.3 ab B	11.7 bc A	5.0 B	6.0 b A	2.7 B
100 ppm	5.7 A	2.0 a B	10.7 abc A	5.3 B	5.0 ab A	3.3 B
500 ppm	5.3 A	2.3 ab B	12.0 c A	5.0 B	6.7 b A	2.7 B
1000 ppm	5.0 A	2.3 ab B	10.7 abc A	5.3 B	5.7 b A	3.0 B
2000 ppm	5.3 A	3.0 b B	11.3 abc A	5.0 B	6.0 b A	3.3 B
3000 ppm	5.0 A	2.7 ab B	10.0 ab A	4.3 B	5.0 ab A	1.6 B
4000 ppm	6.0 A	2.3 ab B	9.7 a A	4.3 B	3.7 a A	1.7 B
Average	**5.43**	**2.41**	**10.87**	**4.89**	**5.44**	**2.61**

*N.S., No significant difference in GA_3 concentrations on first seedling week in seeds with testa, last seedling week in seeds without testa and average seedling week in seeds without testa

**Lower-case letters denote significant differences at the $p<0.05$ level in GA3 concentrations on first seedling week in seeds without testa, last seedling week in seeds with testa and average seedling week in seeds with testa

***Capital letters denote significant differences at the $p<0.05$ level in same GA3 concentrations between seeds with testa and seeds without testa on first seedling, last seedling and average seedling week

According to the results of a study (Sari et al., 2006) supporting this; when seed coat completely removed, time to 50% of final germination took 2 weeks and total germination percentage found 85%. The findings statistically found shorter time consuming than control which is seed without only pericarp (33%) and time to 50% final germination took 7 weeks. In our previous study on mother plant type, seed age and seed coat effect on bay laurel germination (Cavusoglu et al., 2014), we found that first and last germination time showed earliness in naked-seed than seed with coat in both of tested female plant types. In another study on dormancy-breaking and

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germination requirements for seed of *Sorbus alnifolia* (Tang et al., 2019) after removing of pericarp; seeds which are scarified seed coat germination reached 8% when germination of intact seed was 0%. The study on complexities in identifying seed storage behavior of hard seed-coated Lauraceae species (Jaganathan et al., 2019) also emphasized that the lots of reasons of germination barrier of Lauraceae species include seed coat. Baskin and Baskin (2004) emphasized that in most cases, inhibition of protrusion of radicle require longer period to germinate and this results in physiological dormancy which essentially is inability of seeds to germinate despite seed coat is permeable to water. In a detailed study on seed dormancy and dormancy-breaking conditions of 12 West African woody species (Koutouan-Kontchoi et al., 2020), it was emphasized that if scarified coat seeds imbibed more water than intact seeds, then the seeds have an impermeable seed-coat hence physical dormancy. If this is not the case, it means that the seeds are non-dormant or could have another dormancy type and they found two distinct groups of used species in their study.

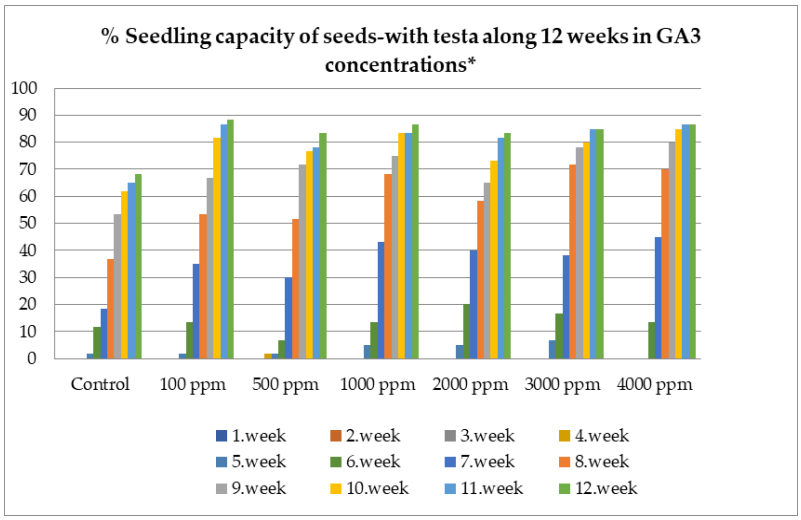

Figure 3. *Seedling capacity (%) of seeds-with testa along 12 weeks in GA_3 concentrations. (*Note that there is no seedling capacity in first 3 weeks)*

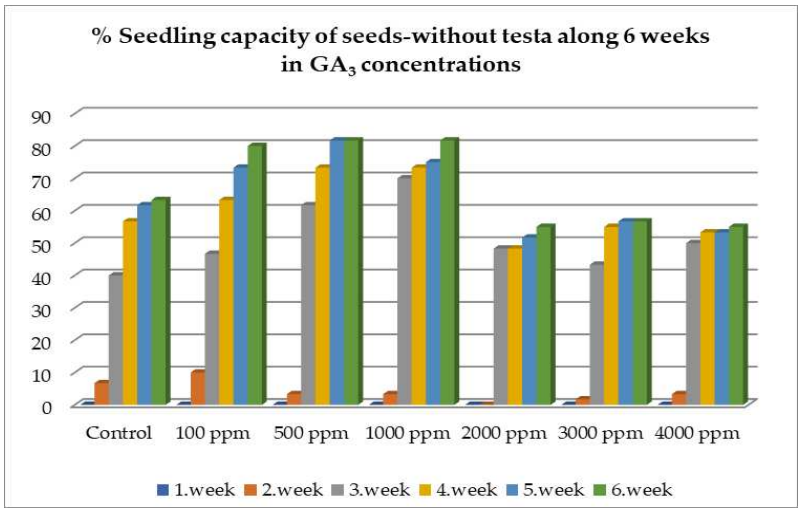

Figure 4. *Seedling capacity (%) of seeds-without testa along 6 weeks in GA_3 concentrations*

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Because of taking very different times in seedling growth of the two used seed types, the parameters on root number, maximum root length, average root length and shoot length were not compared with each other. In addition, when each is evaluated separately in terms of GA_3, only root number in seeds with testa (*Table 4*) and only maximum root length in seeds without testa (*Table 5*) showed statistical difference in the results.

Table 4. *Growth parameters of seedling from seeds with testa treated with different concentrations of GA_3 of Laurus nobilis L. after 12 weeks.*

GA_3 concentration	**Root number** (number/seedling)**	**Maximum root length* (cm/root/seedling)**	**Average root length* (cm/root/seedling)**	**Shoot length* (cm/shoot/seedling)**
Control	7.2 b	11.6	3.2	12.6
100 ppm	7.3 b	9.6	2.6	12.2
500 ppm	7.5 ab	11.5	2.9	12.5
1000 ppm	7.9 ab	9.9	2.6	13.2
2000 ppm	8.2 ab	11.5	2.8	11.9
3000 ppm	8.7 a	12.2	3.1	13.1
4000 ppm	8.2 ab	10.6	2.8	12.5
Average	**7.86**	**8.81**	**2.86**	**12.57**

*N.S., No significant difference in GA_3 concentrations in the parameters of seeds with testa
**Lower-case letters denote significant differences at the $p<0.05$ level in GA_3 concentrations in the parameter of seeds with testa

Table 5. *Growth parameters of seedlings from seeds without testa treated with different concentrations of GA_3 of Laurus nobilis L. after 6 weeks*

GA_3 concentration	**Root number* (number/seedling)**	**Maximum root length** (cm/root/seedling)**	**Average root length* (cm/root/seedling)**	**Shoot length* (cm/shoot/seedling)**
Control	4.5	8.8 ab	2.8	12.2
100 ppm	3.4	7.7 b	3.1	10.3
500 ppm	3.9	6.8 b	2.4	11.9
1000 ppm	4.1	10.5 a	3.9	13.1
2000 ppm	3.5	8.9 ab	3.9	11.3
3000 ppm	2.8	7.0 b	2.9	11.1
4000 ppm	2.6	6.6 b	3.2	10.3
Average	**3.54**	**8.04**	**3.17**	**11.46**

*N.S., No significant difference in GA_3 concentrations in the parameters of seeds-without testa
**Lower-case letters denote significant differences at the $p<0.05$ level in GA3 concentrations in the parameter of seeds-without testa

In seeds with testa, maximum root number reached 8.7 roots per seedling in 3000 ppm GA_3 treatment when control was 7.2 roots/seedling and 100 ppm GA_3 doses (7.3 roots/seedling) show minimum results. When maximum root length was observed in 1000 ppm GA_3 (10.5 cm/seedling) in seeds without testa, higher and lower doses than this showed lesser results. At the end of the seedling growth of seeds with testa, all GA_3 treatments statistically found useful on seedling capacity (88.3% in 100 ppm, 83.3% in

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500 ppm, 86.7% in 1000 ppm, 83.3% in 2000 ppm, 85.0% in 3000 ppm and 86.7% in 4000 ppm GA_3). But interestingly when seeds without testa were used; lesser doses of GA_3 found more useful for seedling capacity (80% in 100 ppm, 81.7% in 500 ppm and 1000 ppm GA_3) than higher doses (55.0% in 2000 ppm, 56.7% in 3000 ppm and 55.0% in 4000 ppm) and than control (63.3%) (*Table 6; Fig. 5*). Moreover, viability capacity of seedling from naked seed after 2 months of acclimatization tended to decrease gradually and statistically from control (95.6%) to 4000 ppm GA_3 (44.9%) when this parameter showed no difference in seeds with testa (*Table 6; Fig. 6*). In a previous study (Sari et al., 2006), when GA_3 was used in high dose (3000 ppm) seeds with testa showed lesser germination than 1000 ppm or control. Besides all, GA_3 and chemical scarification interaction success for *Cyclocarya palirus* (Fang et al., 2006) and for *Loelreuteria paniculata* (Rehman and Park, 2000) has also been demonstrated.

Table 6. *Seedling capacity at the end of the germinations and seedling viability capacity at the end of two months after acclimatization in seeds-with or without testa treated with different concentrations of GA_3 of Laurus nobilis L.*

GA_3 Concentration	**Seedling capacity*** (%)**		**Viability capacity*** (%)**	
	Seed with testa**	**Seed without testa****	**Seed with testa***	**Seed without testa****
Control	68.3 b A	63.3 ab A	92.3 A	95.6 a A
100 ppm	88.3 a A	80.0 a A	88.8 A	88.1 ab A
500 ppm	83.3 a A	81.7 a A	98.0 A	87.5 ab A
1000 ppm	86.7 a A	81.7 a A	96.2 A	87.9 ab A
2000 ppm	83.3 a A	55.0 b B	94.7 A	83.6 ab A
3000 ppm	85.0 a A	56.7 b B	98.3 A	60.1 ab B
4000 ppm	86.7 a A	55.0 b B	92.4 A	44.9 b B
Average	**83.09**	**67.63**	**94.39**	**78.24**

*N.S., No significant difference in GA3 concentrations in viability capacity in seeds with testa
**Lower-case letters denote significant differences at the $p<0.05$ level in GA3 concentrations in seedling capacity in seeds with testa, seeds without testa and viability capacity in seeds without testa
*** Capital letters denote significant differences at the $p<0.05$ level in seedling and viability capacity between seeds with testa and seeds without testa

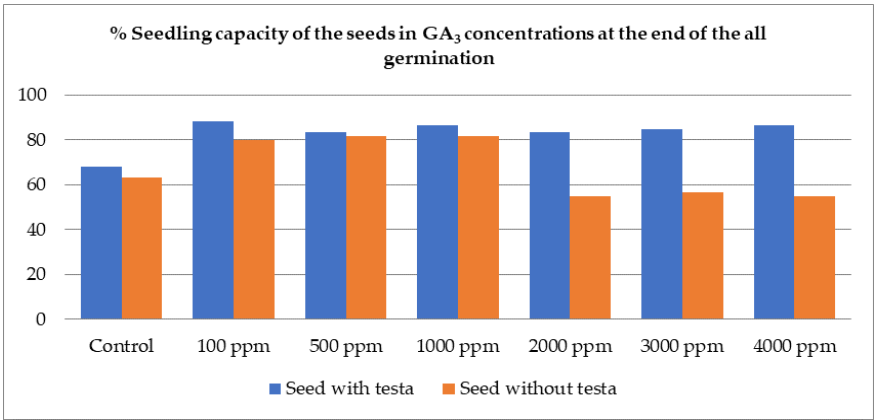

Figure 5. *Final seedling capacity (%) of seeds- with or -without testa in GA_3 concentrations*

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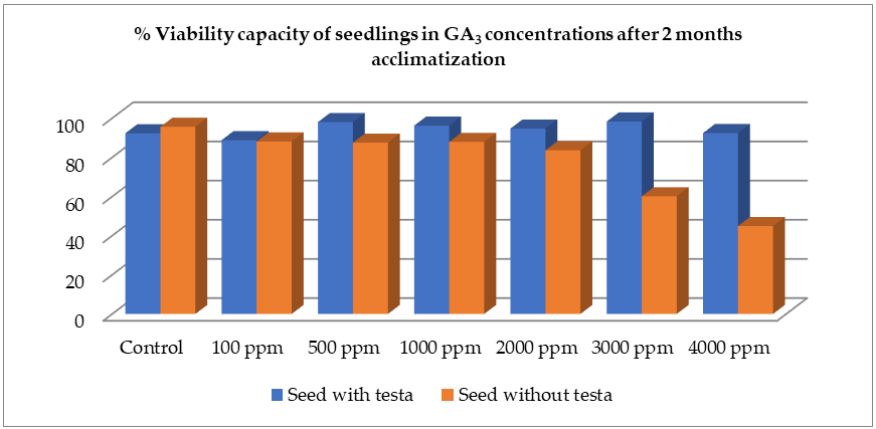

Figure 6. *Final viability capacity (%) of seeds-with or -without testa in GA_3 concentrations after 2 months acclimatization*

Conclusion

Although gibberellin is known in plant physiology and in agricultural practices as a crucial hormone or plant growth regulator from germination to death, the study did not showed effectiveness of this on average root and shoot length, even showed negative effects on germination and viability of seeds without testa under excessive concentrations. This may be due to plant species, gibberellin types, application methods, time and duration of treatments. When we evaluate the results of this study in general terms, it can be said that, if time in obtaining seedlings is the most important thing it may be better to use seeds without testa with relatively lesser GA_3, whereas if the aim is to get healthy seedlings a lot in number it may be better to use the seeds with testa in *Laurus nobilis* L.

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THE EFFECT OF FERTILIZERS ON CROP YIELD, FRUIT QUALITY AND PLANT NUTRITION OF ORGANICALLY GROWN STRAWBERRY (*Fragaria x ananassa* Duch.)

KILIC, N.[1*] – TUREMIS, N. F.[2] – DASGAN, H. Y.[2]

[1]*University of Osmaniye Korkut Ata, Kadirli School of Applied Sciences, Department of Organic Farming Management, Osmaniye, Turkey*

[2]*University of Cukurova, Faculty of Agriculture, Department of Horticulture, Adana, Turkey*

[*]*Corresponding author*
e-mail: neslihankilic@osmaniye.edu.tr; phone: +90-536-320-9556

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Abstract. This study investigated the effects of different organic fertilizer applications on yield and quality of organically grown Albion strawberry variety. The scope of the study covers the use of vermicompost, farm manure and humic-fulvic acid as fertilizer. The yield per plant (g/plant), fruit weight (g), pH in juice, water-soluble-solids/acid ratio in juice, plant leaf area and plant nutrition were investigated. The results indicated that the differences between the applications in yield per plant were statistically significant. The higher total yields were obtained from vermicompost and humic-fulvic acid fertilizer applications with 190.61 g and 182.92 g per plant, respectively and the lowest yield was from farm manure with 95.30 g per plant. The biggest fruits were obtained from the vermicompost application (18.81 g). The difference between applications was not significant for fruit juice pH values. However, the highest total soluble solids (TSS)/acid ratio was obtained with 9.82 from humic-fulvic acid application. TSS/acid ratio in strawberries is an important quality criterion in determining the taste. Nitrogen, phosphorus, potassium and magnesium concentrations were found to be higher in the leaves of strawberry plants fertilized with vermicompost. The vermicompost fertilizer seems promising in organically grown strawberries nutrition.
Keywords: *Organic farming, vermicompost, humic-fulvic acid, manure, low tunnel*

Introduction

Strawberry (*Fragaria x ananassa* Duch.) has a wide growing area since it can be grown in different ecological conditions. Likewise, strawberries are also very important for humans due to health and nutrition benefits. It is fruit rich in minerals and high in vitamin C. It is one of the fruits with the highest antioxidant activity with high ascorbic acid, polyphenols, anthocyanins, and flavonols it contains. Strawberry is usually in high demand by consumers regarding color, taste, aroma, and nutritional facts (Petran et al., 2017; Pradeep and Saravanan, 2018). The total amount of strawberries produced worldwide and in Turkey is increasing every year as it is cultivated in different ecological conditions and yields fruits each year. It has also become an essential part of the food processing industry and fresh consumption; strawberry provides good profit for the producer and has a high market share. According to FAO data, strawberry production worldwide was 7,636,211 tons in 2014 and increased to 8,885,028 tons in 2019. In global strawberry production, China ranks first with 3,212,814 tons in 2019, followed by the United States of America with 1,021,490 tons (Anonymous, 2021a). In this case, Turkey, where strawberry cultivation is possible in almost every region, strawberry production in the Mediterranean and Aegean regions increases day by day in terms of high yield and earliness. According to FAO data 2014-2019 strawberry production volume was on the increase in Turkey. While the total amount of strawberry production was 376,070 tons in

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2014, it rose to 486,705 tons in 2019 (Anonymous, 2021a). Nonetheless, organic strawberry production in the country is very low compared to conventional strawberry production. In 2014, the total amount 3809,4 tons; the figure only raised to 5690,2 tons in 2019 (Anonymous, 2021b). Meanwhile, in 2019, Konya ranked first (4521,3 tons) in terms of strawberry production, which was followed by Bursa (958,3 tons) (Anonymous, 2021b). Due to the rising of environmental problems resulting from inattentive tillage, pesticides, and fertilizer use over the years, interest in organic production and products increased with consumers' interest in consuming healthy products. Therefore, much researches have been conducted to increase the production quantity, quality, and market share of strawberries grown with organic methods (Pokhrel et al., 2015; Esghi and Garazhian, 2015; Petran et al., 2017; Srivastav et al., 2018; Sharma and Negi, 2019).

This study aims to introduce different organic practices with the potential to increase yield and fruit quality and contribute to the popularization of organic strawberry production in Turkey, where organic strawberry production is low. Besides the limited number of studies on vermicompost applications in organic strawberry production, it is also a new type of organic fertilizer for our country. Indeed, it was with this particular study the fertilizer mentioned above was applied in the region for the first time. Therefore, the effects of different organic fertilizer applications on yield and quality of Albion variety were investigated in strawberry cultivation.

Material and Method

Material

Turkey is located between 36°- 42° north latitude and 26° - 45° east longitude. It neighbors Bulgaria, Greece, and Georgia, Armenia in the west, Azerbaijan, and Iran in the east, finally Iraq and Syria in the south. It is also located in the middle belt of the northern hemisphere close to the Equator. Temperate climate conditions are prevalent, and four different seasons are observable. Osmaniye City is in Turkey's southern part and is located in the east of the Mediterranean Region and Cukurova. Although the city's climate differs in mountainous and lowland areas, the Mediterranean climate is still dominant. Although strawberries can be cultivated in almost every region of our country, this study was carried out within the framework of earliness practice with tunnel cultivation method in Osmaniye, where the Mediterranean climate prevails (Anonymous, 2021c,d). The study was carried out on the land owned by Osmaniye Korkut Ata University in 2017-2018. Albion strawberry variety was used in the study. Albion is a day-neutral variety that adapts well to regions with cool and mild climate conditions. It is also very resistant to *anthracnose, verticillium, and phytophthora* (Turemis and Agaoglu, 2013). As organic fertilizers, 1) liquid humic and fulvic acid its trade name "Botanica", 2) solid vermicompost its trade name "Agrosol" and 3) solid farm manure fertilizer its trade name "Eco-flora" were used in the experiment.

Liquid humic-fulvic acid fertilizer (Botanica) is rich in water-soluble plant nutrients. It contains 50% organic matter, 21.3% organic carbon, 3% total nitrogen, and 2.5% water-soluble potassium oxide (K_2O), and it is rich with humic-fulvic acid (Anonymous, 2021e). Vermicompost (Agrosol) was obtained from red california culture worms. It contains 30.33% organic matter, 15.22% total (humic + fulvic) acid, 1.11% total nitrogen, 1.06%, total P_2O_5, 1.55% water-soluble K_2O (Anonymous, 2021f). Farm manure (Eco-flora) is an organic fertilizer produced by the biological fermentation method of bovine manure with organic ingredients of vegetable origin. It contains 40% organic matter,

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1.5% N, 28.2% humic and fulvic Acid, 2% K_2O, 2% P_2O_5 (Anonymous, 2021). Also, black polyethylene mulch was used as mulch material in the experiment to cover the soil surface.

Method

Fresh seedlings were used in the experiment, and soil analysis was done before the experiment. The analysis revealed that the test area's soil was clay-loamy, salt-free, and low in organic mineral (*Table 1*). On October 26, 2017, planting pads were prepared, and a drip irrigation pipe was placed. Following this, the area was covered with black mulch. Seedlings were planted with a triangle planting method at 30x30 cm intervals on 7 November 2017 (*Figure 1*). The trail was set up to consider random parcels with 3 repetitions based on the trial pattern and 32 plants per repetition.

Table 1. *Features of the trial area soil*

Soil Properties	Depth (0 – 20 cm)	Depth (20-40 cm)
Texture	Clay-loam	Clay-loam
pH	7.40	7.40
Saliniy (%)	0.02	0.002
Lime (%)	38.68	38.68
Organic Matter (%)	5.87	5.68
P_2O_5 (kg/ha)	125.6	109.2
K_2O (kg/ha)	712.1	540
Ca (%)	0.0765	0.0728
Mg (%)	0.0533	0.0497
Na (%)	0.0031	0.3327
Fe (mg kg^{-1})	0.92	1.51
Cu(mg kg^{-1})	0.55	0.52
Mn (mg kg^{-1})	4.28	3.74
Zn (mg kg^{-1})	9.79	4.10

Figure 1. *General view of the trial area after the planting process*

In applying vermicompost and farm manure, which were solid fertilizers, the fertilizer company's recommended amount per ha was calculated per plant and then placed into the pits opened as a base fertilizer before planting. The humic-fulvic acid liquid fertilizer was

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applied as top fertilizer with drip irrigation per week. Polyethylene cover sheet was used as the low tunnel to protect the seedlings from the winter's cold.

This study included total yield per plant, fruit weight, pH in juice, water-soluble-solids/acid ratio in juice (Kaska et al., 1986; Ozdemir et al., 2001; Adak et al., 2003) were investigated. In May, in order to investigate plant nutrition status, leaf analyzes for the concentrations of nitrogen (N) (Kacar, 1995), phosphorus (P) (Kacar, 1984), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) (Kacar, 1972) were performed for 15 young leaves randomly selected from each parcel. Three plants were chosen randomly from each parcel for the leaf area analyses, and Digimizer version 5.3.5 was used for measurements. MSTAT-C package program was used in the statistical analysis of the data obtained from the research and the difference between the averages was determined according to LSD.

Results and Discussion

Total Yield and Average Fruit Weight per Plant

The results indicated that the differences between the applications in yield per plant were statistically significant. In *Table 2*, the higher total yield was obtained from vermicompost and humic-fulvic acid fertilizer applications with 190.61 g and 182.92 g per plant, respectively and the lowest yield was from farm manure fertilizer application with 95.30 g per plant. Berk (2013) reported that among the varieties "Camarosa, Kabarla, Festival, Cal Giant 3, Whitney and Sweet Charlie", with farm manure and seaweed application during the growing season, the highest amount of yield was obtained in the first year for Kabarla variety with 189.06 g/plant while it was for Camarosa in the second year with 94.42 g/plant. Eshgi and Garazhian (2015) reported the highest yield of Paros strawberry variety (150 g/plant) in humic acid application. Cay and Kaynas (2016) showed that the yield per plant was higher in the Albion strawberry cultivar with leonardite application (in the first harvest, 189.745 g/plant; in the second harvest, 176.37 g/plant).Weber et al. (2018) stated that applying a mixture of seaweed and SiO_2 increases the total yield during applied the inflorescence and early fruit formation stage of the strawberry. In the study conducted by Srivastav et al. (2018), it was stated that the yield of organic fertilizer applications (farm manure, poultry manure, vermicompost, *Azotobacter*, phosphorus solublizing bacteria) in the Chandler strawberry variety was higher than the control (untreated); however, the highest two yields were obtained from the mix of all treatments (287 g/plant) and vermicompost alone (270 g/plant). Soni et al. (2018), stated that the highest yield in Sweet Charlie strawberry variety was obtained in the combined application of vermicompost, poultry manure, and *Azotobacter* (144.77 g/plant). While in the study of Gecer (2020) it was humic acid application yield per plant in Albion strawberry variety (146.44 g/plant), and he reported that it was higher than the control application (123.39 g/plant). The results obtained in this study are also similar to the current studies. Besides, Yadav et al. (2020) reported that the yield of Camarosa strawberry variety was 94 g/plant in farm manure application, 97 g/plant in vermicompost application, and 85 g/plant in control. In this study, the yield values were higher than some values reported above. Therefore, results showed that yield values per plant in strawberry vary according to cultivars, applications, and ecology of the place where it is grown.

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Table 2. *Effects of organic fertilizer applications on strawberry yield per plant (g/plant) and average fruit weight*

Applications	Yield per Plant (g/plant)	Average Fruit Weight (g)
Humic-fulvic acid (liquid)	182.92 a	16.16
Vermicompost (solid)	190.61 a	18.81
Farm manure (solid)	95.30 b	17.53
Mean	156.28	17.50
$LSD_{0.05}$	25.44	N.S

The averages that differ significantly at the 5% level according to the LSD test are shown in different letters. N.S, Not Significant

According to the results of the study on the effects of different organic fertilizer applications, the highest yield (190.61 g) and the biggest fruits (18.81 g) for the Albion strawberry variety were obtained with the application of vermicompost fertilizer (*Table 2*). However, there was no statistical difference between the total yields obtained from vermicompost and humic-fulvic acid applications with 190.61 g and 182.92 g per plant, respectively and the lowest yield was from farm manure fertilizer application with 95.30 g per plant (*Table 2*). Shehata et al. (2011) found that the weight of compost+mineral fertilizers applied fruits was larger (11.98 g) while Sener and Turemis (2017) observed the fruit weight of the organically grown Albion strawberry variety as 18.03 g. Petran et al. (2017) reported the fruit weight as 14.05 g for the strawberries grown in the low tunnels in the city of St Paul and as 18.25 g for the ones in the city of Morris. Soni et al. (2018) reported that in Sweet Charlie strawberry cultivar, the maximum fruit weight was obtained by applying vermicompost, poultry manure, and azotobacter together (11.83 g). Cabiloski et al. (2014) reported no difference among applications in terms of fruit weight in their study. Strawberry fruit size is a kind of feature, besides the fertilizers environmental conditions, day and night temperature differences could also be effective.

pH in Strawberry Fruit

Fruit juice pH values are given in *Table 3* below. The difference between applications was not statistically significant regarding organic fertilizer applications, as fruit juice pH values varied between 3.55 and 3.65. Gulbag and Ilgın (2016) reported that in organic strawberry cultivation pH values of fruit varied in the range of 3.21 and 3.37. Sener and Turemis (2017) reported that the pH values of organic fertilizer applications Monterey, Albion, Aromas, Camarosa, Sweet Charlie strawberry varieties varied between 3.59 and 3.83. Hoehne et al. (2018) reported the pH value of the Camarosa strawberry variety varied between 3.59 and 3.95. The difference among the applications in terms of pH values of fruits is reported to be insignificant (Berk, 2013; Gecer, 2020).

Total Soluble Solids (TSS)/ Titratable Acidity Ratio in Strawberry Fruit

TSS/acid ratios of different organic fertilizer applications were statistically significant. The highest TSS/acid ratio was obtained with 9.82 in humic-fulvic acid liquid fertilizer application. TSS/acid ratio values obtained in the experiment varied in the range of 8.79-9.82 (*Table 3*). In Sonata strawberry variety, as a result of two solid and two liquid fertilizer application, TSS/acid ratio (8.5-9.0 at 56^{th}-day harvest; 10.7-11.3 at 76^{th}-day

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harvest) was higher than inorganic application (8.5 at 56th day; 10.5 at 76th-day harvest) (Pokhel et al., 2015). The findings obtained in this study are in accordance with the study mention above. TSS/acid ratio in strawberries is an important quality criterion in determining the taste. Regarding the applications, the highest taste content was obtained in the humic-fulvic acid liquid fertilizer application. The high TSS/acid value in the study may have been due to the slower progression of the fruit ripening process in low tunnel cultivation conditions with humic-fulvic acid liquid fertilizer application's positive effect.

Table 3. *Effects of organic fertilizer applications on strawberry fruit pH and total soluble solids (TSS) / titratable acidity*

Applications	pH	TSS/Titratable acidity
Humic-fulvic acid (liquid)	3.55	9.82 a
Vermicompost (solid)	3.63	8.79 b
Farm manure (solid)	3.65	8.96 b
Mean	3.61	9.19
$LSD_{0.05}$	N.S	0.61

The averages that differ significantly at the 5% level, according to the LSD test, are shown in different letters. N.S, Not Significant, humic-fulvic acid was applied to vermicompost and manure applications from above

Plant Nutrient Analysis in Strawberry Leaf

Macro nutrients as nitrogen, phosphorus, potassium, calcium, and magnesium amount of different organic fertilizer applications were provided in *Table 4*. The concentrations of nitrogen and potassium in the leaf was statistically significant, while it was not the case for the amount of phosphorus, calcium, and magnesium. The nitrogen concentration (2.01%) of strawberry leaves of the vermicompost was higher than other applications.

Table 4. *Effects of organic fertilizers on nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) concentarions of strawberry leaves (%)*

Applications	N	P	K	Ca	Mg
Humic-fulvic acid (liquid)	1.85 ab	0.50	0.65 b	0.96	0.65
Vermicompost (solid)	2.01 a	0.73	1.36 a	1.16	0.74
Farm manure (solid)	1.72 b	0.60	0.75 b	1.20	0.67
Mean	1.86	0.61	0.92	1.10	0.68
$LSD_{0.05}$	0.18	NS	0.10	NS	NS

The averages that differ significantly at the 5% level, according to the LSD test, are shown in different letters. NS, Not Significant

Sener and Türemis (2016), in their study on organic strawberry cultivation, found that the nitrogen concentration of the Albion strawberry variety was 2.54%, Pritts (2015) found that the nitrogen adequacy concentration in the leaf was between 2.0-2.8%. Tagliavini et al. (2004) stated that the limit concentration of nitrogen in the leaf is between 0.9-2.02%. Findings in our study also show that nitrogen concentration is sufficient.

The study observed that the leaf phosphorus concentration was between 0.50-0.73%. Hassan (2015), in a study investigating the effects of organic, inorganic, and biofertilizer applications in different ratios in Sweet Charlie strawberry variety, reported that the

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phosphorus value in 100% compost application was 0.56% in 2011/2012 and 0.51% in 2012/2013. Jones et al. (1991) stated that the leaf's phosphorus adequacy level was between 0.25-1.00%. The phosphorus concentration of the fertilizers applied in this study is within the limits of sufficiency and is similar to the findings of Jones et al. (1991).

The highest potassium concentration was detected in leaves (1.36%) applied to vermicompost fertilizer. In our study, the leaf potassium value was between 0.65-1.36%. Sener and Turemis (2016) found the potassium concentration of Albion strawberry variety as 1.50%, Jones et al. (1991) stated that the leaf's potassium adequacy level is between 1.30-3.00%. Considering the data obtained, the concentration t of potassium was found to be sufficient in the application of vermicompost (1.36%), while it was inadequate in humic-fulvic acid liquid fertilizer (0.65%) and farm manure fertilizer application (0.75%).

Leaf calcium concentration in present study was found to be in the range of 0.96-1.20%. Pritts (2015) found that the calcium adequacy concentration in the leaf was between 0.7-1.7%. The calcium concentration of fertilizers applied in this study is also within the limits of sufficiency.

In *Table 4*, it is seen that the total magnesium values in the leaf are in the range of 0.65-0.74%. Jones et al. (1991) stated that the leaf's magnesium adequacy concentration is between 0.25-1.00%. In this study, it can be said that the total magnesium concentration of the leaves is sufficient.

In *Table 5*, differences regarding the concentration of iron and zinc in the leaf were statistically significant, while it was statistically insignificant for the concentration of manganese and copper. The highest concentration of iron was measured on the leaves of strawberries with solid farm manure (127.67 ppm), and the lowest was on the leaves of strawberries with vermicompost with 70 ppm. Jones et al. (1991) reported that the concentration of leaf iron in strawberries was sufficient between 50-200 ppm. The leaf iron concentration we obtained in the study is sufficient.

Table 5. *Effects of organic fertilizers on iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) concentarions of strawberry leaves (mg/L)*

Applications	Fe	Mn	Zn	Cu
Humic-fulvic acid (liquid)	92.33 ab	54.00	16.00 a	3.67
Vermicompost (solid)	70.00 b	37.00	13.00b	2.67
Farm manure (solid)	127.67 a	63.00	13.67 b	2.67
Mean	96.67	51.33	14.22	3.00
$LSD_{0.05}$	44.44	NS	1.20	NS

The averages that differ significantly at the 5% level, according to the LSD test, are shown in different letters. NS, not significant

The highest concentration of zinc was obtained from a humic-fulvic acid liquid fertilizer application (16 ppm). It is determined that the total concentration of zinc in the leaf is 13 ppm-16 ppm. In his study, Jones et al. (1991) reported that the leaf's zinc adequacy concentration was between 20 ppm and 200 ppm. The total zinc value in the leaf we obtained in this study was insufficient in all applications.

The concentration of manganese in the leaf varies between 37 ppm and 63 ppm. Jones et al. (1991), Pritts (2015) stated that the leaf's manganese proficiency concentration is between 50 ppm and 200 ppm. When the data obtained were evaluated, the concentration amount of manganese in the leaf was found sufficient in humic-fulvic acid liquid fertilizer

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(54 ppm) and solid farm manure (63 ppm) and insufficient in vermicompost (37 ppm). The study determined that the leaf's total copper concentration was in the range of 2.67 ppm-3.67 ppm. Jones et al. (1991) reported that the copper proficiency concentration was between 6 ppm and 50 ppm. When the data obtained were evaluated, the concentration of copper was insufficient in all applications.

Effects of Organic Fertilizer on Leaf Area (cm^2/plant)

Leaf area results are given in *Table 6*, and it was observed that the effect of all applications on leaf area was found statistically insignificant. Leaf area values were between 529.10 and 649.10 cm^2/plant. Eshghi and Garazhian (2015) reported that in the Paros strawberry variety, the maximum leaf area in the humic acid application they applied at different rates through the leaf and the soil was 533.4 cm^2/plant as a result of the leaf application (900 mg/L^{-1}). Alkharpotly et al. (2017) reported that the leaf area varied between 295.9 cm^2/plant-566.6 cm^2/plant in 2014/2015 and between 311.2 cm^2/plant - 614.2 cm^2/plant in 2015/2016 in different doses of humic acid and seaweed applications in Festival strawberry variety. Srivastav et al. (2018) reported that in the Chandler strawberry variety, organic and bio-fertilizer applications (farm manure, poultry manure, and vermicompost) yielded higher leaf area compared to control (untreated). The widest leaf area was obtained in the combined application of vermicompost and biofertilizers. The leaf area values in this study are close to the leaf area values of the studies above. However, strawberry leaf area varies based on the cultivars, different types of applications, and the place's ecology where it is grown.

Table 6. *Effects of organic fertilizers on strawberry plant leaf area (cm^2/plant)*

Applications	Leaf area
Humic-fulvic acid (liquid)	649.10
Vermicompost (solid)	529.10
Farm manure (solid)	595.89
Mean	591.36
$LSD_{0.05}$	NS

The averages that differ significantly at the 5% level according to the LSD test are shown in different letters. N.S, Not Significant

Conclusion

Strawberry is a very popular fruit. The organic nature of the strawberry becomes much more attractive and is in high demand by the consumer.

It has been determined that vermicompost and humic-fulvic acid fertilizers are effective in organic strawberry cultivation to obtain high yield. The largest fruits were detected from the vermicompost application. The total soluble solids/ titratable acidity ratio was higher in the humic-fulvic acid application. Considering plant nutrient analysis, it was seen that the amount of nitrogen, phosphorus, calcium, magnesium, and iron was sufficient while the amount of zinc and copper was insufficient in all three different organic fertilizers, namely; vermicompost, humic-fulvic acid liquid fertilizer, and farm manure. While the potassium amount was found to be sufficient in the application of vermicompost, it was found insufficient in humic-fulvic acid liquid fertilizer and farm manure.

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The vermicompost fertilizer seems promising in organically grown strawberries nutrition. In future studies, it is recommended to use solid and liquid forms of vermicompost fertilizers together to increase organic yield and product quality in strawberries, and apply the liquid form via dripping or on the foliage, and try with new biofertilizers.

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EFFECTS OF DIFFERENT RICE PLANTING DURATION ON ORGANIC CARBON COMPONENTS AND CARBON POOL MANAGEMENT INDEX OF SALINE-ALKALINE SOIL IN WESTERN JILIN PROVINCE, CHINA

LIU, Q.[1] – TANG, J.[2*]

[1]*College of Landscape Architecture, Changchun University, Changchun 130012, China*

[2]*College of New Energy and Environment, Jilin University, Changchun 130012, China*

**Corresponding author*
e-mail: hamiqi.365@163.com; phone: +86-181-0431-1389

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Abstract. The soil carbon pool management index is an important quantitative index to characterize soil carbon change. It is of great significance to study the change process of easily oxidized organic carbon in the soil and carbon pool management index can be used in the case of saline-alkali fields of western Jilin province to understand the evolution of soil quality and evaluate its effects during ecological restoration. The temporal and spatial evolution characteristics of soil organic carbon (SOC), readily oxidized organic carbon (ROOC), non-readily oxidized organic carbon (NROOC) and carbon pool activity (A), carbon pool activity index (AI), carbon pool management index (CMI) and carbon pool index (CPI) in saline-alkali paddy fields in western Jilin, China were analyzed. The results showed that the soil layer and rice growth stage were the main factors affecting the changes in soil carbon pool, and the CPI and CMI increased with the increase of cultivation time. The soil ROOC, NROOC is closely related to SOC and is an important part of soil SOC, and obviously depends on the input, fixation, transformation and decomposition of surface carbon sources.
Keywords: *saline-alkali rice field, rice growth stages, easily oxidized organic carbon, carbon pool management index, influencing factors*

Introduction

Soil readily oxidizable organic carbon is an important component of soil carbon pool, and is easily degraded by oxidation in soil (Ya et al., 2013). The temporary fluctuation of soil organic matter mainly occurred in the easily oxidized part, and the ratio of Rooc to organic carbon could indicate the soil quality and the rate of soil organic carbon oxidation (Cao et al., 2019). Therefore, Rooc is an important index of soil fertility change, which can indicate the dynamic change of soil organic matter. It was found that the organic carbon that could be oxidized by 333 $mmol \cdot L^{-1}$ $KMnO_4$ changed the most when growing crops, and the organic carbon that could be oxidized by 333 $mmol \cdot L^{-1}$ $KMnO_4$ was called active organic carbon, while the organic carbon that could not be oxidized was called inactive organic matter (Zhao et al., 2019). Active organic carbon can be used as an indicator of early changes in organic carbon, while non-active organic carbon can be used as an indicator of soil long-term accumulation and carbon sequestration (Liu et al., 2019). Soil carbon pool management index is a quantitative index to characterize soil carbon change. It is based on soil total organic carbon and easily oxidized organic carbon (Lefroy et al., 1993). The soil carbon pool management index is obtained by calculation. Ever since the concept of carbon management index was put forward, it has often been used to monitor the effectiveness of soil carbon pool dynamics and evaluate the management level of soil quality (Tang et al.,

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2014). The effects of different land use (Qiu et al., 2009), tillage management (Lv et al., 2014) etc. on soil carbon pool have been widely used. Therefore, under the background of the sharp change of global climate and the sharp increase of greenhouse effect, it is of great significance to clarify the change characteristics and evolution law of the management index of easily oxidized organic carbon and carbon pool in saline-alkali paddy soil for the rational utilization of saline-alkali soil, soil fertilization and soil carbon sequestration. The saline-alkali land is widely distributed throughout the world, covering more than 30 countries on six continents, with a total area of about 9.56×10^9 ha. Australia accounted for 37%, the former Soviet Union for 18% and China for 10%. The saline-alkali land of Songnen Plain is the concentrated distribution area of saline-alkali land in China. The west of Jilin Province is located on the south side of Songnen Plain, which is a serious disaster area of soda-alkaline desertification. In order to promote the development and utilization of saline-alkali land, rice was planted in part of the land to improve saline-alkali land. (Zhang et al., 2016a). At present, there are few studies on soil carbon pool index of saline-alkalized paddy field under different rice cultivation years. Therefore, the change characteristics of soil carbon pool management index and its evolution law during the maturation of saline-alkali rice field are discussed.

Materials and methods

Study description

The saline-alkali soil area in western Jilin of China was once a large inland lake basin in geological history, and the lake water was abundant and withered many times, which made the soluble salt in the parent rock of the highland in the basin converge with the runoff in the closed-flow lowland, forming a large area of saline-alkali soil deposition (Tang et al., 2012a). Rice plantation is adopted as the main method for the improvement as well as repair and utilization of saline alkali land in this area. Here, the Qianguoerluos County Irrigation District (123°35′-125°18′E, 44°17′-45°28′N) is one of the four major irrigation districts in Northeast China. The annual average number of sunny days is 110 days, the annual average sunshine hours is 2879 h, the annual average temperature is 4.5 °C. The initial frost period is generally in the middle and late September, the final frost period is generally from the end of April to the beginning of May, and the frost-free period is 130-140 days. The average annual precipitation is 400-500 mm. The annual evaporation is more than 1200 mm (Tang et al., 2011). Time series study is a powerful tool to understand the change and evolution of soil texture, so in order to make the sample land more representative and the test results more universal, according to the soil type map and the land use type map, combined with the field investigation, according to the paddy field tillage history, and to determine the management method, soil properties basically involved 5 different cultivation years, one year (S1), 10 years (S2), 20 years (S3), 30 years (S4), 50 years (S5), were determined as test plots based on soil type maps and land use type maps, combined with field surveys and based on paddy field tillage history (*Fig. 1*). The plots were unified into single-planted rice soil, and the management of each plots was consistent. The base fertilizer was urea, phosphate and potash.

Sample collection and analysis

Soil sampling was carried out at different growth stages (seedling stage, tillering stage, heading stage, seed setting stage, mature stage) of rice growth. The soil samples

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of 0-10, 10-20, 20-30, 30-40, 40-50 cm soil layer were collected according to the S sampling method, 180 sampling points were set. The samples of the same soil layer at each sampling point were mixed to remove plant residues and roots from the soil and placed in plastic bags. Then the soil samples were stored in the incubator under 4 °C conditions. After all the soil was collected, the soil was brought back to the laboratory for testing.

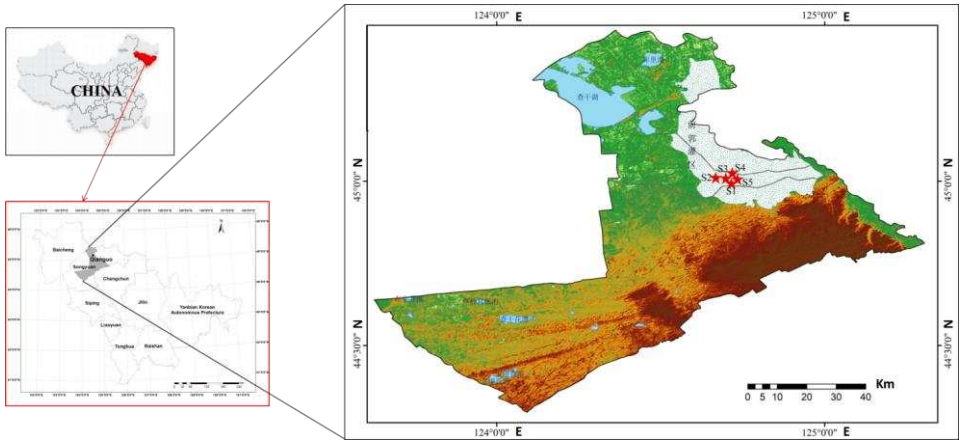

Figure 1. *The location of the study area and the distribution of the sampling points*

Soil organic carbon (SOC) was determined by potassium dichromate external heating (Bao, 2000). Readily oxidized organic carbon (ROOC) was determined by $KMnO_4$ oxidation method (Zhou et al., 2019).

The management index of carbon pool is calculated by Blair and Lefroy, and the calculation formula is as follows:

$$NROOC = SOC - ROOC \quad \text{(Eq.1)}$$

$$A = ROOC / NROOC \quad \text{(Eq.2)}$$

$$AI = TSOCa / RSOCa \quad \text{(Eq.3)}$$

$$CPI = TSOC / RSOC \quad \text{(Eq.4)}$$

$$CMI = AI \times CPI \times 100 \quad \text{(Eq.5)}$$

where SOC is soil organic carbon, ROOC is readily oxidized organic carbon, NROOC is non-readily oxidized organic carbon, A is carbon pool activity, AI is carbon pool activity index, TSOCa is tillage soil carbon pool activity, RSOCa is reference soil carbon pool activity, CPI is carbon pool index, TSOC is tillage soil organic carbon, RSOC is reference soil organic carbon, CMI is the carbon pool management index (Blair et al., 1995).

Statistical analysis

One-way ANOVA method is used to analyze the influence of three factors on soil carbon composition and carbon pool management index under single condition.

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Multivariate ANOVA was used to analyze the comprehensive differences of carbon composition and carbon pool management index caused by three factors: rice cultivation, soil layer and rice growth stage. Pearson correlation analysis is used to analyze the correlation between soil carbon components and carbon pool management indicators.

Results

Distribution of soil carbon content

As can be seen from *Figure 2,* the soil SOC content showed a decreasing trend: S5 > S4 > S3 > S2 > S1. The SOC content of S5 was significantly higher than S1 ($p < 0.05$), and the soil SOC content of S5 increased gradually with the extension of rice cultivation, the results showed that longer rice growing period and lower salt-alkali stress were beneficial to soil SOC accumulation. During the rice growing stage, the soil SOC content decreased firstly and then increased, and the soil SOC content was the lowest in tillering stage.

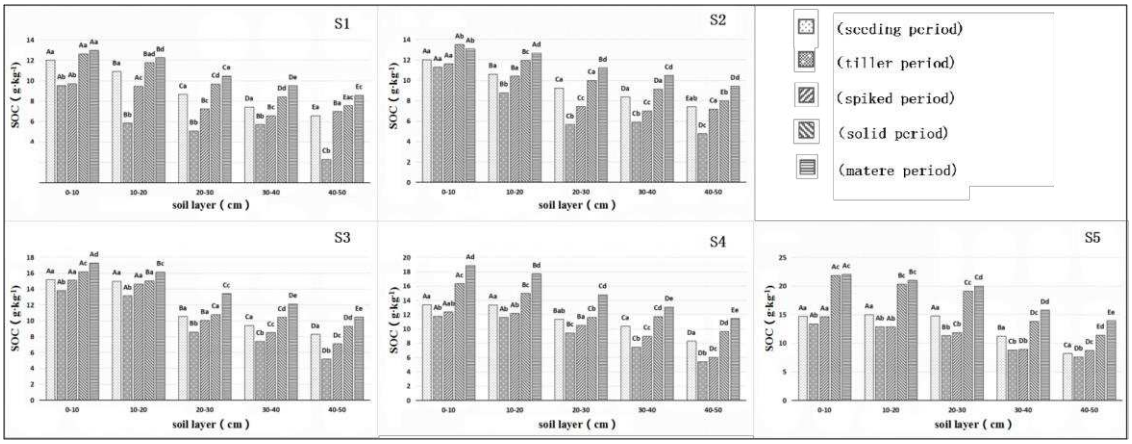

Figure 2. *The soil SOC content and distribution characteristics*

Figures 3 and *4* show that, Soil ROOC and NROOC content increased significantly with planting years. The soil ROOC content of S1, S2, S3, S4 and S5 were 0.47-4.16 g·kg^{-1}, 1.31-5.41 g·kg^{-1}, 2.10-7.89 g·kg^{-1}, 2.05-7.47 g·kg^{-1} and 2.24-7.91 g·kg^{-1}, respectively. The soil ROOC content increased with the increase of rice cultivation years, the soil ROOC content of S5 was significantly higher than S1, the soil ROOC content of S4 was slightly lower than S3. The soil NROOC content was lower than ROOC. The soil NROOC content of S1, S2, S3, S4 and S5 were 1.83-8.94 g·kg^{-1}, 3.46-8.51 g·kg^{-1}, 3.09-9.75 g·kg^{-1}, 3.38-11.39 g·kg^{-1} and 4.75-14.45 g·kg^{-1}, respectively. The soil NROOC content of S5 was significantly higher than S1, and characteristics of small differences between S3 and S4 were observed. The soil NROOC content was lower in tillering stage and higher in spike-pumping stage, and its distribution law was basically consistent with the distribution characteristics of SOC. The results showed that the soil NROOC content of rice was higher in surface soil, the NROOC content of 0-10 cm soil layer was close to 2 times that of 40-50 cm soil layer, and the NROOC content of 10-20 cm soil layer was slightly lower than 0-10 cm, but the decrease was not large.

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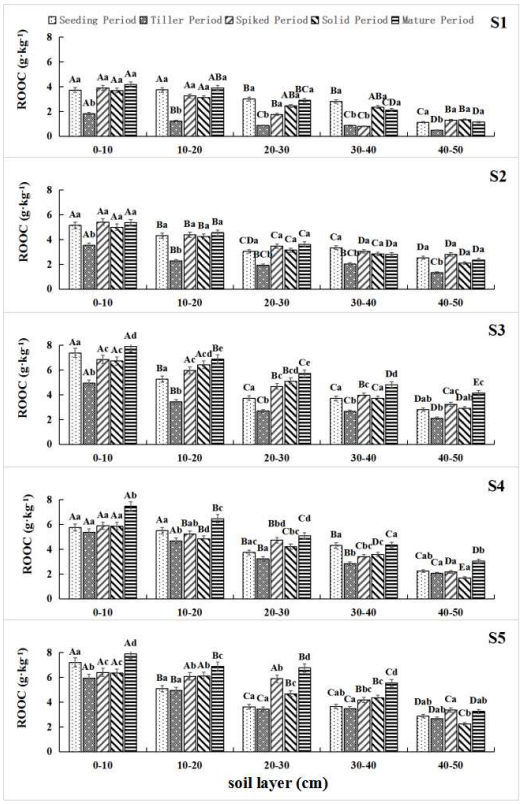

Figure 3. *The soil ROOC content and distribution characteristics*

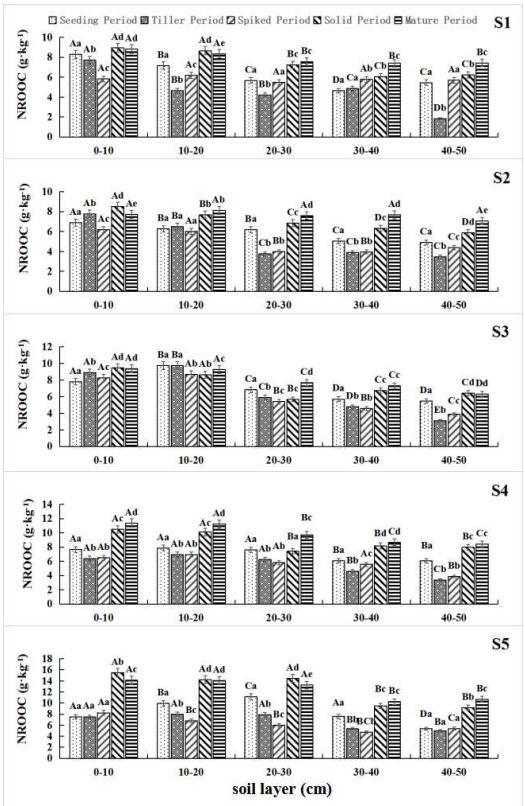

Figure 4. *The soil NROOC content and distribution characteristics*

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Changes in soil carbon pool management indicators

Using saline-alkali wasteland soil in Qianguo County as reference soil, the carbon pool activity (A), carbon pool activity index (AI), carbon pool index (CPI) and carbon pool management index (CMI) of paddy field soil with different rice cultivation years were calculated by space-time substitution method. The results are shown in *Figures 5, 6, 7* and *8*.

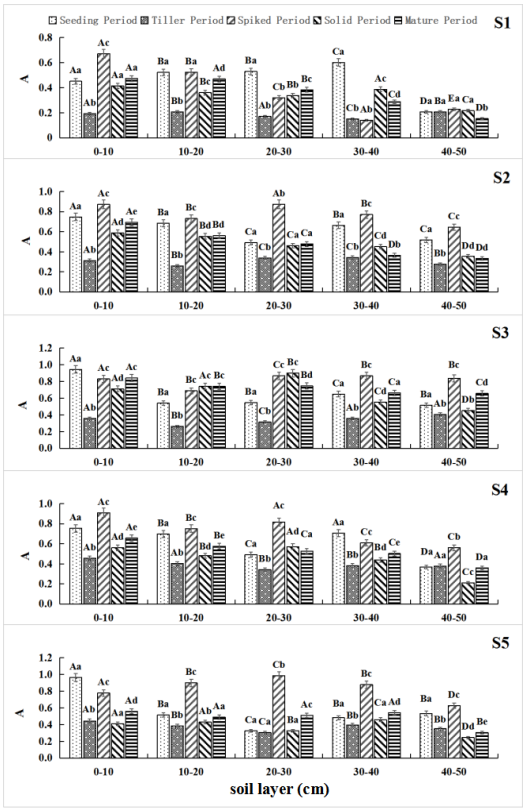

Figure 5. *Distribution of A in rice during different growing stages*

The soil A index values of S1, S2, S3, S4 and S5 were 0.14-0.67, 0.26-0.87, 0.26-0.94, 0.34-0.91 and 0.30-0.96, respectively. During the rice growing stage, the soil A index value ranged from 0.56 to 0.98 at the rice heading stage, which was the highest value in the whole growing stage and more than twice the value of the soil A index at the tillering stage. The change of A index value of S1 soil in rice growing stage is different from other years. This may be closely related to the time of rice planting and human disturbance, a preliminary change in the nature of the land, so that the soil to undertake root exudates type changes. As a result, the formation of soil carbon pool activity will be different (Guo et al., 2014).

The soil AI index value of different rice cultivation years was slightly higher in heading period, but with the extension of rice cultivation years, the fluctuation of AI index was more intense, the law was not obvious, and there was no obvious law between soil layers.

The soil CPI index increased gradually with the increase of rice cultivation years. The values of CPI of S1, S2, S3, S4 and S5 were 0.28-1.04, 0.53-1.10, 0.58-1.36, 0.56-1.44 and

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0.68-1.54, respectively. During the rice cultivation years, the CPI values of mature soil were higher, and those of tillering soil were lower. In addition, the CPI values of 10-20 cm soil layers of S2 and S3 were higher than that of other soil layers, the CPI value of 20-30 cm soil layer of S4 and S5 were higher than that of other soil layers.

The soil CMI index of S1, S2, S3, S4 and S5 were 16.56-222.48, 26.91-323.25, 31.07-130.67, 37.02-129.08 and 27.01-91.65, respectively. With the growing of rice, the soil CMI index of S2 was higher than S1, and the soil CMI index of S3 was similar to S4. The results showed that the soil CMI index of saline-alkali rice field fluctuated greatly. In this study, the maximum value of soil CMI index was reached when rice was planted to 20-30 years, and then began to decrease slowly. During rice growth stages, the soil CMI index of heading stage was higher than that of other growth stages.

Coupling relationship between soil SOC, ROOC and NROOC and carbon pool management index

Table 1 shows the correlation among organic carbon, readily oxidizable organic carbon, steady-state carbon and carbon pool management indicators. The soil SOC, ROOC and NROOC of S1, S2, S3, S4 and S5 were significantly correlated with CMI index, and there were no significant correlation between the soil ROOC and index A. The soil ROOC of S2, S3, S4 and S5 were significantly correlated with index A ($P < 0.05$), and the correlation coefficient was greater between the soil ROOC and index A with the extension of rice cultivation years. The soil SOC of S4 and S5 were significantly correlated with CPI ($P < 0.05$).

Table 1. *Correlation analysis of soil SOC, ROOC, NROOC and carbon reservoir management index*

Sample number	Index	A	AI	CMI	CPI
S1	SOC	-0.240	0.313	-0.402*	-0.419*
	ROOC	-0.080	0.255	-0.473*	-0.529**
	NROOC	0.284	-0.316	0.887**	0.299
S2	SOC	0.311	-0.430*	0.764**	-0.150
	ROOC	0.522**	-0.212	0.502*	0.032
	NROOC	-0.050	-0.447*	0.827**	-0.126
S3	SOC	0.335	0.026	0.629**	0.325
	ROOC	0.645**	0.397*	0.460*	0.499*
	NROOC	0.021	-0.251	0.667**	0.095
S4	SOC	0.314	0.110	0.620**	0.517**
	ROOC	0.672**	0.462*	0.423*	0.465*
	NROOC	0.042	-0.092	0.735**	0.443*
S5	SOC	-0.136	-0.275	0.895**	0.588*
	ROOC	0.883**	0.324	0.413*	0.643**
	NROOC	0.013	-0.312	0.443*	-0.450*

* indicates significant difference ($P < 0.05$), ** represents a very significant difference ($P < 0.01$). SOC, organic carbon; ROOC, readily oxidized organic carbon, NROOC, non-readily oxidized organic carbon, A, carbon pool activity, AI, carbon pool activity index, CPI, carbon pool index, CMI, carbon pool management index

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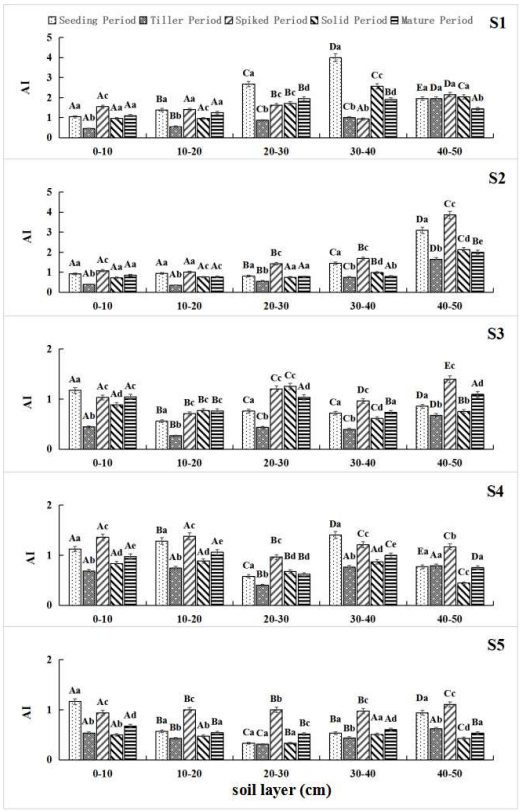

Figure 6. *Distribution of AI in rice during different growing stages*

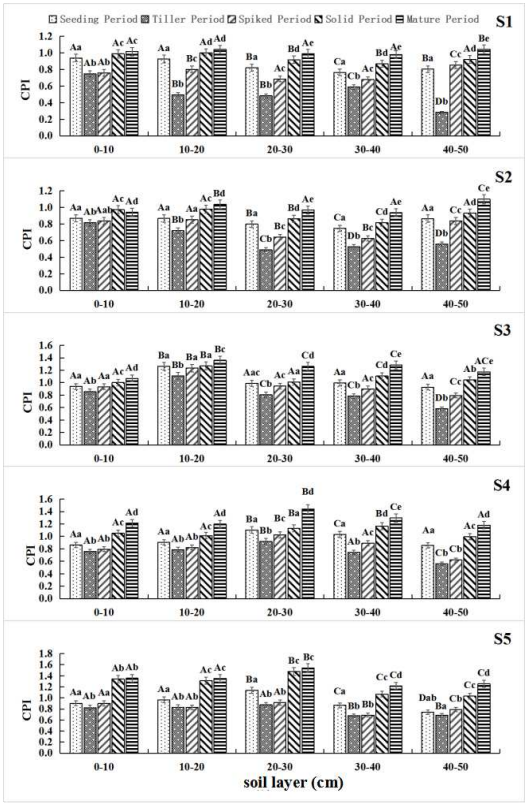

Figure 7. *The soil CPI content and distribution characteristics*

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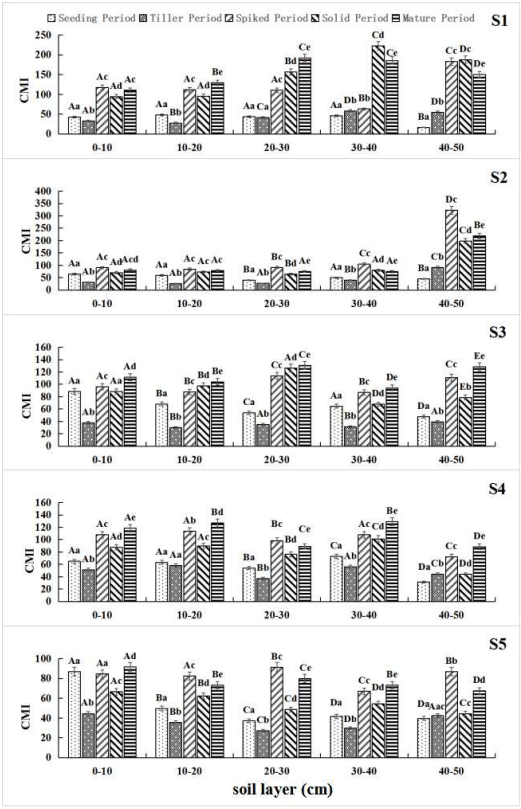

Figure 8. *The soil CMI content and distribution characteristics*

Factors influencing the management index of carbon pool

Table 2 shows the effects of soil layer, the rice cultivation years and rice growth stages on the carbon pool management index. The results showed that soil layer as an independent source of variation had a significant effect on AI and CMI ($P < 0.001$), and the number of rice cultivation years and rice growth stages as the independent source of variation had a significant effect on soil management indexes ($P < 0.001$).

Discussion

Characteristics and contribution of carbon accumulation in soil steady state during saline-alkali rice maturing

The dynamic balance of soil carbon pool is closely related to crop growth and fertilization management and directly affects soil fertility and crop yield (Cuo et al., 2014). Actually, agricultural production (e.g. years of soil tillage, management of fertilization, return of plant residues or organic materials, etc.) causes the initial changes in the soil carbon pool mainly by easy decomposition and mineralization. Currently, some scholars have tried to use soil organic carbon sublibrary to indicate total carbon change (Ma et al., 2019; Sun et al., 2015). The change of soil carbon pool mainly occurs in the active carbon pool (Kong et al., 2019). Therefore, they are important for soil carbon conversion, and it is closely related to soil productivity. Dalal and others think that it is better to use unstable carbon (Labile C) to indicate the change of soil carbon pool (Dalal et al., 1986). According to Su Jing and other studies,

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the main increase is inactive organic carbon content (Su et al., 2005). Distribution of NROOC in general soils is between 75 and 85%, and different ecosystems have different NROOC contents in soil (Ma et al., 2019; Li et al., 2006). In this study, during the rice growth stage, the proportion of the soil NROOC in saline-alkali paddy fields with different rice cultivation years was about 52.4-87.72. With the extension of rice, the soil ROOC and NROOC are increasing, and the NROOC content is higher than ROOC. In the process of rice cultivation years, most of the main carbon in the soil is converted into inactive parts and stored in soil (Mao, 2019). The soil NROOC content of different rice cultivation years also showed seasonal variation law, because of the difference of exogenous carbon input and soil microbial activity in different rice cultivation years.

Table 2. *Multifactorial variance analysis of carbon bank management index*

Index	Source of variation	Sum of squares	Degree of freedom	Mean square	F	P
A	Soil layer	0.558	4	0.139	38.124	0.001
	Reclamation time	1.159	4	0.29	79.2	*
	Growth stage	1.992	4	0.498	136.148	*
	Soil layer * Reclamation time	0.077	15	0.005	1.402	0.377
	Reclamation time * Growth stage	0.293	16	0.018	5.002	0.042
	Soil layer * Growth stage	0.517	16	0.032	8.834	0.012
	Soil layer * Reclamation time * Growth stage	0.555	60	0.009	2.531	0.15
AI	Soil layer	7.321	4	1.83	168.896	*
	Reclamation time	13.354	4	3.339	308.083	*
	Growth stage	7	4	1.75	161.483	*
	Soil layer * Reclamation time	11.159	15	0.744	68.653	*
	Reclamation time * Growth stage	1.895	16	0.118	10.931	0.008
	Soil layer * Growth stage	3.551	16	0.222	20.48	0.002
	Soil layer * Reclamation time * Growth stage	6.266	60	0.104	9.637	0.009
CPI	Soil layer	0.282	4	0.071	11.138	0.011
	Reclamation time	1.123	4	0.281	44.291	*
	Growth stage	3.309	4	0.827	130.548	*
	Soil layer * Reclamation time	0.72	15	0.048	7.574	0.017
	Reclamation time * Growth stage	0.166	16	0.01	1.634	0.308
	Soil layer * Growth stage	0.336	16	0.021	3.313	0.095
	Soil layer * Reclamation time * Growth stage	0.192	60	0.003	0.504	0.906
CMI	Soil layer	18296.604	4	4574.151	62.369	*
	Reclamation time	24126.885	4	6031.721	82.243	*
	Growth stage	112007.641	4	28001.91	381.81	*
	Soil layer * Reclamation time	46784.172	15	3118.945	42.527	*
	Reclamation time * Growth stage	22854.251	16	1428.391	19.476	0.002
	Soil layer * Growth stage	29025.917	16	1814.12	24.736	0.001
	Soil layer * Reclamation time * Growth stage	32085.276	60	534.755	7.291	0.017

* indicates significant difference ($P < 0.001$). A, carbon pool activity, AI, carbon pool activity index, CPI, carbon pool index, CMI, carbon pool management index

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Response of soil carbon reservoir management index to organic carbon accumulation in saline-alkali paddy field

The change of land use mode can lead to the change of tillage system, which will affect the balance of soil organic matter and change the decomposition rate of soil organic carbon (Tang et al., 2012b; Shen et al., 2000). In this study, the effect of planting years on soil carbon pool management index was obvious, and the soil A index value of different rice cultivation years was different. The soil A index value of 50 years was higher than that of the newly planted rice, which indicated that the carbon of saline-alkali paddy soil did not age, which was beneficial to carbon transformation.

The soil CPI index value increased gradually with the extension of rice cultivation years, which was basically consistent with the research of Wu et al (2015). The results showed that the soil CPI value increased after rice cultivation in dry land and wasteland. It shows that rice cultivation can effectively improve the carbon sink function of saline-alkali soil during the ripening process of saline-alkali rice field.

The soil CMI index value combined with soil carbon pool index and soil activity index, it can be sensitive to reflect and monitor soil organic carbon change index (Xu et al., 2006), and it can reflect external conditions on the quantity and quality of each component in carbon pool. The rise in the carbon pool management index indicates an increase in soil fertility, while a decline indicates a decline in soil fertility (Qiu et al., 2009). According to some studies, the initial change of soil carbon pool caused by agricultural production management measures and land use mode is mainly activated carbon part, which further affects soil carbon pool management index (Zhang et al., 2016b).

In this study, carbon pool activity (A) increases gradually with the rice cultivation years from the carbon pool management indices. During the rice cultivation, the soil carbon pool activity in seedling stage and heading stage is large, it shows that the soil quality improved in the process of soil maturation. The return of straw to the field is beneficial to the improvement of soil active carbon content and carbon pool management index in the tillage layer. The soil carbon pool management index is vulnerable to fertilization and farming years according to Wu Jianfu and other studies (Wu et al., 2013). In this study, the soil CMI index values increased gradually with the increase of rice cultivation, S2 above S1 level, and continued to increase with the extension of rice cultivation years, to show that reasonable rice cultivation can effectively improve soil CPI and CMI, but then the soil CMI began to slow down, because of farming provides a more suitable environment for microbes. As a result, the decomposition rate of soil organic carbon increased (Liu et al., 2017), so S5 soil CMI is lower than S4.

Sun Tao and others simulated the effect of planting years on the change of soil carbon pool in secondary saline-alkali soil based on CENTURY model (Sun et al., 2015). It was found that the total organic carbon of soil experienced a rapid rise, rapid decline and gradual steady change after planting Lycium barbarum. Therefore, the renewal and change of soil carbon pool caused by planting years may continue to be dynamic in the early decades of planting. On the other hand, it is found that the L, CPI and CMI of deep soil (40-50 cm) are lower than that of surface soil (0-10 cm), which is similar to that of Li et al. (2008). It shows that the carbon pool transformation and nutrient cycle of deep soil decrease slowly.

The main factors affecting the change of carbon pool management index were analyzed from three aspects: rice cultivation years, soil layer and rice growing stage.

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The results showed that soil layer had only significant effect on AI, CMI ($P < 0.001$), which indicated that the change of carbon pool activity in cultivated soil was obviously affected by section structure. Both rice cultivation years and rice growth stage have significant effects on A, AI, CPI and CMI, indicating that the renewal and evolution of soil carbon pool in saline-alkali rice field is more based on the influence of rice cultivation years and crop growth.

The correlation analysis showed that the soil SOC, ROOC and NROOC content of different rice cultivation years was significantly positively correlated in different rice growth stages, indicating that soil ROOC and NROOC were closely related to SOC, and was an important part of soil SOC, it obviously depended on the input, fixation, transformation and decomposition of surface carbon sources (Zhang et al., 2020). Further, it is clear that soil ROOC is a sensitive index to indicate soil carbon pool, which can reflect the change characteristics of soil organic carbon pool in the short term. Previous studies have shown that the soil CMI index can well indicate the change of soil quality and organic carbon (Zhang et al., 2019). The soil SOC and CMI index showed no significant correlation between tillering stage and heading stage. As a result, the soil CMI index can be used to evaluate the cumulative effect of soil carbon pool in rice cultivation.

Conclusion

The readily oxidized organic carbon to soil rice cultivation years and growing stage is obvious, which indicates that the stability of soil carbon pool is closely related to rice growth. Therefore, the feedback effect of plant and atmosphere on soil material circulation can further be studied, this provides a more comprehensive understanding of the process of the carbon bank cycle.

In the process of maturation of saline-alkali rice field, the content of soil NROOC increased significantly with the extension of rice cultivation years. The soil CMI index and the organic carbon content change obviously, showing that the soil CMI index increases first and then decreases with the extension of rice cultivation years. However, the carbon pool is in dynamic change during the maturation of saline-alkali rice field. The renewal and evolution of soil carbon pool is more based on the influence of rice cultivation and crop growth. Therefore, it is suggested that reasonable rice planting can improve the quality of soil carbon pool in saline-alkali paddy field.

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PREDICTION OF THE POTENTIAL DISTRIBUTION OF *DENDROLIMUS HOUI* LAJONQUIERE IN SICHUAN OF CHINA BASED ON THE SPECIES DISTRIBUTION MODEL

WU, S. J. – ZHU, T. H.* – QIAO, T. M. – LI, S. J. – SHAN, H.

Key Laboratory of Forest Pathology of Sichuan Province, College of Forestry, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

**Corresponding author*
e-mail: zhuth1227@126.com

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Abstract. *Dendrolimus houi* Lajonquiere is one of the main leaf-eating insects of China coniferous wood and causes serious damage to the safety of the forest ecosystem. In this article, the temporal and spatial distribution patterns of *Dendrolimus houi* Lajonquiere in the Sichuan Province of China were analyzed by ArcGIS. Using the band set statistical method in ArcGIS, 28 environmental factors were divided, and it was found that Wind10, Prec7, Bio4, Bio15, Srad3, Prec2 and Srad5, these 7 factors played a key role in the distribution of *Dendrolimus houi* Lajonquiere. Combined with 7 species distribution models, including Artificial Neural Network, Bioclim, GARP, Climate Space Model, Envelope Score, Maximum Entropy and SVM, the potential distribution area of *Dendrolimus houi* Lajonquiere was simulated, and the simulation results were all greater than 0.8, low, medium, and highly suitable average areas are 96227.54 km^2, 66062.81 km^2 and 91549.23 km^2 respectively, occupying 52.23% of the total area of Sichuan, China. According to the simulation results, it is necessary to strengthen the monitoring of the potential suitable areas for *Dendrolimus houi* Lajonquiere to prevent the large-scale damage of pine caterpillars in the forests of Sichuan.
Keywords: *population dynamics, pest prediction, environmental factors, openModeller, spatial distribution*

Introduction

Dendrolimus houi Lajonquiere (Lepidoptera: Lasiocampidae) is one of the most harmful leaf-eating pests of Chinese coniferous wood, parasitizing plants such as *Pinus yunnanensis*, *Pinus densata* Mast, *Pinus kesiya var. langbianensis*, *Cryptomeria fortune* (Liu, 2006; Song et al., 2019; Gou et al., 2003), which are widely distributed in Yunnan, Zhejiang, Fujian, Sichuan, Guangxi, Guangdong, Hunan, Hubei, Guizhou and other provinces (regions). India, Myanmar, Sri Lanka, Indonesia and other Southeast Asian countries also have records of infestations (Yin et al., 2002). *Dendrolimus houi* Lajonquiere mainly eats needles and shoots, which reduces the needles of trees and affects the normal development of plants, even leading to the death of trees, also known as "Smokeless Fire" (Li and Zhong, 2013). *Dendrolimus houi* Lajonquiere is very tolerant, it can safely survive the winter in the form of larvae (eggs or larvae) from January to March (0-5 °C). Old larvae mainly grow in summer (May to July). It can also be baptized with high temperature (>35 °C), successfully completed the development of the larvae and entered the pupal stage. The generations of *Dendrolimus houi* Lajonquiere vary in different regions and occur once or twice a year in Sichuan. From 2010 to 2018, the forestry area in Sichuan Province was affected by an average of more than 315 km^2 each year, and the disaster area involved 51 areas. The severely affected area reached 30 square kilometers, and the occurrence rate reached 100%. The leaves of the trees withered and fell early, which seriously affected the photosynthesis of the

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trees, and affected the growth of the trees and the natural landscape. According to on-site forestry investigations, the worm occurred in Chongzhou City and Chengdu City and the damage rate was 100% (*Fig. 1*). The scenic area dominated by cypress trees suffered huge economic losses and greatly restricts the development of local tourism. *Dendrolimus houi* Lajonquiere not only affects the safety of the forestry resources but also causes severe damage to the forest ecological environment and regional social and economic development (Xu, 2008).

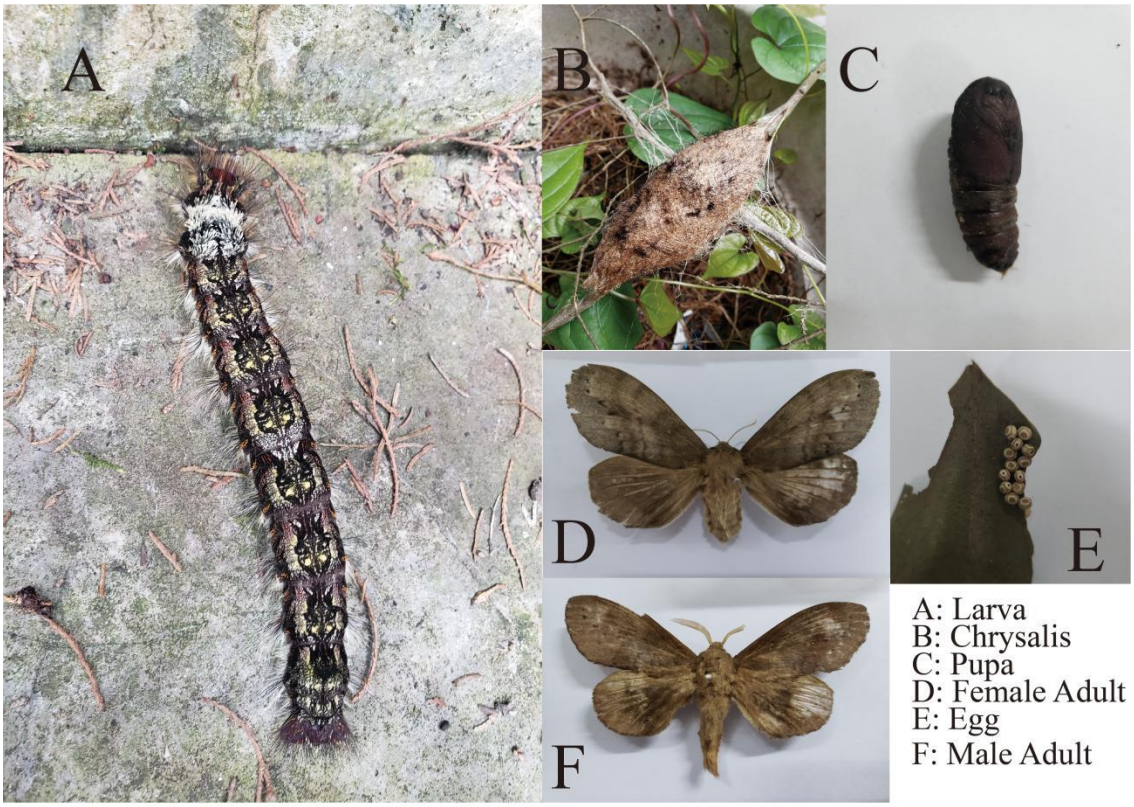

Figure 1. *Five stages of Dendrolimus houi Lajonquiere; A: Larva, B: Chrysalis, C: Pupa, D: Female adult, E: Egg, F: Male adult*

The insect spatial distribution pattern depends on its biological characteristics and the environment and influenced by the interaction of individuals or the distribution of the population in a geographic space, as is typical of spatial heterogeneity (Yu and Liu, 2001). For studying the physiological activities of insects, interspecific competition and breeding diffusion are important and also used as the basis of monitoring and control theory. However, the completeness of the data and the small size of the study area are limitations.

The breeding diffusion of climate factors played a key role in pest distribution. First, the climate factor for pest populations had a direct impact on the ecological and physiological activities; second, the main potential distribution of parasitized plants and the forest ecosystem are also limited by environmental factors (Duan et al., 2015; Sastawa et al., 2002). The current global climate warming trend, including significant changes in temperature and precipitation, has a close relationship with the distribution of pests and their development. Thus, exploring the relationship between pests and

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climate factors can help with effective early warning, monitoring and prediction. However, this approach is still in its infancy, as most of the research involves single simple linear regression models that are roughly associated, with a lack of effective spatial visualization expression; moreover, a deep exploration of the relationship has not yet been undertaken.

A species distribution model (SDM) consists of a collection of many subjects, including ecology, higher mathematics, information science, biology, geography, statistical analysis, and quantitative ecology. Its core aims are to use the distribution data of target species and related environmental variable factors, combining different mathematical algorithms to build a model that meets the ecological needs of the species and to map the results to different times and spaces to simulate the actual distribution of species and to predict the potential distribution (Zhu et al., 2013). Currently, algorithms such as GARP (Sobek-Swant et al., 2012), MaxEnt (Phillips et al., 2006), BIOCLIM (Beaumont et al., 2005), DOMAIN (Xu et al., 2015), SVM (Drake and Guisan, 2005), GAM (Oyafuso et al., 2017), GLM (Ghareghan, 2020), BRT (Yu et al., 2020) and ENFA (Hirzel and Guisan, 2002) are widely used in the potential distribution areas of species prediction, division of suitable areas for conservation of rare species (Mukul et al., 2019), prediction of invasive alien species (Padalia et al., 2014), and determining the future impact of climate on species (Khanum et al., 2013). The current study of *Dendrolimus houi* Lajonquiere focuses on its biolearnability (Hua et al., 2019), sampling investigation (Zhang, 2015), forecasting (Yang et al., 2017), control measures (Zhao et al., 2003), gene sequencing (Han et al., 2019), and other factors. However, with *Dendrolimus houi* Lajonquiere, determining the distribution of a wide range of spatial distribution patterns involves has a high-precision model and is still in the preliminary stages.

The research objectives of this paper are mainly in three aspects: (1) Use Matlab and ArcGIS to find the temporal and spatial patterns of *Dendrolimus houi* Lajonquiere. (2) Determine the key environmental factors that affect the distribution of *Dendrolimus houi* Lajonquiere. (3) Predict the potential distribution of *Dendrolimus houi* Lajonquiere in Sichuan of China. Provide an important reference for the early warning and control of *Dendrolimus houi* Lajonquiere.

Materials and methods

Study area

Our research area is Sichuan Province (26°03’N~34°19’N, 97°21′E~108°12′E), which is located in the southwestern region of China, covering an area of 486,000 km^2 and an altitude of 188 m to 7556 m. The Sichuan Basin belongs to a humid climate zone in the mid-subtropical zone with an average annual temperature of 16-18 °C. The daily temperature range is small. The winter is warm and the summer is hot. At the same time, it has abundant forestry resources, of which the area of cypress is 14520 km^2.

Data collection and processing

Distribution data: The Forestry Harmful Biological Information System (http://sc.30120.org/fpgis/secure/Login.aspx?ReturnUrl=%2ffpgis%2f) was used to obtain the distribution of *Dendrolimus houi* Lajonquiere in Sichuan Province from 2010 to 2018. Forestry workers in each county investigated the occurrence of *Dendrolimus houi* Lajonquiere and uploaded the statistics to the network platform, which was updated

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annually. Through screening and elimination of duplicate sites, 70 distribution sites were ultimately obtained. Query the latitude, longitude and altitude of each site through Google Map, and arrange them by the name of the species, longitude and latitude, and import ArcGIS. And use the distribution Fitter toolbox in Matlab to fit the distribution of latitude and longitude to obtain the distribution law of latitude and longitude.

Environment variable factor

Environment variables: climate data and elevation data mainly come from the global climate interpolation data network (http://www.worldclim.org/). From this network, 28 types of environmental data were selected, including 25 climate factors (Bio01-Srad) and 3 terrain factors (Alt, Slo, Asp). The altitude data (Alt), slope (Slo) and aspect (Asp) were extracted using ArcGIS. These factors were analyzed in detail using a spatial resolution of 30 s (precision for 1 km × 1 km) from the years of 1970~2000 (*Table 1*). The global climate interpolation network WorldClim was downloaded to convert the 28 climate variable factors obtained via ArcGIS to ASC format.

Table 1. *List of 28 environment variables*

Variable data	Description	Unit
Bio1	Annual mean temperature	℃
Bio2	Mean diurnal range	℃
Bio3	Isothermality	-
Bio4	Temperature seasonality	C of V
Bio5	Max temperature of warmest month	℃
Bio6	Min temperature of coldest month	℃
Bio7	Temperature annual range	℃
Bio8	Mean temperature of wettest quarter	℃
Bio9	Mean temperature of driest quarter	℃
Bio10	Mean temperature of warmest quarter	℃
Bio11	Mean temperature of coldest quarter	℃
Bio12	Annual precipitation	mm
Bio13	Precipitation of wettest month	mm
Bio14	Precipitation of driest month	mm
Bio15	Precipitation seasonality	C of V
Bio16	Precipitation of wettest quarter	mm
Bio17	Precipitation of driest quarter	mm
Bio18	Precipitation of warmest quarter	mm
Bio19	Precipitation of coldest quarter	mm
Prec	Precipitation	mm
Tmax	Maximum temperature	℃
Tmin	Minimum temperature	℃
Tavg	Average temperature	℃
Wind	Wind speed	m s^{-1}
Srad	Solar radiation	kJ m^{-2} day^{-1}
Slo	Slope	%
Asp	Aspect	°
Alt	Altitude	m

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openModeller modelling

openModeller (version 1.5.1; http://openmodeller.sourceforge.net/) is used to predict the potential distribution of *Dendrolimus houi* Lajonquiere in Sichuan. The openModeller version (1.1.0) includes the following algorithms: AquaMaps, ANN (Artificial Neural Networks), Bioclim, CSM (Climate Space Model), ENFA (Environment Niche), ES (Envelope Score), ED (Environment Distance), GARP, MaxEnt (Maximum Entropy), NM (Niche Mosaic) and SVM. BIOCLIM and Envelope Scores are classic "climate envelope models" that use the minimum and maximum observations of each environmental variable to define the bioclimatic range. The Climate Spatial Model is a principal component-based algorithm that compares the observed environment with the background data of the study area. GARP is an algorithm based on genetic rules that creates niche models to describe the environmental conditions that the species should be able to maintain the population. Machine learning models are non-parametric, flexible and variable regression models. They are also the most widely used SDM model, including Artificial Neural Network (ANN), MaxEnt and Support Vector Machine.

Input the species distribution data of *Dendrolimus houi* Lajonquiere into openModeller, the threshold is set to 30%, and other specific parameters are set to default values (Santana et al., 2008). The 7 distribution models of Artificial neural network, Bioclim, GARP, Climate Spatial Model, Envelope Score, Maximum Entropy and SVM in openModeller are used to simulate the potential distribution area of *Dendrolimus houi* Lajonquiere in Sichuan, and the fitting results are divided into four levels: high potential area ($0.5 \leq P$), good potential area ($0.3 \leq P < 0.5$), moderate potential area ($0.1 \leq P < 0.3$), and least potential area ($P < 0.1$). The AUC value (the area under the receiver operating characteristic curve ROC) is widely used to evaluate the accuracy of the model (Shabani et al., 2018). Therefore, models were examined by calculating the AUC in the ROC curve (values of 0.5~0.6 indicate failure, 0.6~0.7 indicate poor, 0.7~0.8 indicate worse, 0.8~0.9 indicate better, and 0.9~1.0 indicate good) to determine the models' accuracy (Phillips et al., 2006).

Results

Occurrence in Sichuan Province

From 2010 to 2018, the main affected areas of *Dendrolimus houi* Lajonquiere in Sichuan Province were concentrated in the northeastern plains of Sichuan. Chengdu has the most distribution points among all cities, with a total of 12 points. The distribution range of longitude includes 101.5°E~107.7°E, and the distribution range of latitude includes 27.1°N~32.6°N. Using the probability density toolbox of Matlab to analyze the latitude and longitude of the distribution of *Dendrolimus houi* Lajonquiere, it is found that it conforms to the generalized extreme value distribution law, showing the characteristics of skewed distribution. The peak frequency of occurrence is 104.5°E to 106°E in longitude and 30°N to 31°N in latitude (*Fig. 2*). At the same time, using the density analysis tool in ArcGIS to analyze the nuclear density of the distribution sites, it is found that Chengdu City, Deyang City, Nanchong City, Mianyang City, Guangyuan City, Luzhou City, Dazhou City occur more frequently. Rarely, the outbreaks occurred in other regions with a relatively narrow range of occurrence. The distribution points were the most in 2012 and 2013, while the distribution points were the least in 2011 and

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2018. The overall trend was cyclical changes, which were inseparable from changes in climate factors (*Fig. 3*).

Environmental variable factor division

In order to eliminate the influence of multicollinearity on the accuracy of the model and select variables with higher value to the model, the correlation coefficient of 28 environmental variables was analyzed using the band set statistical method of ArcGIS, and two environmental factors with a correlation greater than 0.8 were analyzed ($|r| > 0.8$). Screen, choose one as the key factor to keep. The selected variables include wind speed in October (Wind10), average precipitation in July (Prec7), Temperature Seasonality (Bio4), Precipitation Seasonality (Bio15), solar radiation in March (Srad3), average precipitation in February (Prec2), solar radiation in May (Srad5) (as seen from *Table 2*).

Table 2. *The correlation coefficient analysis of 7 important environment variables*

Variable	Wind10	Prec07	Bio04	Bio15	Srad03	Prec02	Srad05
Wind10	1						
Prec7	0.09616	1					
Bio4	-0.70683	-0.23695	1				
Bio15	-0.7472	0.00952	0.65299	1			
Srad3	0.0374	-0.32586	0.21193	0.30052	1		
Prec2	-0.59378	-0.54236	0.57145	0.76534	0.3256	1	
Srad5	0.6625	0.30775	-0.55405	-0.64073	-0.56646	-0.57993	1

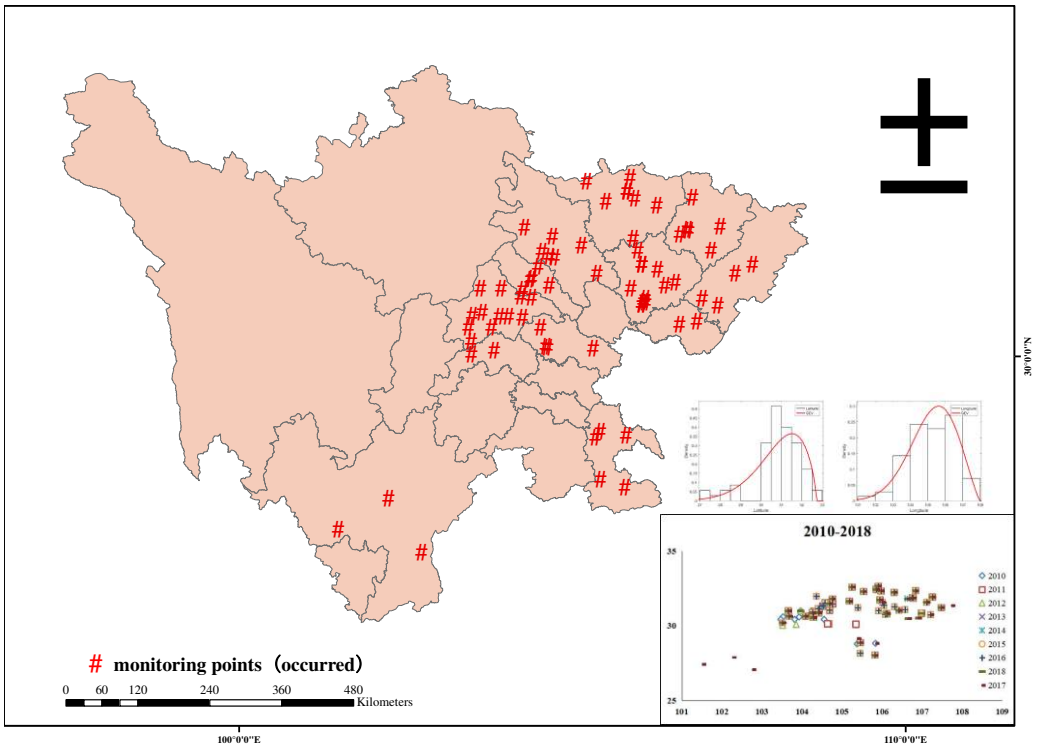

Figure 2. *The map shows the occurrence records of Dendrolimus houi Lajonquiere specimens from 2010 to 2018 and the regularity of their occurrence frequency in latitude and longitude*

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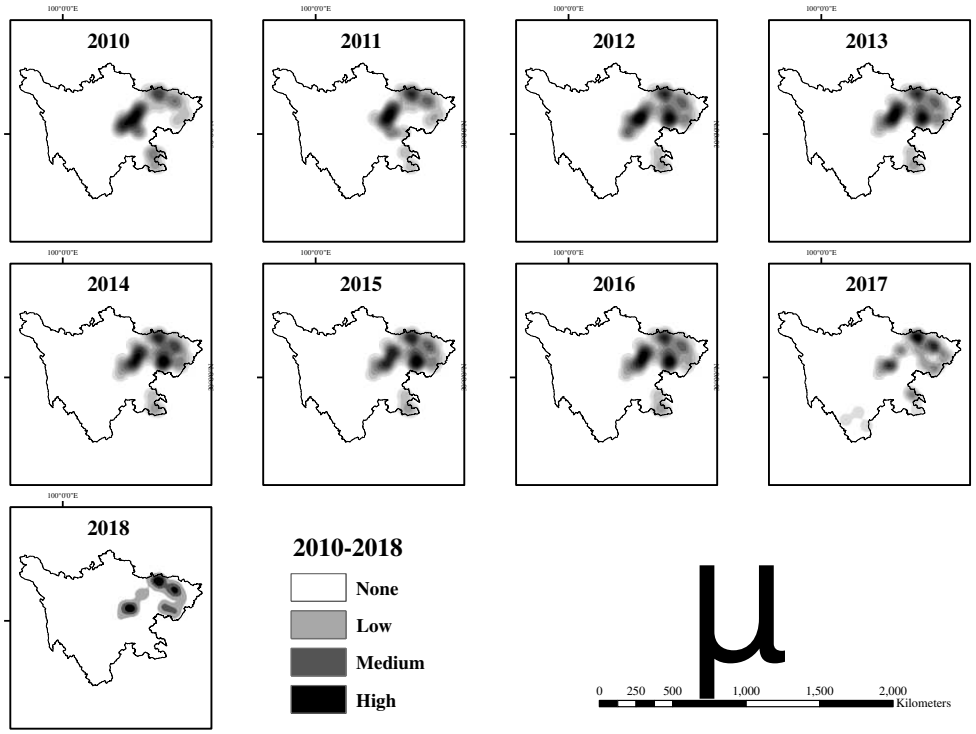

Figure 3. *Kernel density of the Dendrolimus houi Lajonquiere from 2010 to 2018. White indicates that there is no concentration in the center, gray indicates that the range of the species is small, light black indicates the center concentration is medium, and dark black indicates the highest concentration in the center*

Choice of species distribution model

Bio4, Bio15, Prec2, Prec7, Srad3, Srad5 and Wind10 were used as environment layers. Seven species distribution models, Artificial Neural Network, Bioclim, GARP, Climate Space Model, Envelope Score, Maximum Entropy and SVM, were used to simulate the distribution results. The ROC curve was used to determine the accuracy of the model. The experimental results are as follows.

The AUC values were between 0.82 and 0.9, and the accuracy was high. From high to low: SVM (0.9) > GARP = Maximum Entropy (0.88) > Artificial Neural Network (0.85) > Bioclim (0.84) > Envelope Score (0.83) > Climate Space Model (0.82). Among them, the SVM model had the highest AUC value of 0.9, with higher accuracy and stronger credibility (as seen from *Table 3*).

Table 3. *Accuracy, AUC, sensitivity and omission error of 7 species distribution model*

Algorithm	Accuracy	AUC	Sensitivity	Omission error
Artificial Neural Network	80	0.85	0.8	0.0143
Bioclim	100	0.84	1	0
Climate Space Model	84.3	0.82	0.84	0.2
Envelope Score	100	0.83	1	0
GARP	94.3	0.88	0.94	0.0571
Maximum Entropy	98.6	0.88	1	0.0143
SVM	95.7	0.9	0.96	0.0571

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Division of potential areas

The distribution results of the 7 species distribution models, Artificial Neural Network, Bioclim, GARP, Climate Space Model, Envelope Score, Maximum Entropy and SVM, were imported into ArcGIS (*Fig. 4*). The simulated distribution area was divided into four levels by using the natural discontinuity method (*Table 4*). There were 4 types of high potential area ($0.5 \leq P$), good potential area ($0.3 \leq P < 0.5$), moderate potential area ($0.1 \leq P < 0.3$), and least potential area ($P < 0.1$). The average total suitable area simulated by the 7 models is 253839.59 km^2, occupying 52.23% of the area of Sichuan. Among them, the low, medium and high suitable areas are 96227.54 km^2, 66062.81 km^2 and 91549.23 km^2, respectively.

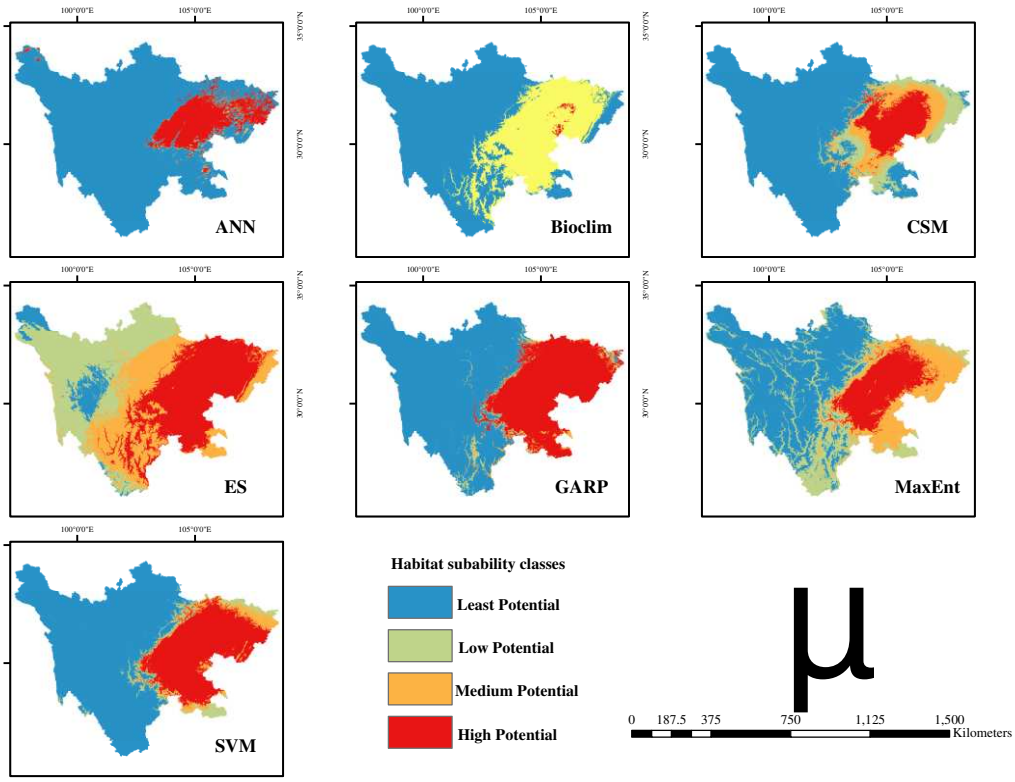

Figure 4. *The potential distribution area of Dendrolimus houi Lajonquiere in Sichuan area simulated by 7 species distribution models. Different colors represent different levels of habitat suitability: red indicates high suitability, with a probability greater than 0.5. Yellow indicates moderate suitability, with a probability of 0.4-0.5, green indicates low suitability, with a probability of 0.1-0.3, white indicates inappropriate, with a probability of less than 0.1*

The potential area for the *Dendrolimus houi* Lajonquiere was mainly concentrated in the northeastern plain of the Sichuan Basin. The high potential area was mainly concentrated in Chengdu, Dujiangyan, Bazhong, Suining, Mianyang, Guangyuan, Deyang City, and Nanchong City. The good potential area was mainly concentrated in Ziyang City, Meishan City, Neijiang City, Zigong City, and Yibin City. The moderate potential area was mainly concentrated in Leshan City, Ya'an City, Luzhou City and South of Liangshan Yi Autonomous Prefecture. The unhealthy areas were mainly concentrated in Ganzi Tibetan Autonomous Prefecture, Aba Tibetan Autonomous

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Prefecture, and Liangshan Yi Autonomous Prefecture. Comparing the temporal and spatial patterns of *Dendrolimus houi* Lajonquiere in Sichuan Province from 2010 to 2018, the simulation results were essentially consistent. Thus, it can be proved that the SVM model is the best model for the distribution of *Dendrolimus houi* Lajonquiere in the Sichuan area.

Table 4. *The suitable area and proportion of Dendrolimus houi Lajonquiere in Sichuan*

Algorithm	Least potential (km^2)		Low potential area (km^2)		Medium potential area (km^2)		High potential area (km2)	
	Rate (%)	Area (km^2)	Rate (%)	Area (km^2)	Rate (%)	Area (km^2)	Rate (%)	Area (km^2)
Artificial Neural Network	83.78%	407189.3	1.36%	6612.5	0.95%	4619.7	13.91%	67578.5
Bioclim	0.00%	0	67.14%	326317.1	32.42%	157570.5	0.43%	2112.4
Climate Space Model	68.57%	333241.1	10.41%	50569.3	9.92%	48232.5	11.10%	53957.1
Envelope Score	6.62%	32152.5	33.13%	160992.2	27.40%	133165.1	32.86%	159690.2
GARP	62.70%	304734.7	2.84%	13788.9	2.29%	11149.5	32.17%	156326.8
Maximum Entropy	48.29%	234709.4	19.28%	93698.7	16.74%	81338.7	15.69%	76253.2
SVM	64.42%	313095.8	4.45%	21614.1	5.42%	26363.7	25.71%	124926.4
Average	47.77%	232160.4	19.8%	96227.54	13.59%	66062.81	18.84%	91549.23

Climate factor variable statistics

Using the Spatial Analyst function in ArcGIS, the niche parameters of the seven contribution factors in the distribution of the different fitness levels were extracted, including the range, maximum, minimum, average, and standard deviation. The results show that among the different grades of suitable regions, the changes of the leading environmental variable factors (Bio4, Bio15, Prec2, Prec7, Srad3, Srad5 and Wind10) showed the same trend: the range gradually decreased, and the standard deviation gradually increased and then decreased, with less variation. Comprehensive analysis showed that *Dendrolimus houi* Lajonquiere has a wide tolerance range for climatic factors. The range occurred when the temperature seasonality was 619.6~810.9, the precipitation seasonality was 162.3~103.2, the average precipitation in February was 6~29 mm, he average precipitation in July was 167~367 mm, the solar radiation in March was 9001~12123 kJ m^{-2} day^{-1} and the solar radiation in May was 14540~16964 kJ m^{-2} day^{-1}, the wind speed in October was 0.8~1.8 m s^{-1} (*Table 5*).

Discussion

This paper analyzes the distribution points of *Dendrolimus houi* Lajonquiere by using ArcGIS and Matlab. It is found that *Dendrolimus houi* Lajonquiere has a high frequency of distribution in the northeastern plains of Sichuan. The distribution range of *Dendrolimus houi* Lajonquiere spans 5 latitudes and longitudes, with a concentration of 104.5°E to 106°E, a latitude distribution of 31°N to 33°N. It is less distributed in other regions, such as northern and western regions, which is inseparable from the distribution of host plants. Insect distribution changes with changes in latitude, longitude, are also limited by host plants and environmental factors (Xin et al., 2019). *Dendrolimus houi* Lajonquiere eat cypress leaves in Sichuan, and Sichuan cypress is mainly distributed in the northeastern plain at an altitude of 350-700 m (Li et al., 2017). This is also an important reason affecting the distribution of *Dendrolimus houi* Lajonquiere in Sichuan. The distribution space overlaps between the two, showing a

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strong saliency relationship, which reasonably explains the extreme distribution of the *Dendrolimus houi* Lajonquiere.

Table 5. *Statistical analysis of variables in different suitable classes of Dendrolimus houi Lajonquiere from SVM model*

Variables	Least potential		Low potential		Medium potential		High potential		Unit
	Range	Mean ±Std	Range	Mean ±Std	Range	Mean ±Std	Range	Mean ±Std	
Bio4	439.4~887.2	636.7 ±85.0	499.3~817.4	703.3 ±57.9	599.9~821.1	739.6 ±45.4	619.6~810.9	733.7 ±30.4	C of V
Bio15	70.1~109.8	89.9 ±6.5	63.1~95.8	78.1 ±7.9	63.1 ~98.3	78.6 ±9.4	62.3~103.2	85.8 ±10.5	C of V
Prec2	2~20	7.9 ±2.3	4~34	14.2 ±3.6	6~29	15.0 ±4.3	6~29	16.3 ±5.0	mm
Prec7	95~236	144.3 ±25.1	110~239	175.5 ±19.7	156~367	196.1 ±17.7	167~367	232.9 ±36.0	mm
Srad3	10174~17299	13722.5 ±1120.3	9909~16172	11795.3 ±908.6	9744~12639	11473.9 ±463.1	9001~12123	10874.1 ±625.3	kJ m^{-2} day^{-1}
Srad5	15291~20412	17322.3 ±984.8	14902~18639	15894. ±613.4	14866~16936	15873.9 ±526.7	14540~16964	15356.4 ±439.6	kJ m^{-2} day^{-1}
Wind10	1~5.1	2.6 ±0.7	0.9~8.8	1.6 ±0.4	0.9~2.3	1.3 ±0.2	0.8~1.8	1.1 ±0.2	m s^{-1}

Studies have shown that terrestrial insects are sensitive to changes in water and temperature; thus, climatic factors have played an important role in the life cycle and geographical distribution of insects (Kingsolver et al., 2011). The occurrence of *Dendrolimus houi* Lajonquiere is concentrated in the northeastern plains of the Sichuan Basin and has expanded in scope over time, in a manner that is inseparable from the climate change caused by global warming. Changes in precipitation and temperature have a significant relationship with the occurrence area of *Dendrolimus houi* Lajonquiere. The 7 factors screened in this study were Bio4, Bio15, Prec2, Prec7, Srad3, Srad5 and Wind10. With reference to the former study (Zhao and Liu, 2007), the highest temperature in December, precipitation throughout the year and the duration of light were considered important climate indicators. At the same time, the life history of *Dendrolimus houi* Lajonquiere was evaluated. In April, *Dendrolimus houi* Lajonquiere are young larvae, and their resistance is poor. The larvae will die if the temperature is too high or too low; September is the high incidence period of *Dendrolimus houi* Lajonquiere cocooning pupae and feathering adults. Excessive precipitation will cause mature larvae to fail to weave on the branches and leaves of trees or shrubs, leading to the mechanical death of pupae or adults. December is the wintering period of eggs. Too low a temperature causes a delay or failure of larval hatching (He et al., 2018; Zhou et al., 2019). This proves that the six climate factors have strong credibility and can be used as important reference indicators for early warning and monitoring of the occurrence of *Dendrolimus houi* Lajonquiere.

With reference to related studies, the use of the AUC value of the ROC curve area to evaluate the precise value of the species distribution model has been widely used (Xu et al., 2015). This paper uses Artificial Neural Network, Bioclim, GARP, Climate Space Model, Envelope Score, Maximum Entropy and SVM to simulate the suitable distribution area of *Dendrolimus houi* Lajonquiere in Sichuan. AUC prediction results ranging from 0.82 to 0.9 were obtained, among which the AUC value of the SVM model was the highest at 0.9. As a supervised machine learning language, the SVM

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model is often used in many fields, such as pattern recognition and regression analysis, but it lacks application experience in ecological modeling (Hoang et al., 2010). Due to their robustness, SVM can correlate the distribution pattern of organisms with abiotic characteristics, thus providing reliable predictions for organisms (Keerthi et al., 2010). In the research for this paper, the SVM model had the highest accuracy value, and it is the most suitable species distribution model compared with the geographical distribution pattern of *Dendrolimus houi* Lajonquiere in Sichuan.

Comprehensive analysis of the niche parameters of 7 key climate factors found that the *Dendrolimus houi* Lajonquiere especially liked a warm and humid environment and had a wide temperature tolerance range. The range occurs when the average precipitation in September is 102~190 mm, the average precipitation in February is 6-29 mm, the minimum temperature in February is 1.4~12.2 ℃, the isotherm is 23.1~44.8, the average temperature in April is 15.7~24.4 ℃, and the maximum temperature in December is 8.8~19.3 ℃. The range of suitable climate factors for the *Dendrolimus houi* Lajonquiere provides an important reference for related prevention and management work. When the temperature and precipitation reach suitable living conditions, it is necessary to strengthen the monitoring and early warning of the forest to prevent a large outbreak of *Dendrolimus houi* Lajonquiere.

Many studies have shown that invasive alien species will be difficult to completely eradicate after entering their habitats and will cause large losses in the local ecological environment (Koyama et al., 2004; Kong et al., 2008). The distribution trend of *Dendrolimus houi* Lajonquiere was analyzed through the spatial distribution pattern of *Dendrolimus houi* Lajonquiere and the map of environmental factors. Central and southern Sichuan are potential areas for future invasion by *Dendrolimus houi* Lajonquiere; in particular, the border between Sichuan and Yunnan has become a hidden danger and shows evidence of radiation. Among the involved factors, transportation has played a role that cannot be ignored. The breeding method of *Dendrolimus houi* Lajonquiere involves the spawning and hatching of adult worms. The flight distance of adult worms is limited, and it is difficult to achieve such a rapid spread. The continuous construction of a large number of infrastructures has accelerated the conversion of logistics between cities and has indirectly facilitated the spread of *Dendrolimus houi* Lajonquiere. Adults invade other urban areas by laying eggs on transportation vehicles or untested seedlings and trees, causing serious disasters.

This paper uses species distribution models to simulate the suitability distribution of *Dendrolimus houi* Lajonquiere in Sichuan Province of China. The simulation results are good, but there are still some limitations. First of all, the time span of climate factors used in this study is from 1970 to 2000. Failure to use future climate data will cause deviations in the prediction of suitable areas for *Dendrolimus houi* Lajonquiere. Secondly, in the actual living environment of species, host plants, interspecies competition, human interference, extreme climate changes, etc. will all have an impact on the potential distribution of predicted species.

Conclusion

The northeastern plains of Sichuan (longitudes 104.5°E to 106°E, latitude 31°N to 33°N) are concentrated high-explosive areas, which are inseparable from the local topographic and climatic conditions. The terrain of the plain in northeastern Sichuan is flat, the climate is a subtropical monsoon climate, warm and humid, with a few extreme

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climates. At the same time, the large number of cypresses in northeastern Sichuan provides a rich source of food for *Dendrolimus houi* Lajonquiere. Comprehensive analysis shows that the core idea for the control of *Dendrolimus houi* Lajonquiere is no longer the single control of pine caterpillars but rather the formation of a "Forest-*Dendrolimus houi* Lajonquiere" ecosystem linkage and the use of ecological principles and probability statistics to analyze and solve the problems. Knowledge of the key climate factors of pine caterpillars, reasonable planning of prevention and control areas, strengthening forestry quarantine law enforcement, and establishing accurate species distribution models can not only effectively prevent *Dendrolimus houi* Lajonquiere from destroying forests but also avoid economic waste and destruction to the environment caused by blind pollution control. In future studies, while adding future impact factor data Therefore, it is necessary to thoroughly explore the impact of global warming on the distribution of *Dendrolimus houi* Lajonquiere, so as to make the prediction results more accurate and reliable.

Disclosure statement. The authors report no conflict of interests.

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SPATIOTEMPORAL DISTRIBUTION PATTERNS OF BENTHIC MACROINVERTEBRATE FUNCTIONAL FEEDING GROUPS IN THE BLYDE RIVER, SOUTH AFRICA

ADDO-BEDIAKO, A.

Department of Biodiversity, University of Limpopo, Private Bag X1106, Sovenga 0727, South Africa (e-mail: abe.addo-bediako@ul.ac.za; phone: +27-15-268-3145; ORCID: 0000-0002-5055-8315)

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Abstract. The impacts of land use changes and differences in seasonality on benthic macroinvertebrate composition were evaluated in the Blyde River of the Olifants River. The highest taxon richness and abundance of macroinvertebrates were at S6 and S5 respectively. These could be due to the high habitat heterogeneity and availability of food resources. The abundance and richness of taxa were greater in winter and spring respectively and both were lower in summer. The composition of macroinvertebrate community and distribution of functional feeding groups (FFG) differed significantly among sites. Collector-gatherers and collector-filterers were the most abundant groups recorded. The least abundant group was the shredders. The low abundance of shredders could be attributed to the enhanced microbial activity replacing shredder activity at high temperatures. The relative abundance and richness of the functional feeding groups did not conform fully to the River Continuum Concept (RCC). This could be due to human activities in the catchment that may be causing a change in the habitat and water quality at various sites. The results suggest that policies governing changes in land use is necessary to conserve the river and the macroinvertebrate community.
Keywords: *bioindicators, distribution, land use, river continuum concept, water quality*

Introduction

Many river systems are continuously undergoing degradation throughout their course of flow due to pollution from various anthropogenic activities, such as mining, agriculture and expanding human settlements (Zhang et al., 2015; Mimba et al., 2018; Chen et al., 2019). Anthropogenic effluents from the catchment of rivers are causing the deterioration of river systems and affecting the biotic communities (Jooste et al., 2015; Jun et al., 2016; Addo-Bediako et al., 2018). Assessment of the ecological status of rivers is a vital in managing river ecosystems in the world.

Biological monitoring is one of the methods used to determine the effects of anthropogenic activities on water quality of rivers and it is very useful to obtain their ecological information (Merritt et al., 2017). Macroinvertebrates are widely used as bioindicators of aquatic ecosystems because they are sensitive to changes in physical and chemical variables of ecosystems and reflect environmental conditions (Rosenberg and Resh, 1993). Benthic macroinvertebrates, for example, provide a more accurate understanding of changes in aquatic conditions when compared to chemical and microbiological data, which rather present short-term fluctuation (Ghasemi and Kamali, 2014). Macroinvertebrates respond to anthropogenic disturbance and natural changes in their habitats by changing their community structure. They serve as a major components of aquatic food webs that link organic matter and nutrient resources (e.g., leaf litter, algae and detritus) with higher trophic levels (Li et al., 2010). Furthermore, macroinvertebrates are made up of many species from different taxonomic levels with a wide range of trophic levels and pollution tolerances (Qu et al., 2013).

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Benthic macroinvertebrates have a high diversity and a differentiated functional form according to the given physicochemical conditions of rivers and therefore respond to changes in the various environmental variables (Jun et al., 2016; Kim et al., 2016). Hence, there has been an increase in the use of functional feeding group (FFG) of benthic macroinvertebrate assemblages to assess environments (Fu et al., 2016). The FFG approach is considered to be more appropriate and rapid for characterizing ecosystem conditions, compared with taxonomical approach (Cummins et al., 2005; Mishra and Nautiyal, 2013; Cummins, 2016). It is used to assess the effect of land use disturbances on river functioning (De Castro et al., 2016), thus FFG provides a further perspective together with other community indices to ensure a better understanding of the relationship between habitat and aquatic fauna (Townsend et al., 1997). The River Continuum Concept (RCC) is widely used to explain the functioning of lotic ecosystems and predicts how relative FFG abundance change along a river gradient (Vennote et al., 1980). The RCC proposes that community structure should shift from an allochthonous in the headwater to an autochthonous in the downstream of the river, as the river widens and algal production increases (Allan and Castillo, 2007). Functional composition is therefore necessary for management actions to enhance ecosystem functioning (Ferreira et al., 2012). This approach is seen to provide more accurate assessment of water quality and ecological integrity of rivers, which indeed could be used for conservation and restoration strategies in managing river ecosystems (Príncipe et al., 2010).

The effect of changes in environmental variability on functional feeding groups of macroinvertebrate communities has scarcely been explored in South African rivers. Most of the studies on macroinvertebrate functional composition to land-use changes have been done in the temperate regions. However, land-use impacts are regionally specific due to the strong influence of cultural, historical, climatic and landscape settings on indicator-disturbance relationships (Zhang et al., 2012). Information regarding the structure and functioning of macroinvertebrates in the Blyde River is rare. The Blyde River was selected for the study because it is one of the few rivers in the Olifants River System known to have good water quality despite the increasing number of anthropogenic activities in the catchment (Ashton and Dabrowski, 2011). The objective of this study was to assess the spatial and temporal structure of aquatic macroinvertebrate community using the FFG approach in the Blyde River and to determine if the macroinvertebrate community structure corresponds to the River Continuum Concept (RCC). It is envisaged that the results of the study would be used to design proper conservation tool in the river system.

Materials and methods

Study area

The Blyde River rises on the western slopes of the north-south trending Drakensberg Mountains and flows northwards towards the escarpment edge where it is dammed. From the dam, the Blyde River cascades down a steep series of rapids to its lower reaches, where the river again flows northwards to join the Olifants River at the town of Hoedspruit in Limpopo Province (DWAF, 2004). The Blyde River sub-catchment is approximately 2000 km^2 in size. Geologically, the northern part of the sub-catchment is made up of crystalline gneissic and granitic rocks of the Basement Complex, underlying the catchment (DWAF, 2004). The sub-catchment lies partly on the escarpment and, as

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a result, experiences higher rainfall considerably than the other sub-catchments in the Olifants River Basin, with mean annual precipitation sometimes exceeding 1000 mm (DWAF, 2004). During the last decade, there has been an increase in human activities in the area, especially agriculture, which are likely to cause environmental pollution in the freshwater systems. Seven sampling sites were selected along the river (*Fig. 1*). S1 (24°30'59.46"S 30°47'56.14"E), S2 (24°30'14.42"S 30°50'08.49"E). S3 (24°25'52.45"S 30°50'03.59"E), S4 (24°24'19.03"S 30°47'54.19"E), S5 (24°19'30.90"S 30°49'52.00"S), S6 (24°23'04.94"S 30°48'22.09"E) and S7 (24°15'30.38"S 30°50'13.22"E). The detailed description of the sampling sites is given in *Table 1*.

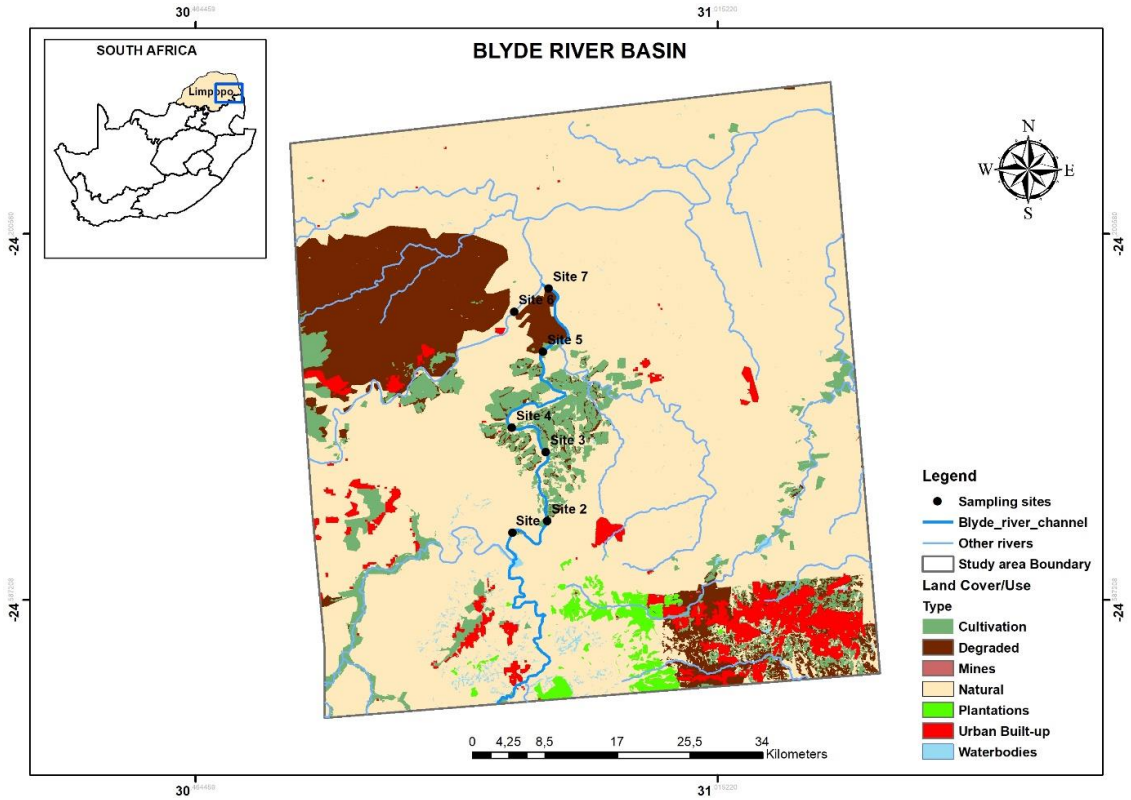

Figure 1. *Map of the study area, showing the locations of the seven sampling sites of the Blyde River*

Physicochemical parameters

The study was carried out in January (summer), April (autumn), July (winter) and November (spring), 2018. Water samples were collected in 500 ml polyethylene bottles (acid pre-treated) and stored in a cooler box in the field using ice packs before being transported to the laboratory. Four water samples were collected from each site during the study. In the laboratory, the samples were stored at 4°C prior to analyses. Three readings of environmental variables, such as pH, water temperature, dissolved oxygen, (DO), total dissolved solids (TDS) and electrical conductivity (EC) were recorded at each site using a YSI Model 554 Data logger. Laboratory measurements were conducted to determine the nutrients (NH_4, NO_2, NO_3 and PO_4) and turbidity, using a spectrophotometer (Merck Pharo 100 Spectroquant™) with Merck cell test kits in the Biodiversity Water Laboratory, University of Limpopo. Percentage riparian vegetation

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cover was visually estimated at each sampling station over a 20 to 30 m riparian width. The following parameters were also measured; Stream width, the distance from bank to bank at a transect representative of the stream channel using a measuring tape; (ii) water depth, the vertical distance from the water surface to stream bottom using a meter ruler and (iii) current velocity at riffles or gliding runs using a flow meter.

Table 1. *Description of the study sites of the Blyde River*

Sites	Watershed features	Riparian vegetation	In-stream features	Mean width (m)	Depth (m)	Type	Substratum		
	Nature and land use	Structure	Canopy cover				Cobble/pebble	Sand	Silt/clay
S1	Lodge/residential	Trees, shrubs, reeds	Moderate (60%)	18	0.28	Riffle	50%	30%	20%
S2	Agriculture	Trees, reeds, grass	Slight (40%)	9.5	0.33	Riffle	30%	50%	20%
S3	Agriculture	Trees, shrubs, reeds	Moderate (50%)	9	0.35	Riffle	50%	30%	20%
S4	Industrial area	Trees, shrubs, grass	Slight (40%)	16	0.32	Riffle	45%	35%	20%
S5	Agriculture	Trees, shrubs, reeds	Moderate (60%)	26	0.25	Riffle, pool	40%	30%	30%
S6	Nature reserve	Trees, reeds, shrubs	Moderate (65%)	17.5	0.20	Riffle	50%	25%	25%
S7	Nature reserve, confluence with the Olifants River	Grass, shrubs	None	8	0.33	Riffle	40%	40%	20%

Sampling of macroinvertebrates

Benthic macroinvertebrate samples were collected at the seven sites of the Blyde River during the four seasons. The samples were collected within a 100-m stretch of the study sites, with substrate of biotopes consisting mainly of mud, sand, gravel or stones. Samples were collected using a 30 cm by 30 cm sampling net with a 500 µm mesh size. Benthic macroinvertebrates were collected using the kick sampling method described by Dickens and Graham (2002), whereby the substrate was disturbed by kicking to free macroinvertebrates. Each site was sampled three times for benthic macroinvertebrates. The macroinvertebrates were then separated from organic and mineral matter, counted and identified to family level using Gerber and Gabriel's field guide manual (2002). Where specimens could not be identified in the field, the samples were preserved in 70% ethanol and transported to the laboratory for further identification, with the aid of a stereomicroscope (Leica EZ4) and magnifying glass. Macroinvertebrates were further classified into functional feeding groups using the key of Cummins et al. (2005), which classified the aquatic macroinvertebrates into the following functional feeding groups: Shredders (Sh) macroinvertebrates that chew conditioned litter or live vascular plant tissue (coarse particulate organic matter); Gathering-collectors (GC), that acquire fine particulate organic matter from interstices in the bottom sediments; Filtering-collectors (FC), that capture fine particulate organic matter from the water column using silken nets and filtering fans; Scrapers (Sc), which feed on algae attached on stable surfaces; Predators (P), which feed on living prey (*Table 2*). Abundance of macroinvertebrate FFGs in each site and season were then calculated.

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Table 2. *Functional group characterization and food resources (from Merritt and Cummis, 1996)*

Type of FFG	Particle size feeding mechanism	Dominant food resources	Range of particle size of food (mm)
Shredders	Chew conditioned litter or live vascular plants tissue, or gouge wood	CPOM – decomposing (or living hydrophytes) vascular plants	> 1.0
Filtering collectors	Suspension feeders-filter particles from water column	FPOM-decomposing detrital particles; algae, bacteria and feces	0.01-1.0
Gathering collectors	Deposits feeders-ingest sediments or gather loose particles in depositional areas	FPOM- decomposing detrital particles; algae, bacteria and feces	0.05-1.0
Scraper	Graze rock and wood surfaces or stems of rooted aquatic plants	Periphyton attached non-filamentous algae and associated detritus, microflora, fauna and feces	0.01-1.0
Predators	Capture and engulf prey or tissue, ingest body fluids	Prey- living animal	> 0.5

FFG - Functional Feeding Group, CPOM - Coarse Particulate Organic Matter, FOPM - Fine Particulate Organic Matters

Statistical analysis

The cumulative values of the four seasons were used as the proportional abundance of each FFG. Analysis of variance (ANOVA) was used to determine differences between the sites and seasons. Where there was a significant variation, the Turkey's Post-Hoc test was performed to determine where the difference occurred. The statistical analyses were conducted using the software package, Statistica v10.0. Canonical Correspondence analysis (CCA), a multivariate method to calculate the relationships between biological assemblages of taxa and the environment was also used (Ter Braak and Verdonschot, 1995).

Results

Physicochemical variables

The mean values of the physicochemical parameters are summarized in *Table 3*. The water depth and width increased from upstream to downstream site. The mean depth varied from 0.25 m (S1) to 0.68 m (S3) and the mean width varied from 4.97 (S1) to 7.07 m (S7). The highest mean velocity was 0.37 m/s at S3 and the lowest was 0.21 m/s at S1. The pH ranged from 8.1 at S5 to 9.0 at S1. The mean temperature varied from 22.28 (S2) to 24.90 °C (S7). The lowest mean dissolved oxygen value of 8.70 mg/l and the highest mean value of 11.85 mg/l were recorded at S5 and S2 respectively. The highest mean conductivity of 442 mS/m was observed at S5 and the lowest mean conductivity of 270.85 mS/m at S1. The mean TDS ranged from 132.9 at S1 to 252 mg/l at S7. There were no significant differences in the physicochemical variables among the seven sites ($p > 0.05$). However, there were seasonal significant differences in temperature (ANOVA, $F = 12.03$; $p < 0.001$), DO (ANOVA, $F = 8.16$; $p = 0.001$), EC (ANOVA, $F = 3.45$; $p = 0.032$), TDS (ANOVA, $F = 18.6$; $p < 0.001$). The nutrient levels were generally higher at the upstream sites than the downstream sites.

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Table 3. *The mean physicochemical variables measured at different sites along the Blyde River*

Water quality parameters	S1		S2		S3		S4		S5		S6		S7		WQG
	AVE	± SD	AVE	± SD	AVE	± SD	AVE	± SD	AVE	± SD	AVE	± SD	AVE	± SD	
Velocity (m/s)	0.21	0.11	0.26	0.12	0.37	0.04	0.32	0.14	0.35	0.12	0.32	0.15	0.35	0.06	
Temp (°C)	22.33	2.01	22.28	2.30	23.53	1.71	22.88	2.54	23.65	2.58	23.95	3.49	24.90	3.49	
pH	8.4-9.0	–	8.4-8.9	–	8.3-8.7	–	8.2-8.8	–	8.1-8.5	–	8.2-8.8	–	8.2-8.8	—	6.5-9.0[3]
EC (mS/m)	270.85	346.6	274.9	361.8	341.4	260.6	338.9	449.8	442.2	547.0	366.7	357.8	333.7	357.8	–
TDS (mg/l)	132.9	32.2	138.5	35.4	147.2	43.7	250.9	158.0	241.2	79.4	158.6	76.64	252.8	76.64	
DO (mg/l)	11.01	1.345	11.85	2.45	9.93	2.45	10.7	1.33	8.70	1.85	9.38	1.31	10.63	0.15	–
Salinity (‰)	0.25	0.34	0.27	0.37	0.35	0.47	0.38	0.51	0.46	0.59	0.38	0.51	0.49	0.63	< 0.5‰[1]
NO_2 (mg/l)	0.02	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.005	0.00	0.06[3]
NO_3 (mg/l)	0.65	0.50	0.55	0.10	0.02	0.00	0.15	0.00	0.25	0.00	0.33	0.05	0.375	0.05	13.0[3]
NH_3 (mg/l)	0.06	0.00	0.05	0.00	0.04	0.00	0.05	0.00	0.03	0.00	0.03	0.03	0.065	0.03	< 0.007[1] 0.354[3]
Ortho-PO_4 (mg/l)	0.22	0.00	0.16	0.32	0.40	0.00	0.25	0.00	0.03	0.00	0.18	0.20	0.13	0.20	–
Turbidity NTU	10.70	8.69	11.20	12.88	6.75	2.05	7.50	2.69	13.25	10.99	7.75	5.24	10.25	3.34	8 - < 50[2]

[1]DWAF (1996) - South African Water Quality Guidelines: Volume 7: Aquatic Ecosystems
[2]BC-EPD (2006) -British Columbia Environmental Protection Division: Water Quality Guidelines
[3]CCME (2012) - Canadian Council of Ministers of the Environment: Water Quality Guidelines-Aquatic Life
[4]US-EPA (2012) - United States Environmental Protection Agency: Water Quality Guidelines-Aquatic Life

Abundance and diversity of macroinvertebrates

A total of 19 797 specimens belonging to 11 orders and 33 families were collected at the various sampling sites and seasons. The greatest abundance (7426) and least abundance (1023) were observed at S5 and S3 respectively. The highest taxa richness (31) and the lowest taxa richness (22) were at S6 and S7 respectively. The family Hydropsychidae (Trichoptera) was the dominant taxa, followed by Caenidae, Baetidae (Ephemeroptera) and Simuliidae (Diptera) (*Table 4*). The abundance of the macroinvertebrates was significantly different among sampling sites (ANOVA, $F = 8.35$; $p < 0.001$). In terms of seasons, the most abundance occurred during winter and least abundance during summer. The highest taxa richness (32) occurred in spring, followed by winter (27), autumn (24) and then summer (23). There was no seasonal significant difference in taxa richness but there was a significant seasonal variation in abundance (ANOVA, $F = 6.03$; $p < 0.001$).

Functional feeding group composition

In terms of the functional feeding groups, 14 predators, 8 collector-gatherers, 6 scrapers, 4 collector-filterers, 3 shredders were collected (*Table 4*). The collector-gatherers and collector-filterers accounted for about 80% of the total abundance (*Fig. 2*). The highest abundance of collector filterers, collector-gatherers and shredders were at S5, the highest abundance of predators and scrapers were at S2 and S7 respectively (*Table 5*). There were significant variations in the FFGs among the sites ((ANOVA: $F = 4.35$, $p < 0.05$). There was a significant difference in the abundance of collector-filterers among sampling sites (ANOVA, $F = 3.88$; $p = 0.009$) and the postdoc showed the significant difference between S1 and S5 (Tukey's test = 0.025), S3 and S5 (Tukey's test = 0.026), S4 and S5 (Tukey's test = 0.017) and S5 and S7 (Tukey's test = 0.015) (*Table A2* in the *Appendix*). The collector-gatherers showed a significant

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seasonal variation (ANOVA, F = 3.06; p = 0.04) and the postdoc showed the significant difference between winter and summer (Tukey's test = 0.048) (*Table A9*).

Table 4. *Order, families and the functional feeding groups of macroinvertebrates from the Blyde River*

Order	Family	S1	S2	S3	S4	S5	S6	S7	Total	FFG
Ephemeroptera	Baetidae	227	594	138	417	501	330	276	2483	CG
	Caenidae	490	722	91	85	612	396	123	2519	CG
	Heptageniidae	7	123	95	270	103	267	40	905	Sc
	Teloganodidae	39	68	13	20	139	101	295	675	CG
	Leptophlebiidae	1	3	20	3		53	76	156	CG
	Tricorythidae	4	3	15	37	532	24	128	743	CG
Trichoptera	Hydropsychidae	207	754	390	131	1308	631	260	3681	CF
	Philopotamidae		11	17	15	226	2	2	273	CF
	Leptoceridae	1	2	3					5	Sh/CG
Coleoptera	Gyrinidae	1	8	1	3	1	8	1	23	P
	Elmidae	23	72	31	52	917	52	13	1160	CG/Sc/Sh
	Helodidae	3		2		9	1		15	Sh
	Psephenidae	24	12	38	21	1	17		113	Sc
Odonata	Libellulidae	10	12	6	5	50	4	10	97	P
	Chlorocyphidae	50	192	53	89	1	75	3	463	P
	Platycnemididae		3	2			1		6	P
	Coenagrionidae	1		1	4	5	3	3	17	P
	Aeshnidae	8	8		2	1	1		20	P
	Gomphidae	68	21	3	2	2	1	14	111	P
Diptera	Athericidae	4	79	7	25	13	40	1	169	P
	Blephariceridae					2	8		10	Sc
	Tabanidae	11	8	5	7	79	19	11	140	P
	Dixidae			1	2	5	3		11	CG
	Chironomidae	59	66	37	58	975	246	62	1503	CG
	Muscidae		1			3	2		6	P
	Simuliidae	11	26	11	80	1223	854	12	2217	CF
Plecoptera	Perlidae	1	5	2	3	9	6		26	P
Annelida	Hirudinea					36	57		93	P
	Oligochaeta	18	7	3	2	4	32	42	108	CG
Mollusca	Physidae					1		2	3	Sc
	Planorbidae	1	3		2		17		23	Sc
	Thiaridae	38	45	41	3	23	152	642	944	Sc
	Corbiculidae	3	14	5	43	635	239	140	1079	CF
Total of individuals		1309	2862	1031	1381	7416	3642	2156	19797	
Taxa richness		24	24	27	26	29	31	22		

FFGs: collector–filterers (CF), collector–gatherers (CG), predators (P), scrapers (Sc) and shredders (Sh)

Relationships between macroinvertebrates (FFG) and physicochemical variables

The CCA ordination of macroinvertebrate FFGs with water quality variables and nutrient concentrations showed distinct patterns, where the variables correlated with specific macroinvertebrate FFG under different levels of disturbance (*Fig. 3*). Sites 6 and 7 were less affected by temperature, EC, TDS, turbidity and nitrate. There was an association of shredders with the upstream sites, S1 and S2, while scrapers were associated with S3 and S4. The predators and the collectors (filterers and gatherers)

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were well distributed at all the sites. The eigenvalues were 0.090, 0.058, 0.046 and 0.032 for the axis 1, axis 2. axis 3 and 4, and explained 34.7%, 58.4%, 74.0% and 86.8% of variance respectively (*Table 6*).

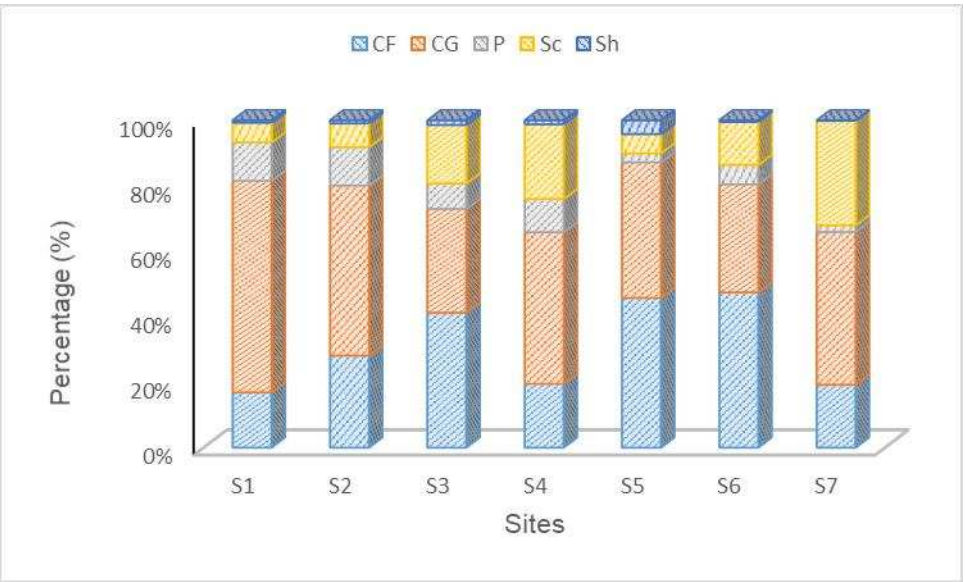

Figure 2. *Composition of the FFGs of aquatic insects at the different sites in the Blyde River (CF-collector filterer, CG- collector gatherer, P- predator, Sc- scrapers, Sh- shredders)*

Table 5. *Abundance of the functional feeding groups (FFGs) of macroinvertebrates in the seven study sites of the Blyde River*

	CF	**CG**	**P**	**Sc**	**Sh**	**Relative abundance (%)**	**Total**
S1	221	846	154	78	10	6.6	1309
S2	805	1486	337	207	27	14.5	2862
S3	425	327	80	184	15	5.2	1031
S4	269	640	140	315	17	7.0	1381
S5	3392	3073	200	436	315	37.4	7416
S6	1727	1202	217	478	18	18.4	3642
S7	414	1006	44	688	4	10.9	2156
Relative abundance (%)	36.6	43.3	5.9	12.1	2.1	100	19797

FFGs: collector–filterers (CF), collector–gatherers (CG), predators (P), scrapers (Sc) and shredders (Sh)

Table 6. *The canonical correspondence analysis (CCA) between functional feeding groups and environmental variables for the Blyde River*

Axes	**1**	**2**	**3**	**4**	**Total**
Eigenvalues	0.090	0.058	0.046	0.032	0.247
Taxa-environment correlations	0.99	0.99	0.99	0.99	
% variance explained					
- of taxa data	34.7	58.4	74.0	86.8	
- of taxa-environment relation	34.7	58.4	74.0	86.8	

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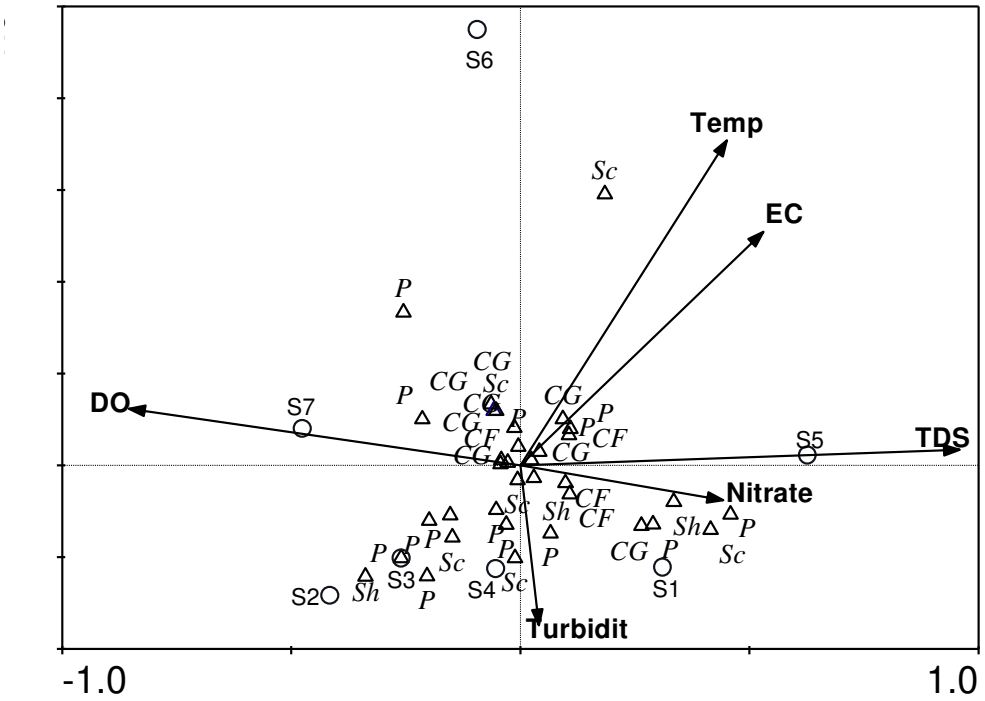

Figure 3. *CCA tri-plot of the relationship between physicochemical variables and functional feeding groups of macroinvertebrates (CF-collector filterer, CG- collector gatherer, P-predator, Sc- scrapers, Sh- shredders)*

Discussion

Physicochemical variables

Generally, the high levels of dissolved oxygen and low nutrient levels indicate that the water in the Blyde River was of good quality and that the river was only slightly disturbed by human activities. Higher EC and TDS were recorded in the midstream and downstream (from S4 to S7) than upstream (S1 and S2) of the river. The dissolved oxygen was high throughout the river, an indication that the river is in good condition. The nutrient levels were generally low at all sites. Thus, the physicochemical variables and nutrients recorded at all the sites were within the guideline limits (DWAF, 1996; CCME, 2012; USEPA, 2012).

Abundance and diversity of macroinvertebrates

The highest richness and abundance of macroinvertebrates were at S6 and S5 respectively. The high abundance of macroinvertebrates at site S5 could be attributed to increased input of organic nutrients from agriculture and high habitat heterogeneity of the site. High abundance of macroinvertebrates is known to increase with higher habitat heterogeneity due to the available stable and diverse microhabitats (Braccia and Voshell, 2006). The highest macroinvertebrate taxon richness at S6 could be due good habitat condition and the canopy cover. Many studies have found higher macroinvertebrate taxon richness at shaded sites than sites with low canopy cover (Dalu et al., 2017). The improved habitat condition at S5, though at the downstream of the river further supports the importance of conservation of water resources. The observed

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differences in taxa compositions across the sites were most likely due to different land use characteristics (Cortes et al., 2011, 2013).

Functional feeding group composition of macroinvertebrates

The collectors (filterers and gatherers) were dominant at all the sites. The high abundance across the sites could be due to the wide range of food resources consumed by this group. The predominance of collectors throughout the river has been reported in other studies of tropical streams (Tomanova et al., 2006; Jiang et al., 2011). Collectors are commonly abundant in streams and typically increase in abundance with stream size (Vannote et al., 1980). Generally, the high abundance of collector-gatherers in the river could be attributed to enrichment of organic matter in the water (Rosenberg and Resh, 1993). The highest abundance of collector-filterers was at S5, followed by S6. This may be attributed to the relative abundance of fine particulate organic matter transported in the water column (Strand and Merritt, 1999).

The high taxa richness of predators along the whole longitudinal gradients of the river may be due to availability of food and less competition. Predators are more abundant in small intermittent streams where fishes are scarce (Rieradevall et al., 1999). The river continuum concept (RCC) proposed that the abundance of predators depend on prey availability and abundance (Vannote et al., 1980). Predators normally have a similar proportion along the entire river, according to the river continuum concept or, alternatively, their abundance may depend on prey availability (Vannote et al., 1980). In this study, the relative abundance of predators was not significantly different at all sites except at S3 and S7. The highest abundance of scrapers at S7 could be due to sufficient algal production (periphyton), which serves as their food (Grubaugh et al., 1996). The shredders abundance was generally low especially at S7. Generally, the low abundance of shredders could be attributed to the enhanced microbial activity replacing shredder activity at high temperatures. The highest abundance at S5 could be due to the presence of riparian canopy which created conditions with plenty of feeding material that supported the shredders. The shredders utilize leaf litter from the riparian zones as an energy source. On the contrary, the lowest abundance of shredders at S7 could be due to low availability of leaf litter. Similar results have been reported that shredders are intimately related to the riparian vegetation, because of their reliance on allochthonous feeding resources and as well contribute much in the degradation of leaf materials dropping into aquatic systems from overhanging vegetation (Brasil et al., 2014; Masese et al., 2014).

The variation of the different FFG at the sampling sites can be explained by the availability of the food resources and changes in the environmental variables. Studies have shown that macroinvertebrate fauna can be altered by land use practices (Miserendino and Masi, 2010; Egler et al., 2012; Fierro et al., 2015). The shredders and scrapers were very low in the river and this could also be due to the fact that these two groups are more sensitive to disturbances, while collector-gatherers and collector-filterers are more tolerant to pollution that might alter the availability of certain food (Barbour et al., 1996). Thus, the functional groups can potentially be used to assess aquatic ecosystem health (Bhawsar et al., 2015). Studies on the distribution of FFGs and benthic macroinvertebrate assemblages according to environmental variables are increasingly being conducted (Fierro et al., 2015; Fu et al., 2016).

Seasonally, the taxon richness and abundance were highest in spring and winter respectively than in other seasons. These are the drier seasons of the year and therefore

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the river receives less runoff draining from the catchment. In general, factors driving macroinvertebrate seasonal variation include precipitation/discharge, temperature, and photoperiod, and each of these factors can influence disturbance regimes in streams (Bêche et al., 2006). Seasonal variation of macroinvertebrate distribution can also be caused by changes in current velocity, substrate type and organic matter. The collector-gatherers were the most dominant functional group in autumn, winter and spring, while the collector-filterers were the most dominant group in summer. According to the continuum river concept, low order rivers such as Blyde River should contain mainly collector-gatherers and shredder organisms (Vannote et al., 1980). However, in this study the predominant group was the collector-gatherer. This study supports other studies which found high abundance of only collectors' group in some rivers (Miserendino and Pizzolon, 2003).

Relationships between macroinvertebrate assemblages and water quality variables

The distribution of macroinvertebrate structural and functional assemblages was influenced by physicochemical variables and nutrients across the sites. Canonical correspondence analysis (CCA) indicated that specific site categories correlated with specific water quality variables which in turn affected the distribution of the macroinvertebrates. Shredders were mainly found in the sites with riparian influence. Increase in nutrient levels and reduction in water quality negatively impacted the distribution of the sensitive macroinvertebrate taxa. The deteriorating physicochemical variables (EC and TDS) at S4 and S7 contributed to the low richness and diversity of macroinvertebrate taxa in these sites. Macroinvertebrate distribution closely followed the observed land-use changes in the various sites which induced changes in water quality with the least disturbed sites being associated with macroinvertebrate communities that were different from sites with poor water quality. These results are consistent with many other studies that have also found abiotic factors to be significantly affecting variation in macroinvertebrate communities (Kasangaki et al., 2008; Masese et al., 2014).

Conclusion

The study determined the influence of physicochemical variables on the distribution of benthic macroinvertebrate FFG. The collector-gatherers and collector filterers dominated across all the sites. The shredder and collector co-dominance in the headwaters was not observed as predicted by RCC. The RCC predicts that shredders will decrease in abundance from headwaters to the mouth and that collector-gatherers, collector-filterers and scrapers will increase downstream (Vannotte et al., 1980). Generally, low abundance of shredders and scrapers were recorded in this study. Thus, the distribution of the functional groups did not conform fully to RCC pattern and it could be due to land use changes (degradation) occurring in sections of the river. The high richness of macroinvertebrates at S6 further supports the importance of conservation. The FFG pattern shows the influence of changing environmental conditions on macroinvertebrates along the river and therefore confirm that FFG is an effective tool to assess ecological integrity of rivers. The findings suggest that conservation and protection of the river catchment including the riparian zone are important for preservation of ecological integrity and biodiversity of rivers. Future

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studies should consider how the increasing land use changes and climate change will affect the structure of macroinvertebrates in the river.

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Conflict of interests. The author declares no conflict of interests.

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APPENDIX

Results of analysis of variance (ANOVA) for sites

Table A1. *Collector-filterers*

	SS	df	MS	F	p
Intercept	1878786	1	1878786	21.6258	0.00014
"Site"	2023574	6	337262	3.88207	0.00918
Error	1824415	21	86877		

Table A2. *Post-hoc analysis of the collector-filterers*

	Var1	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	S1		0.919330	0.7064	0.59351	0.44503	0.69865	0.55829
2	S2	0.91933		0.9992	0.99440	0.06773	0.99905	0.99116
3	S3	0.70639	0.999194		0.99999	0.02610	1.00000	0.9999
4	S4	0.59351	0.994395	0.999		0.01737	0.99999	1.0000
5	S5	0.44503	0.067731	0.026	0.01737		0.02537	0.01531
6	S6	0.69865	0.999053	1.000	0.99999	0.02537		0.99998
7	S7	0.55830	0.991155	0.999	1.00000	0.01531	0.99998	

Table A3. *Collector-gatherers*

	SS	df	MS	F	p
Intercept	1198958	1	1198958	27.38833	0.000003
Site	475145	6	79191	1.80899	0.116774
Error	2145035	49	43776		

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Table A4. *Predators*

	SS	df	MS	F	p
Intercept	14088.0	1	14088.0	19.2434	0.00003
Site	4000.20	6	666.70	0.91067	0.49099
Error	66620.8	91	732.10		

Table A5. *Scrapers*

	SS	df	MS	F	p
Intercept	95238.1	1	95238.1	6.89483	0.01274
Site	47219.6	6	7869.93	0.56975	0.75150
Error	483454	35	13812.9		

Table A6. *Shredders*

	SS	df	MS	F	p
Intercept	7581.00	1	7581.00	1.74152	0.20812
site	25782.67	6	4297.111	0.987139	0.470084
Error	60943.3	14	4353.1		

ANOVA for seasons

Table A7. *Collector-filterers*

	SS	df	MS	F	p
Intercept	3287876	1	3287876	11.79258	0.004949
Season	1585194	3	528398	1.89520	0.184258
Error	3345706	12	278809		

Table A8. *Collector-gatherers*

	SS	df	MS	F	p
Intercept	2098176	1	2098176	13.41930	0.001028
Season	1441322	3	480441	3.07275	0.043881
Error	4377942	28	156355		

Table A9. *Post-hoc of collector-gatherers*

	Season	{1}	{2}	{3}	{4}
1	A		0.933996	0.855422	0.241845
2	Sp	0.933996		0.996930	0.079585
3	Su	0.855422	0.996930		0.048130
4	W	0.241845	0.079585	0.048130	

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Table A10. *Predators*

	SS	df	MS	F	p
Intercept	24654.02	1	24654.02	15.18067	0.000281
Season	7822.91	3	2607.64	1.60565	0.199266
Error	84450.07	52	1624.04		

Table A11. *Scrapers*

	SS	df	MS	F	p
Intercept	166666.7	1	166666.7	6.382639	0.020068
Season	15363.3	3	5121.1	0.196117	0.897798
Error	522250.0	20	26112.5		

Table A12. *Shredders*

	SS	df	MS	F	p
Intercept	13534.08	1	13534.08	3.294568	0.107053
Season	5044.92	3	1681.64	0.409357	0.750719
Error	32864.00	8	4108.00		

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THE RELATIONSHIP BETWEEN THE MORPHOLOGICAL AND STRUCTURAL CHANGES OF *POPULUS EUPHRATICA* OLIV. AND ENDOGENOUS HORMONE CONTENTS

HAN, X. L.[1,2,3] – ZHAI, J. T.[1,2,3] – GAI, Z. S.[1,2,3] – LI, Z. J.[1,2,3*]

[1]*Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin Xinjiang Production and Construction Corps, Alar, Xinjiang 843300, China*

[2]*Desert Poplar Research Center of Tarim University, Alar, Xinjiang 843300, China*

[3]*College of Life Science, Tarim University, Alar, Xinjiang 843300, China*

**Corresponding author*
e-mail: lizhijun0202@126.com; phone: +86-997-468-1202

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Abstract. *Populus euphratica* Oliv. exhibits heterophylly, which is regarded as a manifestation of evolutionary adaptation to extremely arid environments. To clarify the relationship between Morphological and structure changes of *P. euphratica* (*Populus euphratica* Oliv) and its physiological characteristics, we studied the changes in the morphological structures, carbohydrates, endogenous hormone levels and their ratios, as well as their relationships during the developmental stages, and heterophylly at crown height. Correlation analysis shows, IAA and GA^3 contents, as well as the ratios of IAA/ABA, GA_3/ABA and ZR/ABA, were negatively correlated with LW and LA and were positively correlated with LL and LI. IAA, ZR, IAA/ABA, and ZR/ABA were negatively correlated with LT. GA_3, IAA, GA_3/ABA, and IAA/ABA were significantly or extremely significantly correlated with ECN, PTCL, PTT and PSR. The content of soluble sugar, starch and soluble protein were positively correlated with the LI and negatively correlated with LA, PL, LW, ECN and PTC. *P. euphratica* regulates the drought resistance at different developmental stages by changing the content and ratio of 4 hormones in heterogeneous leaves and the synergistic changes of some anatomical structures, and adapts to arid environment.

Keywords: *ecological adaptation strategy, heterophylly, morphological anatomy, physiological characteristics*

Introduction

Plants are constantly subjected to various environmental stresses throughout their whole life cycle, especially in desert ecosystems, and plant responses to adverse conditions may be complex combinations of physiological, morphological, and long-term adaptation strategies. Air contact generally do not affect plants leaves negatively but its extremes such as very high temperature or very low air humidity could lead to stresses, Leaves are extremely sensitive to environmental changes during species evolution and individual growth and development, showing strong plasticity (Yang et al., 2019a). Leaf traits directly affect the basic behaviour and functions of plants, have the closest relationship with plant biomass and plant resource acquisition, utilization and utilization efficiency, and can reflect the survival strategies formed by plants adapting to environmental changes (Huang et al., 2010a). For example, the leaves in young plants of *Sabina chinensis* are needle-shaped, while the old ones are scales. This phenomenon of having leaves of different shapes on the same plant is called heterophylly. Heterophylly is a genetic mechanism that accumulates during the long-

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term adaptation process of plants to the environment (Zhang et al., 2017) and the leaf structure also best reflects the adaptability characteristics of plants in adversity (Cao, 2005; Xu, 2018). *P. euphratica* has biological characteristics of heterophylly, which are successively manifested in the appearance of strip, lanceolate, ovate, and broad-ovate leaves at different developmental stages from seedling and sapling to adult trees (*Plate 1* in the *Appendix*). Studies have confirmed that there is a significant negative correlation between the age of *P. euphratica* and the leaf index (LI) and a positive correlation between age and leaf area (LA) (Huang et al., 2010a, b).

More recent studies have also revealed that plant hormones, including abscisic acid (ABA), gibberellin (GA), auxins (IAA) and zeatin-riboside (ZR), can affect heteromorphic leaf formation in many plant species. For example, analysis of the different developmental stages of *Ludwigia arcuata* showed that ABA played an important role in the changes in leaf morphology in underwater and above water conditions (Hokuto et al., 2017). The increased levels or responses of the plant hormone gibberellin (GA) in tomato leaves also led to the simplification of leaf shape (Yanai et al., 2011). Homologous members of the AUX/indole-3-acetic acid (IAA) gene family mediate the action of auxin in determining leaf shape by repressing the growth of regions with low auxin concentrations during simple and complex leaf development (Koenig et al., 2009). The growth and development of different types of leaves are also the result of the combined action of multiple hormones (Kalve et al., 2014; Morillon and Chrispeels, 2001). Previous studies showed that the hormone contents in leaves of *P. euphratica* also change with the age of the tree and morphological changes of heteromorphic leaves, however, it has not been reported how the changes in the hormone content and ratio of the heteromorphic leaves coordinate with the morphological structure to adapt to drought.

Leaf structure is sensitive and adaptable to the environment, and the change of leaf structure is an important mechanism for plants to adapt to environmental changes (He et al., 2018) For example, the leaves of plants grown in arid environments for a long time usually exhibit increased leaf thickness, thickened cuticle, increased thickness of palisade tissue, decreased or degraded sponge tissue thickness. (Li et al., 2016). Leaf morphology is formed by plants constantly adapting to changing environments in the process of evolution and selection, which can better reflect the adaptability of plants and affect the functions and performance of plants. (Yang et al., 2019b; Leigh et al., 2011), heteromorphic leaves that differ in their morphology exhibit various functional characteristics to adapt to environmental stress; thus, heteromorphic leaves may play a crucial role in plant adaptations to environmental changes. For example, the size of the leaves directly affects the degree to which plants receive light resources. Generally, larger leaves can enable plants to obtain more light energy. At the same time, leaf area affects leaf heat and water balance by affecting leaf temperature and transpiration rate (Wright et al., 2017). Several studies showed that from the base of the trunk to the top of the same individual of *P. euphratica*, the number of epidermal cells of heteromorphic leaves increased, the mesophyll cells were more closely arranged, the palisade tissues were increasingly developed, the sponge tissues were increasingly degenerate (Ding et al., 2010; Li et al., 2005), the xeric structure of broad-ovate leaves was more developed than that of lanceolate leaves, and stronger resistance was shown (Yang et al., 2005; Wang et al., 1997). During different developmental stages, there are gradual increases in the LA, leaf thickness (LT), number of leaf epithelial and hypodermal cells, epidermal cell length (ECL), and palisade tissue thickness (PTT) in *P. euphratica* from the base to the top of the crown, but there are gradual decreases in the leaf length (LL), LI, and sponge tissue

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thickness (STT) from the base to the top of the crown. Additionally, there is a significant correlation between morphological characteristics and anatomical structures (Zhao et al., 2016). Studies have shown that the contents of soluble sugar, starch and soluble protein in heteromorphic leaves are closely related to the morphological formation and ontogenetic stage of heteromorphic leaves (Li et al., 2015). The levels and ratios of endogenous hormones of heteromorphic leaves of *P. euphratica* were significantly different at different canopies (Li et al., 2017). However, studies on the changes in these morphological characteristics, anatomical structures, and carbohydrate and hormone levels, as well as their relationship during the development of heteromorphic leaves and how these ecological adaptation strategies are formed, are lacking.

P. euphratica was researched during different developmental stages in the same ecological environment. We studied the changes in the morphological characteristics, anatomical structure, carbohydrate, soluble protein and endogenous hormone levels and their ratio in different developmental stages and the vertical spatial distribution of the same individual crowns. The current study aims to clarify the relationship between various endogenous hormones, soluble sugars and the morphological and structural changes of *P. euphratica* heteromorphic leaves and establish a foundation to further reveal the regulatory mechanism of the morphological and structural changes of heteromorphic leaves at the molecular level.

Materials and methods

To minimize the environmental effects, we selected *P. euphratica* at different developmental stages but under the same site conditions. The conditions of the test site were introduced in previous studies (Zhai et al., 2019). The study site was located in the *Populus euphratica* forest (81°17'56.52" E, 40°32'36.90" N, 980 m above sea level) in the upper reaches of the Tarim River on the northwestern margin of the Tarim Basin, Xinjiang, China. This region has a hot, dry summer with little rainfall all year round. The average annual precipitation, potential evaporation and temperature are 50 mm, 1900 mm, and 10.8 °C, respectively, with average annual sunshine of 2900 h, representing a typical temperate desert climate (from the local meteorological bureau). The *P. euphratica* forest at the study site covered an area of 180.6 ha, with 355 individual *P. euphratica* trees.

Experimental design and sampling

Using the diameter at breast height (DBH) and 2 cm as the interval, the forest was divided into nine diameter classes, indicating nine developmental stages of *P. euphratica.* Three sample trees with uniform crowns were selected from each diameter class, with 27 trees in total. During the leaf maturity period, the sampling canopy height was divided into 5 equal parts (total stations to measure the tree heights and under branch heights of trees in the different diameter classes from base to top was defined as 1 to 5 layers) (*Table A1*). At each sampling layer, twelve one-year-old branches were collected from the four directions of east, south, west and north. Sixty branches were collected from each sampling tree, and sample leaves were taken at the third node from the base of the branch. A total of 60 leaves per sampling were used to measure the leaf morphological, anatomical and physiological characteristics. The leaves used for the physiological characteristics were immediately stored in liquid nitrogen after collection.

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Measurements of leaf morphological and anatomical parameters

The leaf length (LL), leaf width (LW), leaf area (LA) and petiole length (PL) were measured using a scanner (CanoScan LiDE 700F, Canon) and LA-S plant image analysis software. The leaf index (LI) was calculated from the blade length/blade width ratio.

We used the paraffin section method to observe the leaf lamina anatomy. The blade was cut transversely at its widest part (Slicer:RM2135, Leica Instruments GmbH). The material that retained the primary vein and leaf margin was selected and fixed in a formalin-acetic acid-alcohol (FAA) solution. Tissue sections were prepared as 8-μm-thick paraffin sections, double-stained with sarranine-fast green, and mounted with neutral resin. The epidermal cell number (ECN), epidermal cell length (ECL), epidermal cell width (ECW), palisade tissue thickness (PTT), palisade tissue cell number (PTCN), palisade tissue cell length (PTCL), palisade tissue cell width (PTCW) and spongy tissue thickness (STT) were determined. The palisade tissue/sponge tissue ratio (PSR) was calculated. Five fields of view were observed for each leaf, and 20 values were obtained for each field of view (Digital stereo microscope, SMZ1500, Nikon). The average values for the leaf structural parameters in five fields of view were collected as the anatomical parameters of each leaf.

Determination of carbohydrate and soluble protein contents

The content of soluble sugar was determined by the anthrone-sulfuric acid method as described previously, starch content was determined by the anthrone-colorimetric method, and soluble protein content was determined by the Coomassie blue G-250 method (Juan et al., 2015; Song et al., 2017; Zhou et al., 2014).

Determination of endogenous hormone content changes

The stem tips of the same layer of branches in the sampled tree canopy were mixed as test samples. Test samples were accurately weighed to 200 mg, and the contents of indoleacetic acid (IAA), zeatinriboside (ZR), gibberellin (GA_3) and abscisic acid (ABA) were determined by enzyme-linked immunoassay as described previously (Duan et al., 2009; Li et al., 2019).

Statistical analysis

One-way ANOVA was used to compare the differences in the morphological, anatomical and physiological characteristics of the heteromorphic leaves. In SPSS 20.0, Duncan's new multiple range method was used for data analysis of variance, and the significance level was 0.05.

Results

Morphological characteristics of heteromorphic leaves change with development stage and tree height

As shown in *Figure 1,* with the increase in diameter and crown level, the leaf length and leaf shape index showed a decreasing trend, while the leaf width, leaf thickness, petiole length and leaf area showed an increasing trend. The results showed that the morphology of the heteromorphic leaves varied with changes in diameter and canopy

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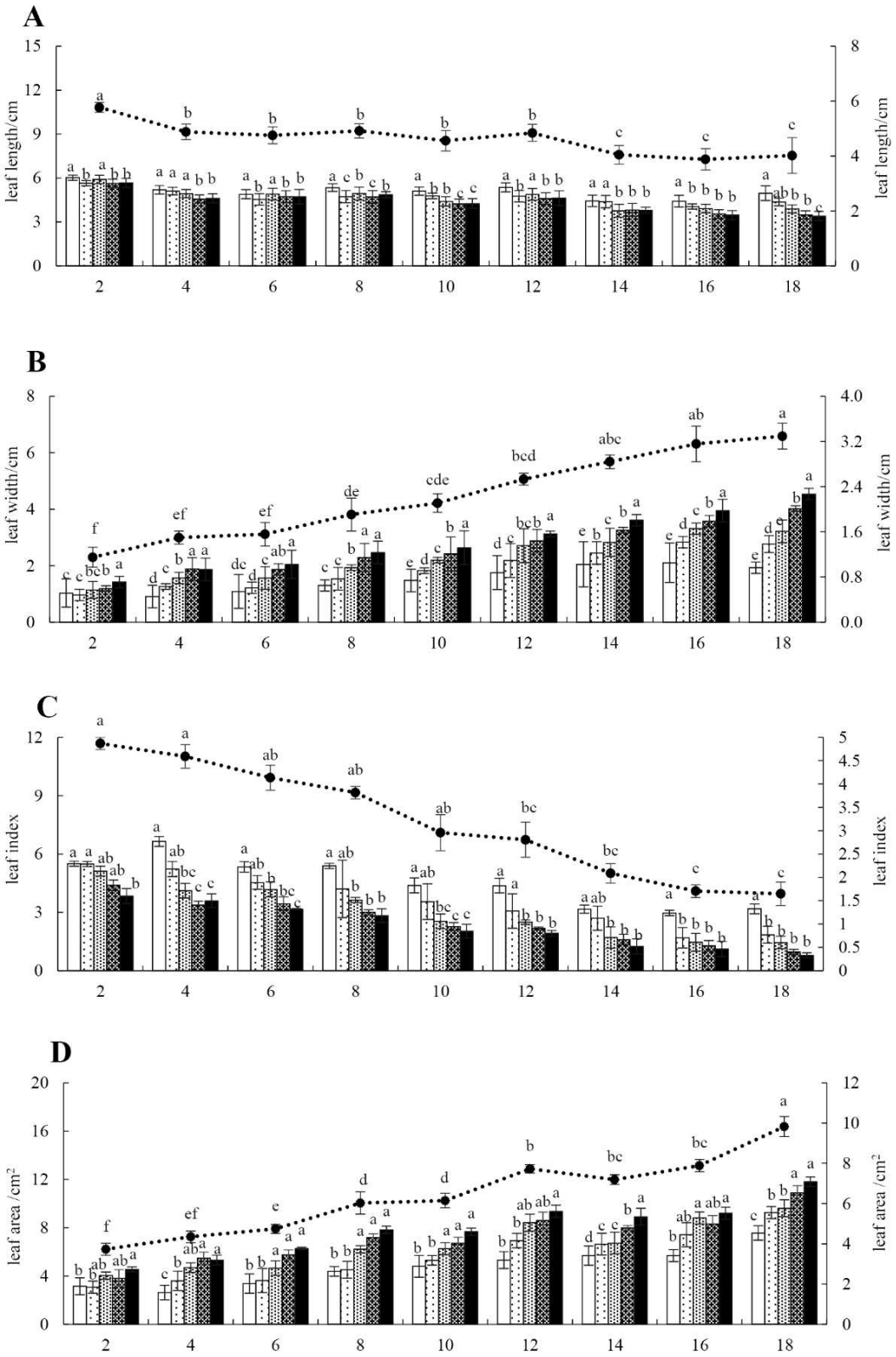
level. The heteromorphic leaves were distributed in the first to fifth layers of the crown of each diameter of *P. euphratica*, the leaf area was obviously larger, the leaves were thicker and the petioles became longer.



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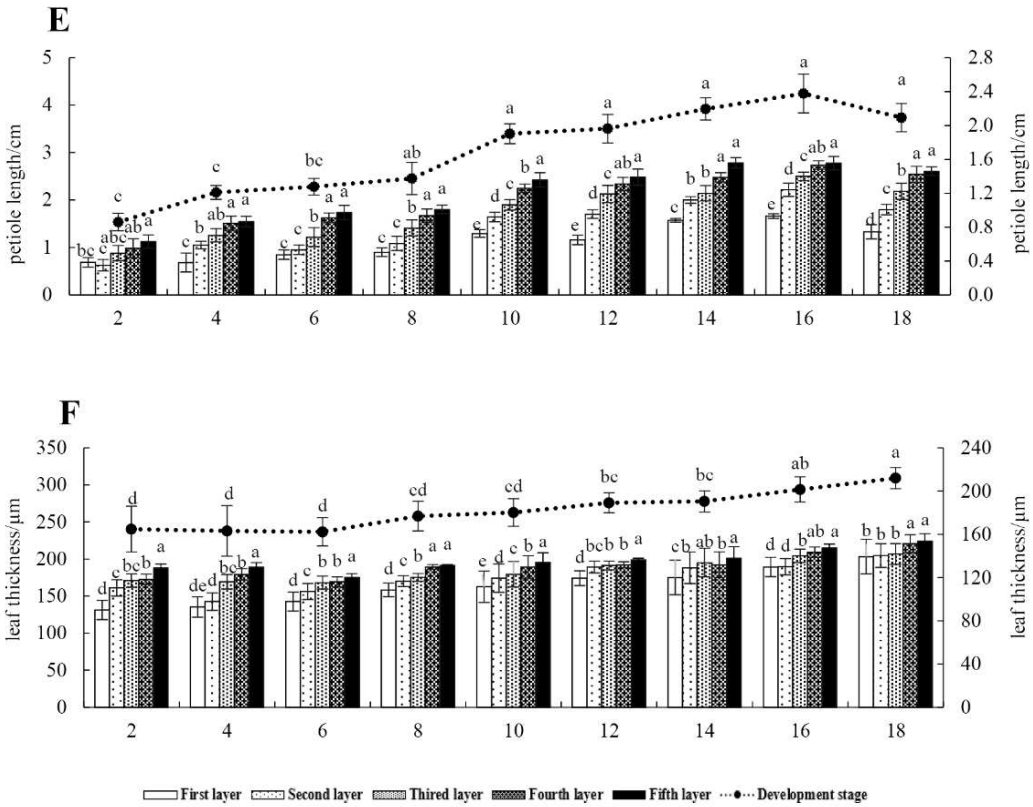

Figure 1. *Variations of heteromorphic leaf morphology with developmental stage and tree height. (A) leaf length/cm,(B) leaf width/cm,(C) leaf index,(D) leaf area/cm^2,(E) petiole length/cm,(F) leaf thickness/μm. Lowercase letters represent significant differences, the same letters indicate that the differences are not significant, and different letters indicate significant differences. Same below*

Variation in anatomical structure of heteromorphic leaves with development stage and tree height

The epidermal structure of the widest transverse section of the heteromorphic leaves in each diameter class and crown level was observed, and the results showed that the number of epidermal cells and the width of epidermal cells significantly increased with the increase of diameter class and canopy levels, and the first layer of the crown in each diameter class was significantly smaller than the fifth layer ($P < 0.05$) (*Figs. 2* and *A1*), suggesting that the number of epidermal cells and the width of epidermal cells of *P. euphratica* heteromorphic leaves increase with increasing diameter class and canopy level.

The cell number, cell length and palisade tissue thickness in the cross section of *P. euphratica* heteromorphic leaves increased with increasing diameter class and canopy layer, and the spongy tissue thickness showed a decreasing trend. They all showed significant differences between the 2 diameter class and the 18 diameter class. ($P < 0.05$). The width of palisade tissue cells in the 12-18 diameter class increased with increasing canopy level, and the fifth layer of the canopy was significantly larger than the first layer ($P < 0.05$) (*Figs. 2* and *A1*), indicating that the anatomical structure of heteromorphic leaves changed significantly with the increase in diameter class and canopy levels. Specifically, the palisade tissues were increasingly developed, and the sponge tissues were increasingly degenerated.

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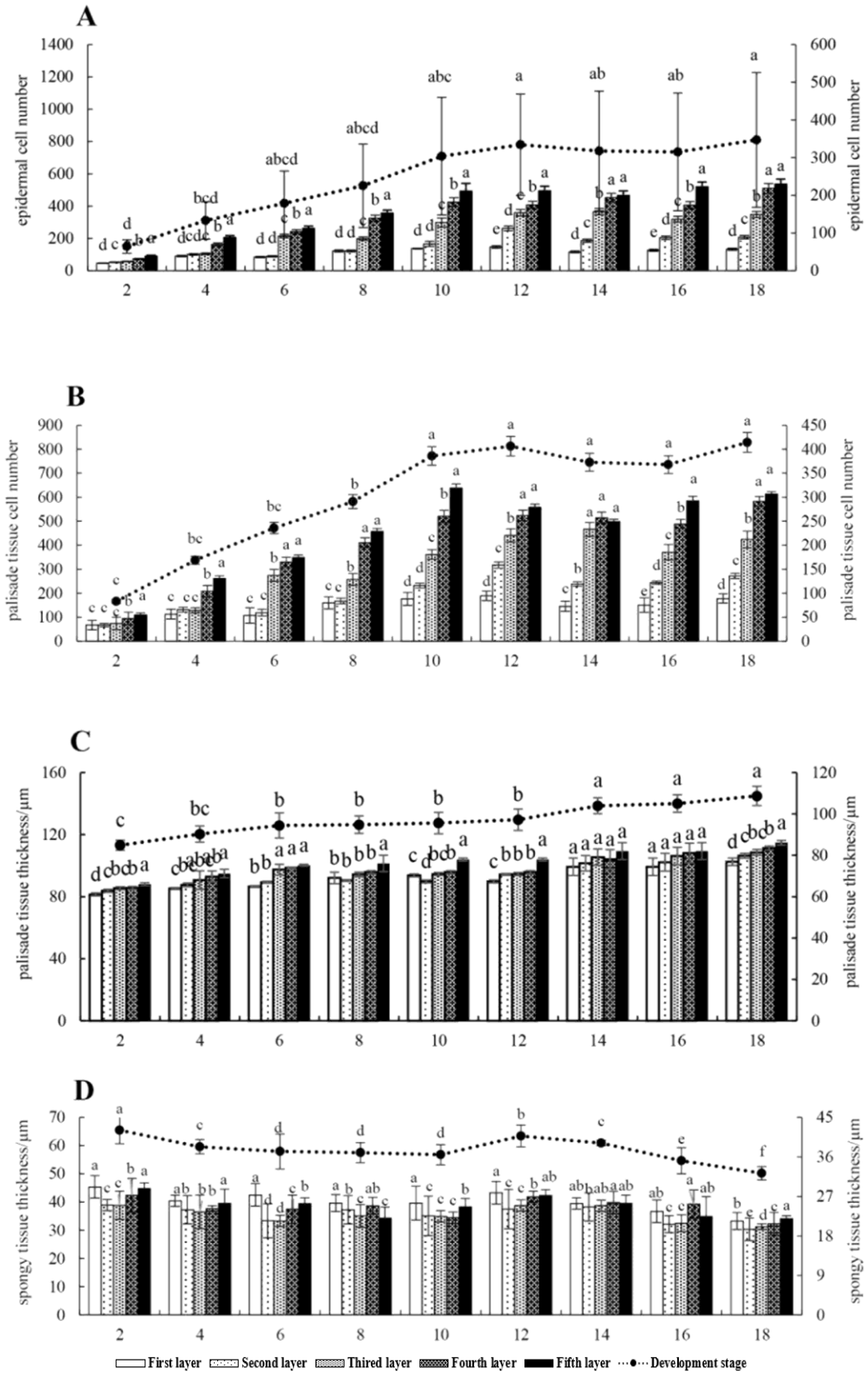

***Figure 2**. Variation in epidermal tissue structure of P. euphratica heteromorphic leaves with developmental stage and tree height. (A) epidermal cell number,(B) palisade tissue cell number,(C) palisade tissue thickness/μm,(D) spongy tissue thickness/μm*

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Variation in endogenous hormone content and ratio of heteromorphic leaves with development stage and tree height

Figure 3 shows, the levels of GA_3 and IAA demonstrated a decreasing trend with increasing diameter class. Among them, the levels of GA_3 in the fifth layer of the 2 diameter class crown were significantly higher than those in the other layers. The levels of IAA were higher in the first layer than in the fifth layer of the 10-18 diameter class. The levels of ZR increased from the 2 to 6 diameter classes. The level of ZR was not significantly different between the 2 diameter class and other diameter classes, and there was no significant difference among different levels of the crown of each diameter class. The levels of ABA gradually increased from the 2 to 6 diameter classes, with no obvious difference from the 8 to 18 diameter classes, and there were differences between the different levels of the crowns of the 10 and 14 diameter classes, indicating that the levels of 4 endogenous hormones in *P. euphratica* heteromorphic leaves showed different variations with increasing diameter class and crown level.

The hormone ratio of heteromorphic leaves was determined, and the results showed that IAA/ABA, GA_3/ABA, and ZR/ABA showed a decreasing trend with increasing diameter class, and IAA/ABA significantly decreased with increasing canopy levels in the 12-14 diameter class (*Fig. A2*). GA_3/ABA showed an increasing trend with increasing canopy level in the 2 diameter class, while the 14 diameter class showed a decreasing trend. ZR/ABA showed an increasing trend with the increase in the canopy layer in the 10 diameter class, and the 14 diameter class showed a decreasing trend. ZR/IAA gradually increased with increasing diameter class and increased with increasing canopy layers in the 4, 6, 12 and 18 diameter classes. There was a significant difference between the fifth or fourth layer and the first layer. GA_3/IAA showed a trend of first decreasing and then increasing with increasing diameter class, and it increased with the increase of crown level in the 2 diameter class. ZR/GA_3 increased first and then decreased with increasing diameter class, while it only showed a decreasing trend with increasing crown layer in the 2 diameter class, and there was significant difference between the fifth layer and the first layer. The results demonstrated that the hormone ratio of heteromorphic leaves also showed different variations with increasing diameter class and canopy level.

Variation in carbohydrate and soluble protein of heteromorphic leaves with developmental stage and tree height

Figure A3 shows, the contents of soluble sugar and starch decreased with increasing diameter class. Among them, the content of starch in the 10-18 diameter class increased with increasing crown layer. The first layer of the crown was significantly larger than the fourth and fifth layers. The soluble protein contents significantly decreased in the 2-6 diameter class, and there was no significant difference in the 8-18 diameter class, but in the 6-18 diameter class, the protein contents showed a decreasing trend with the increase in the canopy layer. The first layer of the canopy was significantly larger than the fifth layer. This suggests that the contents of soluble sugar, starch and soluble protein in heteromorphic leaves tended to be stable during the later stage of growth, and the contents of starch and soluble protein showed obvious differences in the vertical space of the crown.

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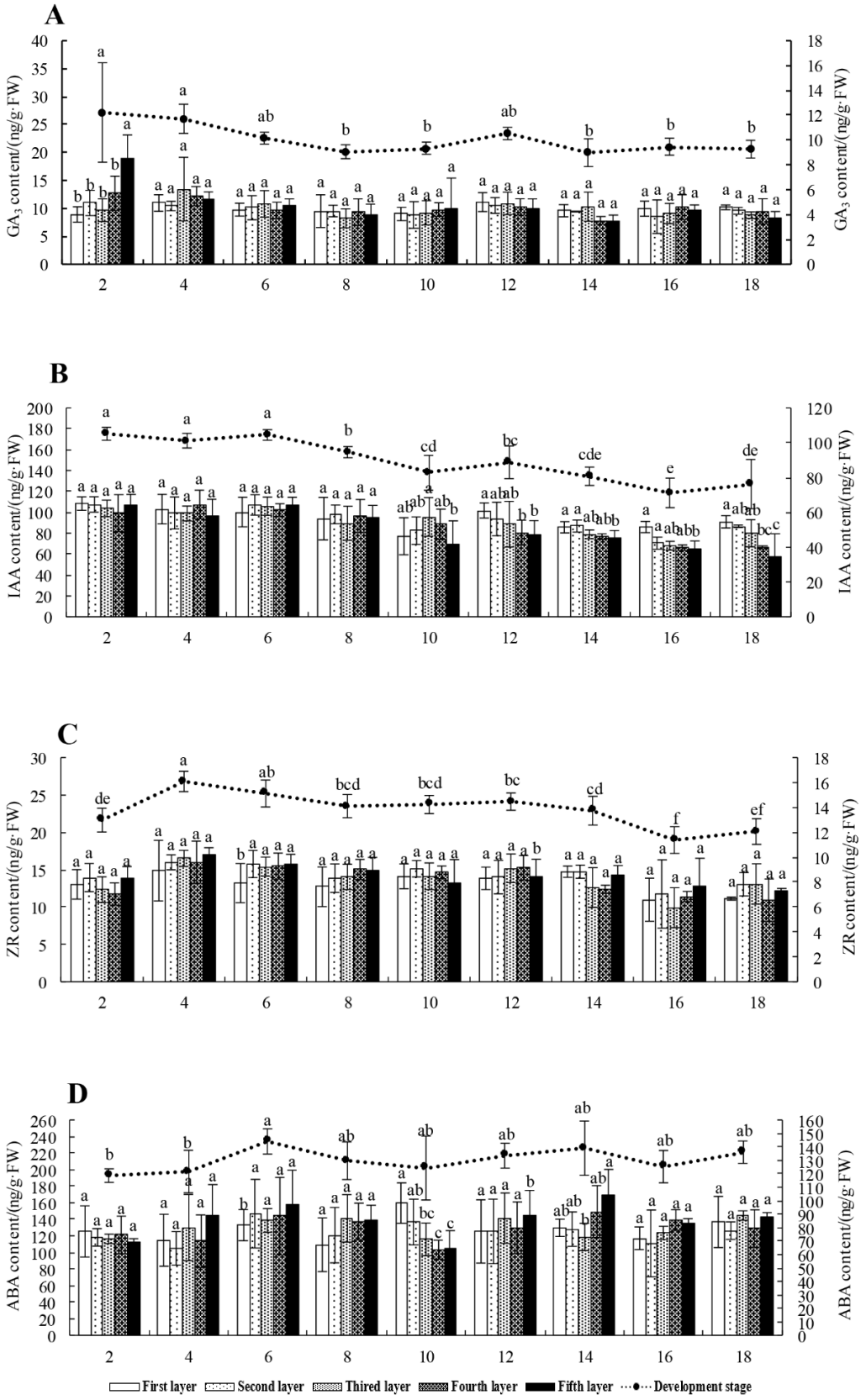

Figure 3. *Variation in the endogenous hormone content of heteromorphic leaves with developmental stage and tree height. (A) GA_3 content, (B) IAA content, (C) ZR content, (D) ABA content*

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Correlation analysis between morphological structure parameters and hormone content and ratio of heteromorphic leaves

Correlation analysis between Morphological structure parameters and hormone contents and ratios of heteromorphic leaves was conducted (*Table 1*). The results showed that the IAA content, IAA/ABA, ZR/ABA and GA_3/ABA were significantly negatively correlated with diameter class or crown height ($P < 0.01$). IAA content and IAA/ABA, GA_3/ABA, ZR/ABA, and ZR/IAA were significantly or extremely negatively correlated with petiole length, leaf width, leaf thickness and leaf area and were significantly or extremely positively correlated with leaf length and leaf shape index. In addition, the GA_3 content was significantly positively correlated with leaf length ($P < 0.01$), and the ZR content was significantly negatively correlated with leaf width and leaf thickness and significantly positively correlated with the leaf shape index ($P < 0.05$). The ABA content of leaves was only significantly positively correlated with leaf width and leaf area ($P < 0.01$)

GA_3, IAA and GA_3/ABA, IAA/ABA were significantly negatively correlated with palisade tissue cell length, palisade tissue thickness, palisade tissue cell number, and palisade tissue/sponge tissue ratio. IAA and IAA/ABA were also significantly negatively correlated with palisade tissue cell width and epidermal cell width, while GA_3 and IAA/ABA were significantly positively correlated with sponge tissue thickness ($P < 0.05$) The results demonstrated that the Morphological structure parameters of heteromorphic leaves was closely related to the hormone content and ratio with increasing diameter step and crown height. The relationship between Morphological structure parameters and hormone contents and ratios of heteromorphic leaves reflects the consistency of the morphological changes and physiological changes of *P. euphratica.*

Table 1. *Correlation analysis between Morphological structure parameters and hormone content and ratio of heteromorphic leaf*

	GA_3	IAA	ZR	ABA	IAA/ABA	GA_3/ABA	ZR/ABA	GA_3/IAA	ZR/IAA	ZR/GA_3
DC	-0.75*	-0.94**	-0.63	0.4	-0.97**	-0.73*	-0.77**	0.54	0.71*	0.04
CH	-0.88**	-0.88**	-0.47	0.52	-0.97**	-0.87**	-0.69*	0.32	0.76*	0.3
PL	-0.72*	-0.96**	-0.52	0.31	-0.93**	-0.68*	-0.63	0.58	0.83**	0.13
LL	0.77**	0.87**	0.42	-0.44	0.92**	0.76*	0.64*	-0.43	-0.76*	-0.22
LW	-0.70*	-0.94**	-0.64*	0.35	-0.95**	-0.68*	-0.75*	0.59	0.70*	-0.02
LI	0.75*	0.96**	0.64*	-0.36	0.97**	0.71*	0.76*	-0.57	-0.73*	-0.04
LA:	-0.68*	-0.87**	-0.59	0.38	-0.90**	-0.68*	-0.71*	0.52	0.65*	-0.01
LT	-0.63	-0.92**	-0.74*	0.23	-0.89**	-0.58	-0.77**	0.64*	0.6	-0.15
ECN	-0.79**	-0.88**	-0.39	0.43	-0.92**	-0.77**	-0.56	0.85**	0.4	0.29
ECL	-0.4	0.14	-0.21	0.55	-0.17	-0.51	-0.47	-0.41	-0.55	0.21
ECW	-0.45	-0.72*	-0.61	0.29	-0.75*	-0.48	-0.73*	0.43	0.59	-0.23
PTCN	-0.81**	-0.85**	-0.34	0.45	-0.90**	-0.79**	-0.52	0.85**	0.33	0.36
PTCL	-0.74*	-0.74*	-0.34	0.41	-0.80**	-0.73*	-0.51	0.72*	0.3	0.26
PTCW	-0.58	-0.95**	-0.56	0.09	-0.83**	-0.48	-0.56	0.79**	0.70*	0
PTT	-0.78**	-0.88**	-0.58	0.5	-0.97**	-0.79**	-0.79**	0.65*	0.44	0.1
STT	0.63*	0.6	0.41	-0.25	0.63*	0.59	0.53	-0.46	-0.26	-0.13
PSR	-0.72*	-0.78**	-0.57	0.38	-0.84**	-0.71*	-0.74*	0.54	0.4	0.05

*$P < 0.05$, **$P < 0.01$. PL: petiole length. LL: leaf length. LW: leaf width. LI: leaf index. LA: leaf area. LT: leaf thickness. DC: diameter class. CH: crown height. ECN: epidermal cell number. ECL: epidermal cell length. ECW: epidermal cell width. PTCL: palisade tissue cell length. PTCW: palisade tissue cell width. PTT: palisade tissue thickness, PTCN: palisade tissue cell number. STT: sponge tissue thickness. PSR: palisade tissue/sponge tissue ratio

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Correlation analysis of morphological structure parameters and physiological and biochemical parameters of heteromorphic leaf

Correlation analysis between the morphological structure parameters and carbohydrate and soluble protein levels of heteromorphic leaves was conducted (*Table 2*). The results showed that the contents of soluble sugar, starch and soluble protein were significantly or extremely negatively related to diameter at breast height, crown height, petiole length, leaf width and leaf area and significantly or extremely positively correlated with the leaf shape index. In addition, the starch content and soluble protein content were significantly or extremely positively correlated with leaf length.

The contents of soluble sugar, starch and soluble protein were significantly or extremely negatively correlated with epidermal cell number, epidermal cell length, and palisade tissue thickness. The contents of soluble sugar and starch were significantly or extremely negatively correlated with palisade tissue cell width. The starch content was significantly negatively correlated with the palisade tissue/sponge tissue ratio ($P < 0.05$). The results showed that the morphological structure of heteromorphic leaves was closely related to carbohydrate and soluble protein levels with increasing diameter steps and crown height.

Table 2. *Correlation analysis between morphological structure parameters and physiological and biochemical parameters of heteromorphic leaf*

	Soluble sugar	**Starch**	**Soluble protein**
DC	-0.79**	-0.87**	-0.72*
CH	-0.81**	-0.96**	-0.87**
PL	-0.88**	-0.88**	-0.74*
LL	0.62	0.82**	0.75*
LW	-0.78**	-0.85**	-0.67*
LI	0.83**	0.85**	0.70*
LA:	-0.77**	-0.84**	-0.68*
LT	-0.75*	-0.76*	-0.55
ECN	-0.93**	-0.95**	-0.85**
ECL	0.19	-0.11	-0.26
ECW	-0.33	-0.47	-0.37
PTCN	-0.92**	-0.96**	-0.87**
PTCL	-0.65*	-0.82**	-0.79**
PTCW	-0.84**	-0.73*	-0.54
PTT	-0.64*	-0.83**	-0.72*
STT	0.26	0.53	0.54
PSR	-0.46	-0.69*	-0.63

*P < 0.05, **P < 0.01. PL: petiole length. LL: leaf length. LW: leaf width. LI: leaf index. LA: leaf area. LT: leaf thickness. DC: diameter class. CH: crown height. ECN: epidermal cell number. ECL: epidermal cell length. ECW: epidermal cell width. PTCL: palisade tissue cell length. PTCW: palisade tissue cell width. PTT: palisade tissue thickness, PTCN: palisade tissue cell number. STT: sponge tissue thickness. PSR: palisade tissue/sponge tissue ratio

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Discussion

P. euphratica heteromorphic leaf morphological structure change and ecological adaptation

Plants have the ability to change their shape according to environmental conditions, and this phenomenon is called phenotypic plasticity (Alpert et al., 2002; Zotz et al., 2011). Phenotypic plasticity manifests as changes in leaf morphology in response to environmental conditions, such as light intensity and quality, environmental temperature, and water utilization (Hokuto et al., 2017). Studies have shown that *P. euphratica* exhibits different leaf shapes at different developmental stages or different leaf shapes at different levels of the same plant and at different leaf nodes on the same branch, and they all demonstrate typical heteromorphic leaf characteristics related to individual developmental stages. To adapt to the arid environment, plants in desert areas try their best to change their own morphological characteristics, such as reduced leaf area or leaf degradation to assimilated branches, stomatal sinking, and leaf thickening, to resist the adverse effects of drought stress on plants. A previous study found that compared with lanceolate leaves, the xerophytic structure of broad-ovate leaves was more obvious, characterized by a thicker stratum corneum, a denser arrangement of mesophyll cells, developed palisade tissue, and more mucus cells among mesophyll cells (Yang et al., 2005). Broad-ovate leaves with a larger leaf area on the same adult plant had a more developed xerophytic structure than ovate and lanceolate leaves with a smaller leaf area (Ding et al., 2010; Li et al., 2005; Yang et al., 2005; Wang et al., 1997). The anatomical structure of heteromorphic leaves changes with increasing breast diameter at different developmental stages of *P. euphratica* (Zhao et al., 2016).

Our study showed that the morphological characteristics and anatomical structure of heteromorphic leaves changed regularly with increasing diameter class, such as larger leaf areas, longer petioles and thicker leaves. The anatomical structure of heteromorphic leaves coordinated with development in the direction of more developed palisade tissue, more degraded sponge tissue, and a larger palisade tissue/sponge tissue ratio, which was consistent with previous studies. A longer petiole is more conducive to the movement of the petiole, which receives more light to improve the photosynthetic efficiency. Thicker leaves have a better ability to store water and resist drought than thinner leaves. In addition, Levitt (Levitt et al., 1980) believed that the palisade tissue/sponge tissue ratio (PSR) was one of the important indicators to evaluate drought resistance (Zhang et al., 2017). The PSR was greater than 1 at different diameter classes, indicating that the drought resistance of *P. euphratica* was enhanced with increasing diameter class and crown height. The synergetic changes between leaf morphology and anatomical structure of heterotypic leaves not only met the energy needs of individual development, but also gradually enhanced the stress resistance of *P. euphratica* and improved the adaptability of the species to the growth environment.

The role of endogenous hormones in the morphological and structural changes of heteromorphic leaves

More recent studies have also revealed that plant hormones, including abscisic acid (ABA), gibberellin (GA), auxins (IAA) and zeatin-riboside (ZR), can affect heteromorphic leaf formation in many plant species (Hokuto et al., 2017; Li et al., 2019). For example, GA is thought to be a key factor in the regulation of heterophylly in *Rorippa aquatica* (Nakayama et al., 2014), and GA can reduce leaf complexity in *S.*

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lycopersicum (Yanai et al., 2011) and induce cell length growth of hydrophytes (Rijnders et al., 1997). Several studies have demonstrated that auxins affect leaf morphology and development (Barkoulas et al., 2008), regulate the cell wall structure, promote stem cell elongation, increase cell volume and affect the formation of veins and vascular tissue (Donner et al., 2009; Avsian et al., 2002). ABA can induce the formation of heterophylly in *P. octandrus* (Kuwabara et al., 2003). ZR can promote cell division of stems and leaves and is beneficial to the formation of leaves (Debnath et al., 2009).

Macroscopically, the morphology of the heteromorphic leaves of *P. euphratica* changed with the ontogenetic developmental stage—with the increase in tree age, heteromorphic leaves with a smaller leaf index and larger leaf area gradually appeared on the plant (Huang et al., 2010a, b; Li et al., 2017, 2015; Zhao et al., 2016). Our research shows that the 4 hormone contents and ratios of heteromorphic leaves varied with increasing diameter class and crown levels, they have different roles in the morphological and structural changes of *P. euphratica*, in terms of morphological changes of heteromorphic leaves, IAA, GA3, IAA/ABA, GA3/ABA and the ZR/ABA ratio were significantly negatively correlated with petiole length, leaf width and leaf area and positively correlated with leaf length and the leaf index. The ZR/IAA ratios showed significant positive correlations with leaf width, leaf area and petiole length and extremely significant negative correlations with leaf length and the leaf index. The contents of IAA, ZR and IAA/ABA decreased with increasing leaf thickness, and the content of ZR decreased with increasing leaf width. In terms of anatomical structure changes of heteromorphic leaves, GA_3, IAA, GA_3/ABA, and IAA/ABA have a significant or extremely significant negative correlation with the palisade tissue cell number, epidermal cell number, palisade tissue cell length, palisade tissue thickness and palisade tissue/sponge tissue ratio. GA_3 and IAA/ABA were significantly positively correlated with sponge tissue thickness, and ZR/IAA was significantly positively correlated with epidermal cell number, palisade tissue cell number, palisade tissue cell width and palisade tissue thickness. The changes in the number of epidermal cells, palisade tissue thickness, palisade tissue cell number, palisade tissue/sponge tissue ratio and sponge tissue thickness represented the drought resistance of plants (Zhang et al., 2017; Li et al., 2017). For example, as the DBH and height gradient of the profiled leaves increase, palisade tissue thickness, sponge tissue thickness, and cuticle thickness also become thicker, indicating that the leaf anatomical structure has become more xerophyte (Zhai et al., 2019). ABA of *P. euphratica* heteromorphic leaf has long been considered a plant stress hormone and is considered a signaling molecule for drought stress in plants. ABA induction can increase a plant's ability to resist drought stress, for example, by increasing waxy deposits on the cuticle (Jana et al., 2013; Cui et al., 2016), But in our research, leaf ABA was not related to changes in the morphological structure of heteromorphic leaf, but the ratio of it to the hormones GA3, IAA, and ZR participates in the changes in the morphological structure of heteromorphic leaf. According to the analysis, the content of GA_3, IAA, ZR and ABA and the ratio of IAA/ABA GA_3/ABA, ZR/ABA, and ZR/IAA of *P. euphratica* heteromorphic leaf play a role in petiole length, leaf width, leaf area, leaf thickness, leaf length and the leaf index changes, *P. euphratica* regulates the drought resistance of *P. euphratica* at different developmental stages through the synergistic changes of the content and ratio of GA_3, IAA, ZR and ABA in the heteromorphic leaves with the number of epidermal cells, palisade tissue thickness, sponge tissue thickness and other anatomical structures to adapt to the drought environment.

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The role of soluble sugar, starch and soluble protein in the changes of morphological structure of heteromorphic leaves

Plants under adverse stress can maintain their osmotic balance to address damage by accumulating various osmotic regulatory substances that have a regulatory role in the growth, development, maturation and senescence of plants (Shen et al., 2016). Starch, as a nutritive polysaccharide, is hydrolyzed into soluble sugar under the action of amylase. The soluble sugar content reflects the supply basis of available substances and energy in plants (Jiang et al., 2011). Studies have shown that the contents of carbohydrates and soluble proteins change regularly to adapt to environmental changes with changes in leaf morphology and ontogenetic stage (Yue, 2009). A study on the developmental process of heteromorphic leaves in *P. euphratica* found that the content of soluble sugar was always higher than that of starch, except at the initial stage of leaf development, which may be an adaptation to the arid environment (Xu et al., 2007).

Other studies on the relationship between the leaf carbohydrate and soluble protein levels and leaf morphology of *P. euphratica* at different developmental stages showed that the changes in leaf morphology were closely related to the soluble sugar and soluble protein contents (Li et al., 2015). Correlation analysis results show that the contents of soluble sugar and starch in heteromorphic leaves decreased with increasing leaf width and decreasing leaf index, while the content of soluble protein decreased with decreasing leaf index and leaf length. Meanwhile, the contents of carbohydrates and soluble protein decreased with increasing palisade tissue cell number, epidermal cell number, palisade tissue cell length and palisade tissue thickness. We speculate that the metabolism of soluble sugar and soluble protein may play a regulatory role in changes in leaf length and width, as well as changes in the number of palisade tissue cells, cell length and palisade tissue thickness. In summary, *P. euphratica* regulated drought resistance during different ontogenetic stages through synergistic changes between soluble sugar and soluble protein levels and the morphological structures of heteromorphic leaves.

Conclusions

P. euphratica exhibited different heteromorphic leaves in different developmental stages and canopy heights. The xerophyte structural characteristics of heteromorphic leaves were obviously increased during the process. The close relationship between the 4 endogenous hormones and ratios, carbohydrate and soluble protein contents of heteromorphic leaves and the morphological characteristics and anatomical structure of heteromorphic leaves reflected the consistency of the morphological changes and physiological changes of *P. euphratica*. *P. euphratica* forms an ecological adaptation strategy that can cope with environmental pressure through the coordinated changes of its leaf morphology, anatomy and physiological characteristics, which provides a theoretical basis for further research on the molecular mechanism of how leaf morphology, anatomy and physiological characteristics can resist extreme environments through coordinated changes.

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APPENDIX

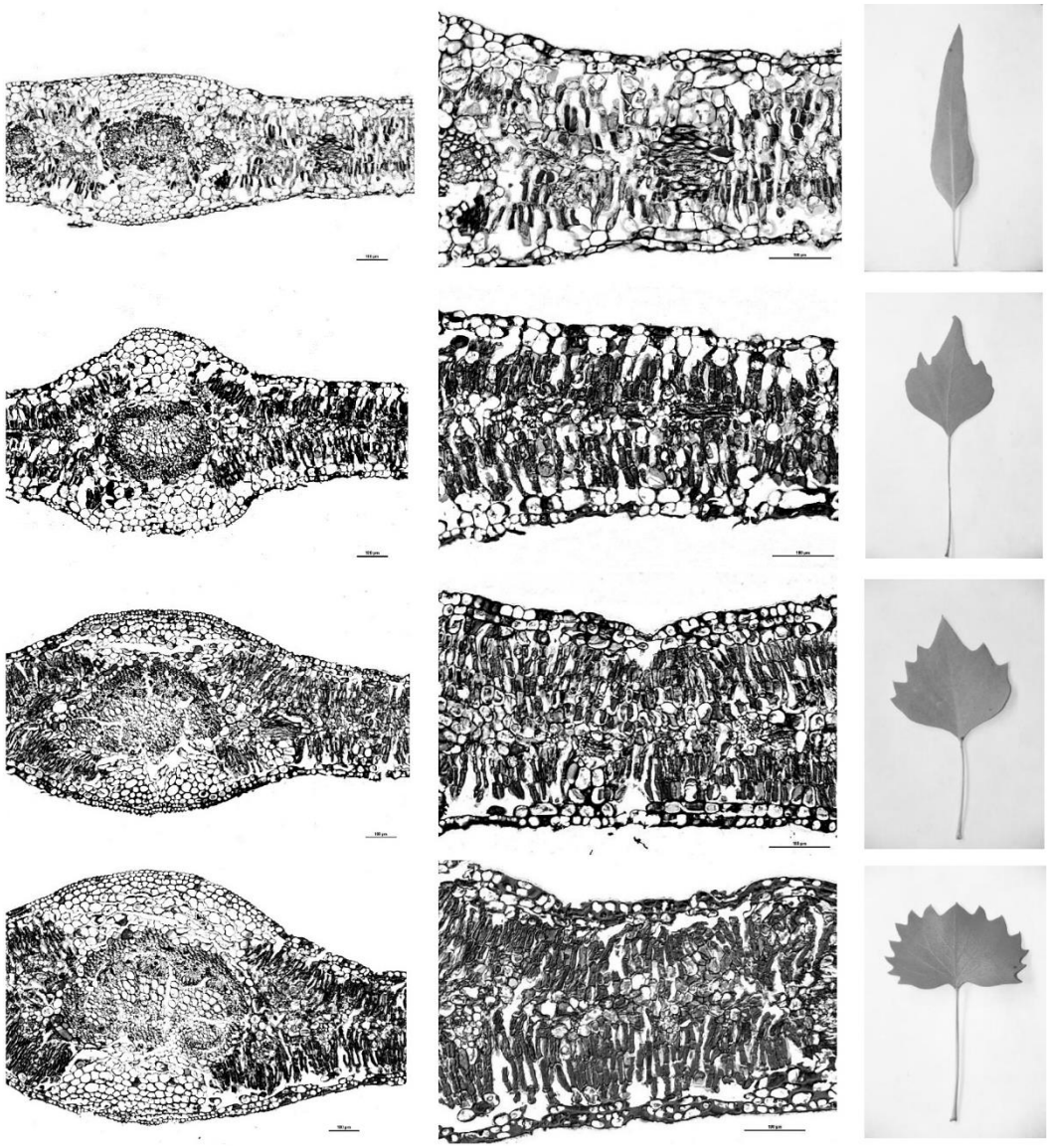

Plate 1. *Anatomical structure and epidermal stomata of Populus euphratica in strip, lanceolate, ovate, broad ovate leaves. From top to bottom are the first to fifth layers of the crown of Populus euphratica heteromorphic leaf D stage, corresponding to the anatomical structures of striped leaves, lanceolate leaves, ovate leaves, and broad-ovate leaves; The magnification and leaf shape photos are respectively 10X10 times, 10X20 times, and leaf shape photos*

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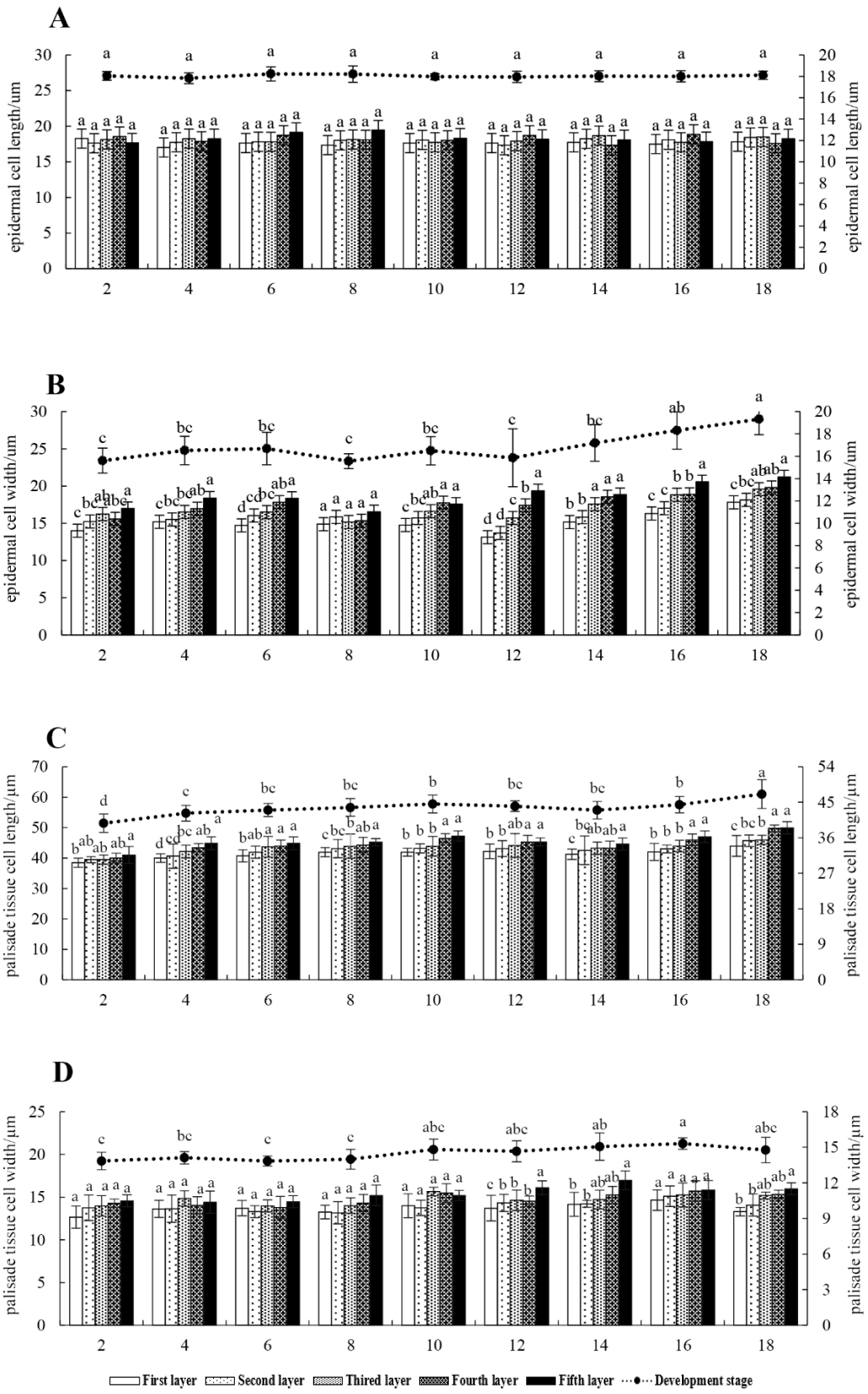

Figure A1. *Variation in heteromorphic leaf anatomical structure with development stage and tree height. (A) epidermal cell length/μm, (B) epidermal cell width/μm, (C) palisade tissue cell length/μm, (D) palisade tissue cell width/μm*

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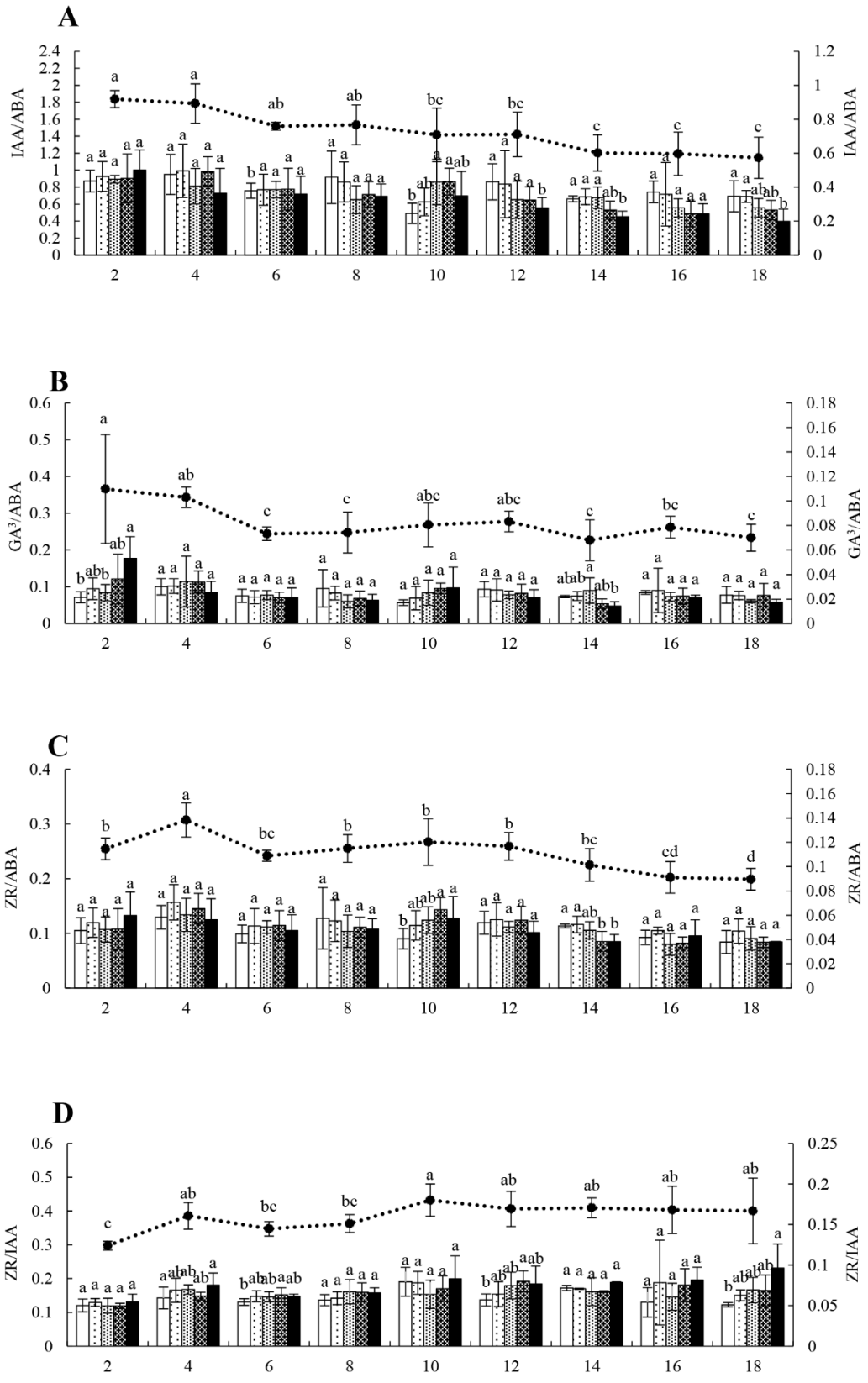

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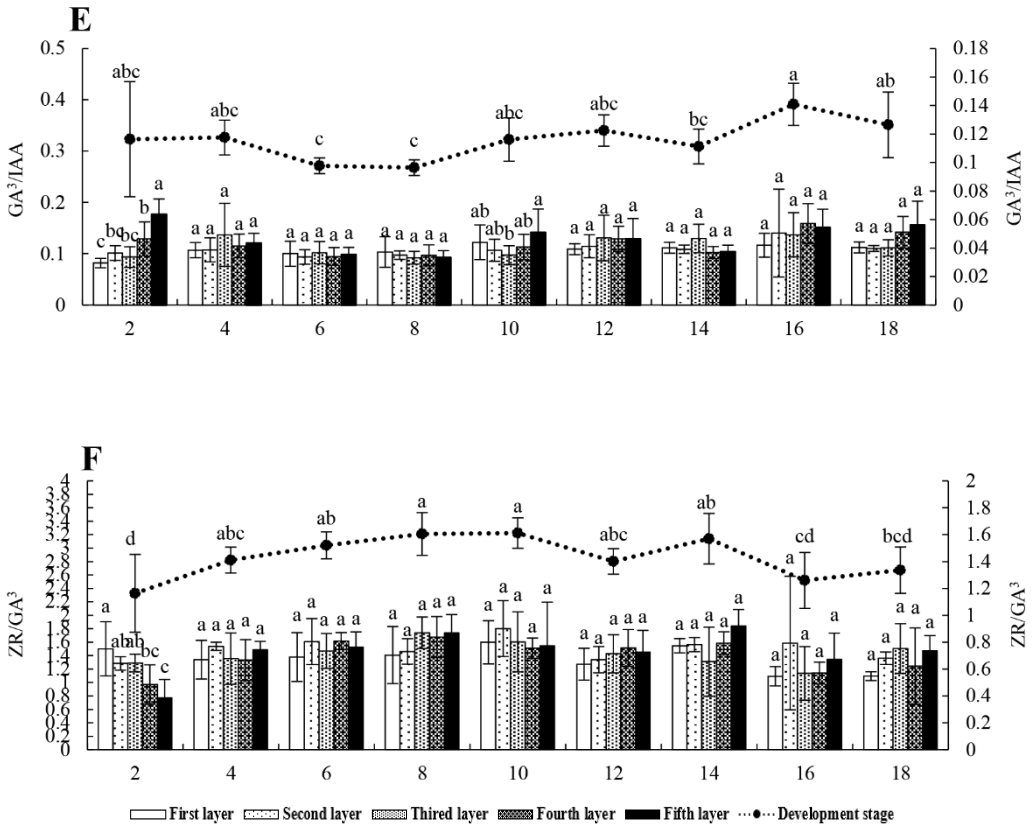

Figure A2. *Variation in the endogenous hormone content ratio of heteromorphic leaves with developmental stage and tree height. (A) IAA/ABA, (B) GA_3/ABA, (C) ZR/ABA, (D) ZR/IAA (E) GA_3/IAA, (F) ZR/GA_3*

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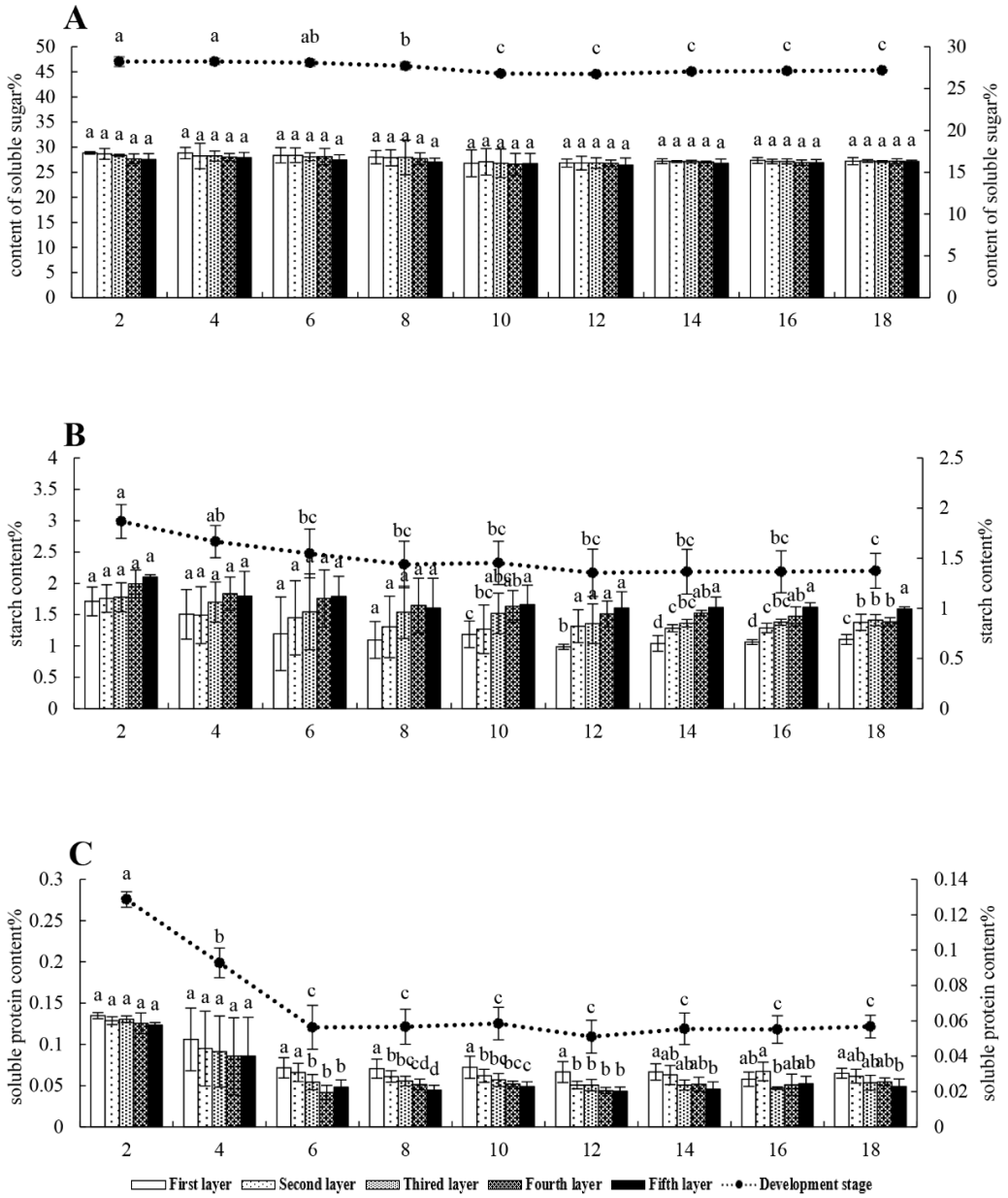

Figure A3. *Variation in the carbohydrate and soluble protein of heteromorphic leaves with developmental stage and tree height. (A) content of soluble sugar%, (B) starch content%, (C) soluble protein content%*

Table A1. *Basic information of P. euphratica*

Diameter classes	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**
Breast diameter range	0-2cm	2-4cm	4-6cm	6-8cm	8-10cm	10-12cm	12-14cm	14-16cm	16-18cm
Number of plants	20	78	67	59	48	43	29	8	3
Mean breast diameter (cm)	2.3	3.9	5.9	7.9	9.8	12	14.1	15.7	17.4
Average tree height (m)	6.3	8.8	8	8.1	8.7	9.7	10	9.6	11.1
Average crown height (m)	2.4	3.9	5.7	6.8	7.2	7.5	8.4	8.6	9.4
Average tree age (year)	4.1	5.2	6.6	7.6	8.6	10.3	10.5	11.3	11.6
Number of samples (pcs)	9	31	23	21	20	19	11	4	3

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THE TOLERANCE OF AN EXTENSIVE COLLECTION OF GARLIC (*ALLIUM SATIVUM* L.) GERMPLASMS TO SALT STRESS – A SUSTAINABLE SOLUTION TO SALT STRESS

AHMAD, J.[1] – WANG, H.[1#] – SONG, J.[1] – NUERAWUTI, M.[1] – ZHANG, X.[1] – YANG, W.[1] – MA, L.[2] – LI, X. X.[1*]

[1]*Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences; Key Laboratory of Biology and Genetics Improvement of Horticultural Crops, Ministry of Agriculture, Beijing 100081, China*
(e-mails: jalil.ahmad1300@gmail.com – J. Ahmad, wanghaiping@caas.cn – H. Wang, songjianping@caas.cn – J. Song, 2780812743@qq.com – M. Nuerawuti, zhangxiaohui01@caas.cn – X. Zhang, wenlongyang@caas.cn – W. Yang)

[2]*Shandong Engineering and Technology Research Center for Garlic, Shandong Jining, 272200, China*
(e-mail: 52569370@qq.com- L.Ma)

[#]*Co-first author*

*****Corresponding author*
e-mail: lixixiang@caas.cn

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Abstract. Salinity is a serious problem that limits growth and yield of many agricultural crops worldwide. There is a need for a sustainable long -term solution to this problem. In this study, 354 diverse garlic germplasms were evaluated for their salt -tolerance at the seedling stage by being exposed to NaCl (0.25 mol/L) stress. The salt injury index (SII) along with several plant growth parameters were investigated. The results showed a wide variation of SII from 16.51 to 98.15 among these accessions. All accessions were classified into five groups according to their SIIs, corresponding to different grades of the tolerance to salinity. Among them, two highly tolerant and twenty-four salt-tolerant accessions were screened out. The response of distincit germplasms to salt stress was somewhat different in various trait indices. Moreover, an extremely significantly negative correlation was observed between SII and agronomic traits (PH, LL, LW and RHL) and physiological traits (gs, A, E, Ch). This study provides sustainable solution, original salt-tolerance evaluation technology and valuable materials for the garlic salt-tolerant genetic improvement.
Keywords: *Allium sativum L., NaCl stress, seedling growth, salt injury index, clustering analysis, correlation analysis*

Introduction

Various plants show variable responses to different environmental stresses including abiotic and biotic stresses. Abiotic stresses are a result of non-living components of the ecosystem, such as temperature, drought, waterlogging, salinity, nutrient deficiency, gaseous pollutions, metal toxicity, and so on. Of these, salinity stress is a severe threat to agriculture and food security as one-third of the irrigated land on the earth is affected by salinity (Machado and Serralheiro, 2017). The saline or sodic soils comprise about 6% of the total land all over the world (Munns, 2002). Salinity stress highly declines the crop growth and yield of many field crops (Teshika et al., 2019) and some vegetables, such as garlic, pea, okra, tomato, eggplant, peppers, carrot, cauliflower and potato (Francois, 1994; Shahbaz et al., 2012; Tanveer et al., 2020). Secondary salinization is

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becoming more and more serious in the continuous cropping protected vegetable production (Shahbaz et al., 2012).

Secondary soil salinization is typically caused by an imbalance between transpiration and water inputs from rainfall and irrigation. This imbalance comes in combination with soil characteristics that impede leaching (Cocks, 2001; Mateo-Sagasta and Burke, 2011). Wrong irrigation practices (e.g., waterlogging) and misplanning (e.g., temporal over irrigation) are major drivers of soil salinization (Cocks, 2001; Wichelns and Qadir, 2015). Other factors include the use of unlined canals and reservoirs, and vegetation clearing, in combination with inadequate drainage that filters salts into the groundwaters (Ritzema, 2016), from where the dissolved salts can be remobilized to the upper layers of the soil by means of upward water flows during dry periods (Crescimanno and Garofalo, 2006; Bhutta and Smedema, 2007). The quality of the irrigation water and rational application of fertilizer are also very important to avoid secondary salinization.

Salinity excess affects plant growth through many aspects of physiological and biochemical processes in the plant. Saline stress results in osmotic pressure and ionic stress, which impair the pivotal cellular function. Osmotic stress minimizes the water availability, causes dehydration and stomatal closure and slows down the rate of biochemical reactions (Acosta et al., 2017; Munns, 2002). Salinity stress alters some of the physiological process of plants, such as respiration rate, mineral distribution, membrane stability and turgor pressure (Hasegawa, 2013; Garrote et al., 2015). Shoot growth and early flowering are also influenced by salinity due to the inhibitory effect of salt in growing points on cell division and cell enlargement (Alom et al., 2016).

Plants withstand the salt stress by several mechanisms including high complexity or low complexity mechanisms. The former one is thought to be involved in alterations of many biochemical pathways. The later one is believed to induce coordination for the preservation of complex processes (Nasri et al., 2015). The high-complexity mechanism protects the main processes such as respiration and photosynthesis and retains important features such as plasma membrane interactions, cytoskeleton, cell wall (Gupta and Huang, 2014) and chromatin structural changes such as polyploidization, DNA methylation, DNA removal or amplification of unique sequences (Walbot and Cullis, 1985). Anyway, the genetic mechanism may be the major controller behind the different mechanisms.

Economic reasons or scarcity of freshwater are the major limitations to the soil salinization recovery in many situations. The only possible feasibility is the development of salinity tolerant varieties (Hoque et al., 2015), which could be developed through the germplasm selection and genetic improvement. Still, the success is related to the extent of genetic variation of tolerance to salinity among available germplasm for a crop species. Simultaneously the complexity of traits, limited knowledge about physiology and genetics of tolerance related attributes, and shortage of efficient selection domain are the major constrains to salinity tolerance breeding programs. What is more, optimizing saline conditions in the field and greenhouse for identification could be temporary and expensive.

Genetic characterization of useful germplasm is the first step towards releasing tolerant cultivars. Many researchers have demonstrated that evaluating the salt tolerance at the vegetative stage of a plant species is important to determine the ultimate tolerance of the species (Aslam et al., 1993; Uddin and Hossain, 2018; Kakar et al., 2019; Sikder et al., 2020). The early vegetative stage of a crop is regarded as the most dangerous stage, where plant yield is determined by (Uddin and Hossain, 2018) reported that

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selection of a crop is noted to be important at the vegetative stage for at least two reasons. Firstly, under controlled conditions in limited space, vegetative growth rates can be calculated easily and relatively in a short time (4-6 weeks). Secondly, under saline conditions, rapid vegetative development reflects a plant response to the stress environment and its capacity to produce additional resources for growth. The difference in salinity tolerance at vegetative stage has been reported in vegetables crops (Blum, 2018). Furthermore, a more significant yield loss has been reported when plants were exposed to salinity at the early growth stage than the divulgence at a later part of growing (Machado and Serralheiro, 2017).

A few researches have been done on salt tolerance of garlic. Francois (1994) reported that yield components (bulb weight and diameter) were reduced with increasing salinity, as well as the percent of solids which is a major component of bulb quality. Shoot dry weight was less susceptible to salinity stress than bulb weight, but higher concentration of chlorine, sodium and calcium accumulated in leaf tissues than in bulb by a small-scale experiment. Saline water irrigation significantly decreased the number of leaves and plant height of garlic (Shama et al., 2016).

Therefore, the main objective of this study was to evaluate the salt-tolerance of garlic germplasm at the seedling stage based on agronomic characteristics in a pot culture system on a large scale for the first time and understand the genetic variation of salinity tolerance and the relationship between the salt-tolerance and the agronomic/physiological characteristics for garlic genetic improvement of salt-tolerance and effective utilization of salt-tolerant garlic varieties in saline soil exploitation.

Materials and methods

The garlic germplasm of 354 accessions collected from in 15 province of China and 30 other countries were conserved in the national field gene bank of vegetative propagation vegetables on Lang fang farm of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences in the suburb of Beijing, China. The experiment was conducted in a solar greenhouse using a pot culture method during early Spring and were arranged according to a completely randomized block design for each accession under stress and control with three replicates. Ten garlic cloves for each replicate were sowed in plastic pots (8 × 8 cm) containing peat and vermiculite (1:1). Hoagland's solution was used as a source of nutrient. Each pot with garlic cloves was irrigated with irrigation water in the first week and with the 1/2 fold Hoagland's solution prepared with irrigation water during the second week and the third week after sowing once a week. Each pot for salt stress was subjected to 1.5% concentration of NaCl (0.25 mol/L) prepared with irrigation water starting from the beginning of the fourth week after sowing, continuously four weeks, once in each week. In contrast to salt treatment, each pot under the control was irrigated with irrigation water at the same frequency.

Investigation of salt injury grades and calculation of salt injury index

Visual salt injury grades for each plant in different accessions was determined after one week after finishing the salt treatment according to the following standard we modified based on the method by IRRI (2014) (*Table 1*).

Eight plants per replicate for each accession were scored. Salt injury index (SII) were calculated by the following formula (*Eq. 1*):

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$$SII = \frac{\sum(s \times n)}{N \times s} \times 100 \quad \text{(Eq.1)}$$

where: ∑ = The sum of the product of each grade value and the number of plants at each grade; s = the value of each grade; n = the number of plants at each grade; N = the total number of plants investigated; S = The highest value of injury grade.

Salt tolerance was classified according to clustering analysis result based on the average SII value of three replicates for each accession.

Table 1. *Modified standard of visual salt injury of each plant at the seedling stage*

Grade	Observation of visual salt injury (VSI)
1	Plant grows normally, and only the leaf tips of 2 leaves, or less than 2 leaves of a plant are yellowing
3	Plant grows basically normal, and the leaf tips of 3~ 4 leaves are yellowing or curly slightly
5	Plant growth is retarded partly; 5 ~ 6 leaves yellowing or curly
7	Plant growth is severely retarded or even ceases; 6 ~ 7 leaves of a plant are yellowing or becoming dry
9	More than 7 leaves are yellowing and severe, the whole plants are dead or dying

Measurement of agronomic traits

Plant height (PH), leaf length (LL), leaf width (LW), total leaves number and healthy leaves number of 354 accessions were measured individually in the eighth week after sowing. The ratio of healthy leaves number to total leaves number per plant was calculated by the formula (*Eq. 2*):

$$RHL = \frac{Healthy\ leaves\ number}{Total\ leaves\ number} \times 100 \quad \text{(Eq.2)}$$

In order to understand the effect of salt stress on plant growth change, the relative value of plant height (RPH), leaf length (RLL), leaf width (RLW) and ratio of healthy leaves (RRHL) under salt stress and under control were calculated referred to the following formula (*Eq. 3*):

$$RPH = \frac{Plant\ height\ under\ stress}{Plant\ height\ under\ control} \quad \text{(Eq.3)}$$

Measurement of physiological indexes

Sub-stomatal CO_2 concentration (Ci, μmol mol^{-1}), assimilation/respiration (A, μmol CO_2 m^{-2} S^{-1}), stomatal conductance (gs, mmol H_2O m^{-2} S^{-1}), transpiration (E, mmol H_2O m^{-2} S^{-1}) of 81 accessions selected randomly from 354 accession as representatives was determined by CIRAS-3 portable photosynthesis system (PP system, Amsbury, MA, USA) after seven weeks of sowing in order to shorten measurement time and minimize experiment error. Data were automatically recorded by the CIRAS-3 every 5 s. The CO_2 concentration (380 μ mol mol^{-1}), relative humidity (60%), and leaf temperature 28 °C were maintained using an automatic control device on the CIRAS-3. Red- blue light (90%: 10%) was provided by the LED light unit in the CIRAS3.

The Soil and Plant Analyzer Development (SPAD) values can present the relative content of chlorophyll and be measured by the portable SPAD-502m emitting red light (650 nm) and infrared light (940 nm) via transmission. By the difference of optical

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density between the two wavelengths, the relative content of chlorophyll (SPAD value) can be obtained. For the measurement, three plants for each replicate in each accession and three leaves in the middle of each plant were selected, and each measurement was repeated three times.

Statistical analysis

Statistical analysis of basic data is carried out in Excel 365. Cluster analysis was performed based on the unweighted pair-group method with arithmetic means (UPGMA), using the SPSS for windows. The correlation analysis among the index values of these traits under stress was performed using Pearson's Correlation Coefficient using R studio software version 1.2.500.1.

Results

Variation in salinity tolerance of garlic germplasm from salt injury observation

Based on the performance of 354 garlic germplasm under salt stress, the SII of all tested germplasm under salt stress were calculated and distributed from 16.51 to 98.15. A cluster analysis based on the un-weighted pair group method assigned the 354 germplasm into five main groups. Group I represents two accessions of 8N327 and 8N825, recognized as highly tolerance to salt stress with SII range from 16.51 to 17.59. Group II exhibits 24 germplasms, having the SII from 21.83 to 34.72 regarded as tolerance to salt stress. Group III illustrates 269 germplasm of moderately tolerance, which SII distributed from 35.45 to 57.01. Group IV covers 54 accessions recognized as susceptible to salt stress having the SII from 57.41 to 81.48. Group V include 5 accessions with SII range of 91.53 to 98.15 known as highly susceptible to salt stress (*Table 2; Figs. 1* and *2; Table A1* in the *Appendix*).

Table 2. *Classification of the germplasms on the base of SII*

Tolerance	Germplasm name	No of germplasm
Highly tolerant	8N327 8N825	2
Tolerant	8N850 8N847 8N724 8N869 8N908 8N911 8N325 8N360 8N032 8N128 T-167 8N167 8N364 T-141 8N141A 8N141B T-261 8N038A 8N719 8N261 T-17 T-258 8N026B 8N748	24
Sensitive	8N422 8N503 8N406 8N312 ZS-9 8N556 8N423 8N587 8N002 8N764 8N512 8N273 8N421 8N560 8N930 8N514 JX-1 8N372 8N076 8N566 8N654 8N786 WQS 8N780 8N629 8N037 JX-4 8N002B 8N410 8N728 8N249 8N975 8N254A 8N761 8N218 ZSS 8N324 8N013 8N529 8N427 8N332 8N561 8N675 8N526 8N950 8N030A 8N1040 8N545 8N760 8N559B 8N1046 8N970 8N676 8N1042	54
Highly sensitive	8N952 8N649 8N953 8N972 8N954	5

Agronomic traits response of different garlic germplasm to salt stress

NaCl salt stress decreased the growth performance of seedlings in most of 354 garlic germplasm to a different extent. The plant height showed a dispersing and great variation in the growth reduction of most germplasm under stress as compared to the control. Similarly, larger decreases in leaf length were observed under stress for most accessions. The leaf width and the ratio of healthy leaves number of germplasms also displayed a certain degree of difference between salt treatment and the control (*Fig. 3*). In more details, RPH varied from 0.42 to 1.00 with a mean of 0.73 and the frequencies

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of germplasm resources with different RPH values were almost normally distributed. Some germplasm such as 8N327 (0.87), 8N570 (0.93), 8N654 (0.94), 8N738 (0.95) T-167 (0.96) 8N222 (0.97) and T-17 (1.00) had the RPH value over 0.9, indicating these germplasms were more salt tolerant than other germplasm based on plant height reduction. Lower RPHs was found in 8N830 (0.47), 8N808 (0.44), 8N675 (0.44), etc., indicating these germplasms were highly susceptible to salt stress based on plant height reduction (*Fig. 4a*). RLL values ranged between 0.41 and 0.97 with a mean of 0.64 and the frequencies of germplasm resources with different RLL values were also nearly normally distributed. T-258 (0.89), 8N424(0.91), 8N239 (0.92), 8N992 (0.96) and 8N069 (0.97) had higher RLL values, indicating that they were salt-tolerant based on leaf length change; whereas, 8N586(0.45), 8N888 (0.44), MS No1 (0.44) 8N643 (0.43), and 8N1008(0.43) were highly susceptible to salt stress (*Fig. 4b*). RLW values varied from 0.43 to 1.00 with a mean of 0.86 and the frequency distribution of germplasm resources with different RLW values was skewed, indicating the leaf width of most accessions was relatively stable under salt stress. Higher relative values of leaf width were recorded for T-17 (0.86), 8N826 (0.87), 8N032 (0.93), 8N325 (0.96), T-141 (0.97) 8N847 (0.99), 8N519 (0.97), and 8N078B (1.00). Lower RLWs were found for 8N780 (0.43), 8N953 (0.49), 8N1005 (0.50) and 8N1022 (0.50) (*Fig. 4c*). RRHL values ranged between 0.71 and 0.99, within a mean of 0.88 and the frequencies of germplasm resources with different RRHL values were normally distributed. 8N758 (0.91), 8N249, (0.92), 8N876 (0.93), 8N808 (0.94), 8N066 (0.95), 8N922 (0.96), 8N1005 (0.97), 8N1004 (0.98), 8N126 (0.99) had the higher RRHL, indicating that these germplasms were more tolerant to salt based on RHL reduction than other germplasm. Lower RRHLs values were found for 8N586 (0.71), 8N675 (0.75) and 8N676 (0.75), showing that these germplasms are more susceptible to salt stress based on the RHL reduction (*Fig. 4d*). From the results above, it is obvious that the seedling growth of most accessions was decreased to different extent in the four parameters. However, the tolerance performance of different germplasm affected by salt stress is different in various agronomical traits. The SII for each accession is a comprehensive index, which is contributed by different single traits in different ways.

Correlation among agronomic and physiological traits affected by salt stress

By using 81 accessions as a pilot study, correlation analysis exhibited the association among different agronomic traits and physiological traits of garlic germplasm (*Fig. 5*). SII as an important comprehensive agronomic trait is extremely significantly negative correlated with PH (r = -0.65), LL (r = -0.64), LW (r = -0.53), RHL (r = -0.53), gs (r = -0.29), A (r = -0.44) and E(r = -0.60) at $P_{0.001}$, highly significant with Ch (r = -0.20) at $P_{0.05}$. Among other morphological and physiological traits, PH is extremely significant positive correlated with LL (r = 0.76), LW (r = 0.66), RHL (r = 0.47), A (r = 0.42), E (r = 0.50) at $P_{0.001}$ and highly significant positive with gs (r = 0.25) at $P_{0.05}$. LL is extremely significantly positive related with PH, LW, RHL, A, E and Ch at $P_{0.001}$. LW is highly significantly positive with RHL, A and E at $P_{0.05}$ except for the relationship with PH and LL. RHL is also extremely significantly positive related with A (r = 0.50), E (r = 0.64) and Ch (r = 0.48). Ci is extremely significantly positive with gs (r = 0.41), A (r = 0.34) and E (r = 0.34). gs is extremely significantly positive with A (r = 0.35), E(r = 0.26) and Ci. A is extremely significantly positive with E(r = 0.65), Ch (r = 0.27) besides PH, LL, RHL, Ci and gs. E is extremely significantly positive with Ch (r = 0.37) LL.RHL and A.

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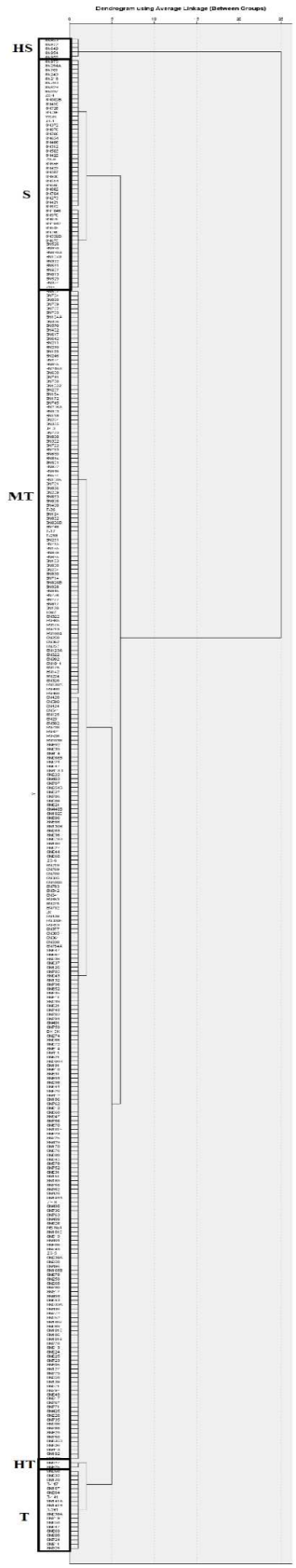

Figure 1*. Cluster analysis of the garlic germplasm based on the salt injury index (SII) using unweighted pair-group method (UPGMA)*

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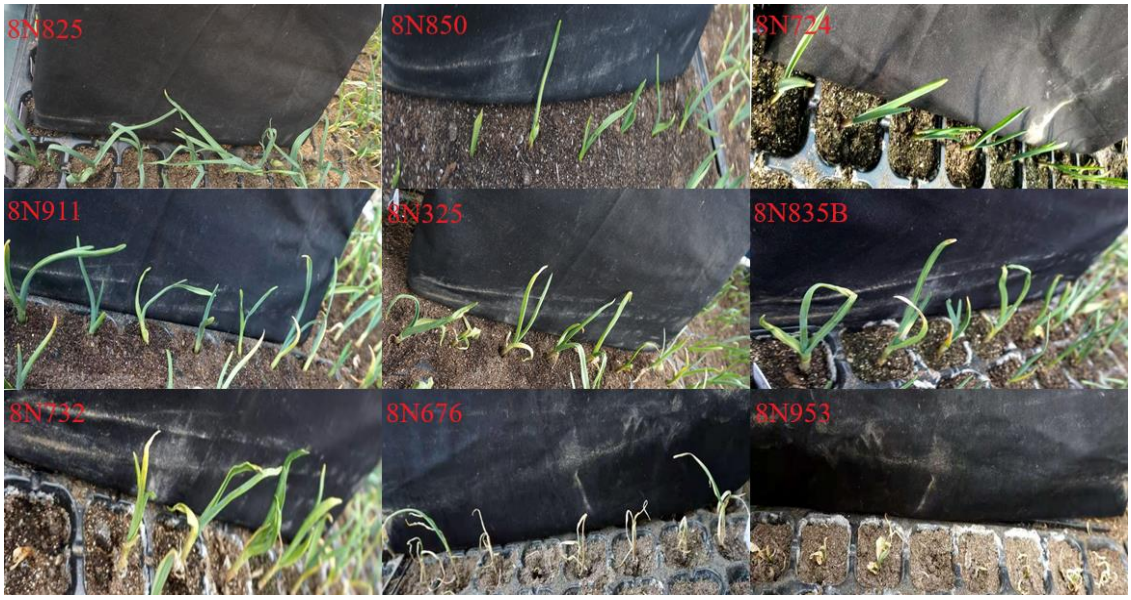

Figure 2. *Growth performance of representative germplasm with different tolerance to salt stress*

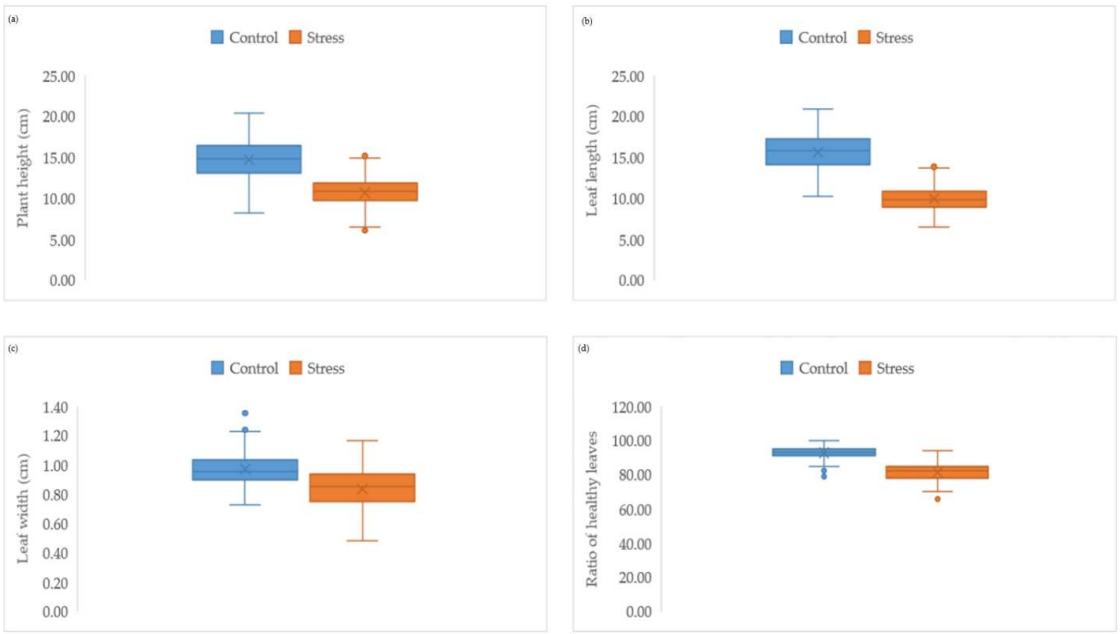

Figure 3. *The value distribution and comparison of agronomic traits of all garlic germplasm under salt stress and under control. (a) Plant height (cm); (b) leaf length (cm); (c) leaf width (cm); (d) ratio of healthy leaves*

Discussion

Tolerance to salt is important target trait for garlic breeding. Reasonable identification method is the basis of successful salt tolerance evaluation of garlic germplasm or breeding materials. Mass screening for salt tolerance of crops is complex, even in the soil culture or directly in the field because of multiplicity limitations like the

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status of soil fertility, irrigation management (Perez-Harguindeguy et al., 2016), salinity type (Munns and Tester, 2008) meteorological aspects like temperature and humidity) and as well as natural variation in fields (Hasana and Miyake, 2017). Field screening techniques have been reported confronting the main problem of soil heterogeneity, and a limited number of genotypes could be handled (Aslam et al., 1993).

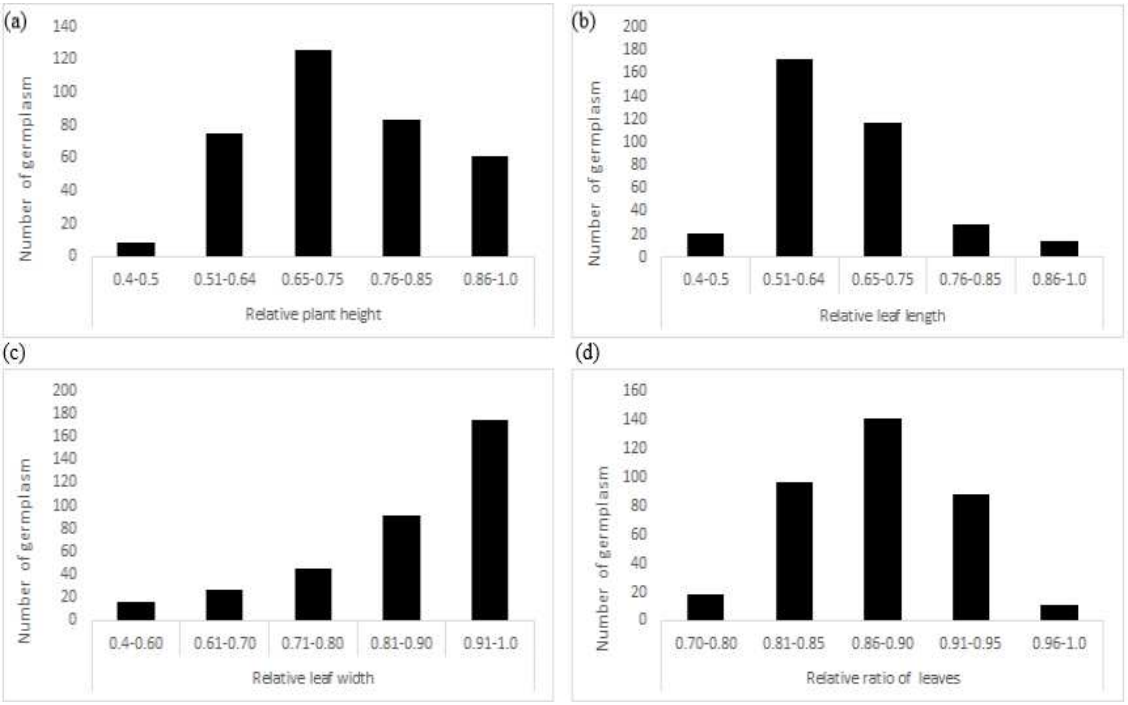

Figure 4. *Germplasm frequency distribution in different relative value range of each growth parameters under salt stress to under control. (a) Relative plant height; (b) relative leaf length; (c) relative leaf width; (d) relative ratio of leaves*

In the present study, we designed a pot culture method to characterize saline tolerance of garlic germplasm at the seedling stage. We screened out two highly tolerant and 24 tolerant garlic germplasm which are valuable for breeding and further study. The SII from visual salt injury observation is a comprehensive performance of plants under salt stress. It is reflected in many aspects of plant growth. The method is rapid and economical, and can easily meet the experimental conditions with the following advantages: (i) the salinity is standardized in the pot, or among pots, (ii) the irrigations quantity could be controlled, (iii) problems associated with salt depletion have been overcome. The effectiveness of this method was also confirmed by previous study in other crops such as rice (Kakar et al., 2019) and cotton (Sikder et al., 2020).

A reliable technical standard and index system for salt injury grading and tolerance classification is also a key to successfully develop a target-specific variety for salt tolerance. Visual symptoms of salt stress are mainly chlorosis of leaves, leaf tips burning, plant stunted growth and wilting (IRRI, 2014), and visual salt injury grades in rice. Sabra et al. (2012) studied the salt injury in three different Echinacea species by observing the appearance of leaves to develop a five-point scale according to the severity of necrotic tissues and number of injured plants. In the present study, we successfully distinguished garlic germplasm with the extensively distribution of SII

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value from 16.51 to 98.15 based on the modified grading standard of visual salt injury of leaves and plants in garlic (*Table 1*). For the salt tolerance evaluation, we did not use directly the arbitrary man-made resistance grading standard as in previous studies (Kopittke et al., 2009; Bolton and Simon, 2019). By the aid of cluster analysis based on the SII of each germplasm, we divided all germplasm into five groups, which exhibits high homogeneity within a cluster and high heterogeneity between clusters and help to setup a reasonable salt tolerance classification system according with the actual tolerance distribution in garlic and some other crop (Pradheeban et al., 2015).

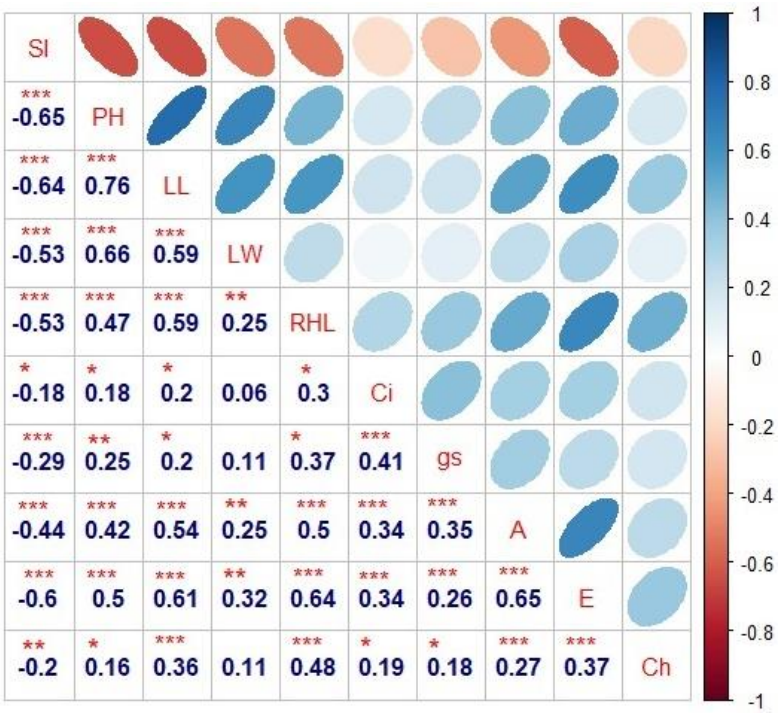

Figure 5. *Correlation coefficients among the growth attributes of garlic. The upper diagonal represents the correlation coefficient with significance levels at *p < 0.05, **p < 0.01, and *** p < 0.001, respectively; PH (plant height), LL (leaf length), LW (leaf width), RHL (Ratio of healthy leaves) Ci (Substomatal CO_2 concentration), gs (Stomatal Conductance), A (Respiration), E (Transpiration), Ch (Chlorophyll content)*

Salt stress reduces plant growth and productivity by affecting morphological, anatomical, biochemical and physiological characteristics, processes and functions. Reduced plant height and other morphological characters are the most distinct and obvious effect of salt stress. Depressed growth due to salinity is attributed to several factors such as, water stress specific ion toxicity and ion imbalance stress or induced nutritional deficiency. Our findings show that, plant height of all the germplasms deceased by the salinity stress. The reduced plant height might be attributed to the direct effect of excess salt on plant tissues and poor intake of minerals. Reduced plant height under saline conditions has been observed in garlic (Shama et al., 2016) and quinoa (Cai and Gao, 2020).

Leaf area represents the plant growth measurement (leaf length and width), which can be affected by salt stress. Our results showed a decrease in leaf length and width with NaCl stress. These results agree with Mathur et al. (2006); they reported that the

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moth bean plant (*Vigna aconitifolia* L.) with increasing concentration of sodium chloride, led to a decrease in leaf area. This reduction was inversely proportional to the concentration of NaCl. Also, a significant decrease in leaf area of sugar cane (*Beta vulgaris* L.) in response to salt stress using concentration zero, 50, 100, 150 mmol of sodium chloride, has been reported (Jamil et al., 2007). Other supporting results include those of Zhao et al. (2007), with their study on oat (*Avena sativa* L.) Yilmaz and Kina (2008), with their study on *Fragaria x anassa* (L.).

NaCl salt stress decrease ratio of healthy number of leaves in plants, compared with the control plant. The results have been confirmed by the results of Karen et al. (2002), with their study on *Cirer arietinum* L. and Lopez et al. (2003), with their study on the teprary bean (*Phaseolus acutifolius* L.), cowpea (*Vigna unguiculata* L.), and wild bean (*Phaseolus filiformis* L). They mentioned that, the treatment of NaCl reduced the number of leaves compared with control plants. Shama et al. (2016) reported that saline water irrigation significantly decreased the number of leaves and plant height of garlic. The decrease of leaves number may be due to the accumulation of NaCl in the cell wall and cytoplasm of the leaves. At the same time, their vacuole sap cannot accumulate more salts and, thereby decreases the concentration of salt inside the cells, which ultimately leads to their quick death (Munns, 2002).

Salinity tolerance has been measured based on both the absolute and relative values of plant growth, which are very important in assessing plant material of diverse origin (Ashraf and Waheed, 1990; Ding et al., 2018). The 354 accessions here were assessed for their ability to sustain growth under saline conditions, as absolute values under stress and relative values compared with control. These results were in line with other previous studies that salinity suppressed agronomic traits in *Triticum durum* Desf (Noori and McNeilly, 2000) and sweet sorghum (Ding et al., 2018).

From the correlation analysis, although some correlation coefficients were not very high, they were significant. This is mainly because of the large-scale samples, some of which may contribute interference to the final result. The reliability of the weaker correlation results from large-scale at an enhanced significance criterion of $P_{0.001}$ besides $P_{0.01}$ *and* $P_{0.05}$ are equally recognized as the strong correlation in small samples (Abdelghany et al., 2020; Azam et al., 2021). SII as a comprehensive index are involved in many aspects of plants which may be affected and interacted with each other. In our study, among indexes of the observed agronomic traits at seedling stage, it was found that SII was extremely significantly negative correlated with all the measured agronomic traits under stress, and that there was also extremely significantly negative correlation between the physiological traits affected by salt stress, which provides important clue to assess the plant response to salt stress. These findings are consistent with the previous study in pistachio (Karimi and Roosta, 2014).

NaCl stress can decrease in physiological activities and consequently hinder the photosynthetic mechanism of the plant (Khan, 2016). In the current study, NaCl stress is a negative correlation with physiological traits such as sub-stomatal CO_2 concentration (ci), stomatal conductance (gs), transpiration (A), respiration (E), and relative chlorophyll content (Ch). Generally, CO_2 exchange was regarded as an important indicator of the growth of plant, because of its direct link to net productivity (Asharf, 2004). It was proven that sub-stomatal CO_2, stomatal conductance, transpiration and rate of photosynthesis of all the parameters are affected by salt stress. These results agree with previously report in rocket (*Eruca sativa* (L.) *Mill.*), where there were negative correlation between salinity and gas exchange parameters (Hniličková et al.,

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2017). The decrease in gaseous exchange attributes in the current study might be associated with salinity-induced osmotic stress that rendered the growing plants out of the water and hampered the rate of transpiration, which further excavated water and CO_2 supply for normal photosynthesis (Shahzad et al., 2019). They might also be due to osmotic and hormonal imbalances created by the generation of reactive oxygen species (ROS) in plant cells that impaired carbohydrates metabolism and hence the photosynthetic efficiency (Bergmann et al., 2008).

The impact of photosynthesis can be evaluated from the photosynthetic pigments. With increasing salt stress, the SPAD value decreased. Previous studies have described that salinity stress declined photosynthetic pigments of plants (Anand and Byju, 2008) and have also shown that the SPAD value measured by the SPAD chlorophyll meter is highly correlated with chlorophyll content (Anand and Byju, 2008). In the present study the chlorophyll content are negative correlation with salt stress. It was reported that the effect of salt stress on chlorophyll was varied species to species, and some studies have shown that salt stress can inhibit the chlorophyll synthesis of plants (Wang et al., 2015). Heidari (2012) reported that salt stress is a negative correlation with chlorophyll content in *ocium basilicum* (L.). Gouveianeto et al. (2011) found the main reason for the decrease in chlorophyll content caused by high salt concentration was the blocking of electron transport.

Conclusions

This study revealed a widely variation of salt tolerance during the seedling stage in a collection of diverse garlic germplasms based on an improved identification method and evaluation system. The discovery of salt tolerant accessions could not only serve as potential materials for identification of salt tolerant associated QTLs/genes, but also were promising for breeders to develop salt-tolerant cultivars. Although the respective agronomic traits of different germplasm had somewhat different response to salt stress, there were significantly or extremely significantly positive correlation among the most concerned agronomic and physiological traits affected by salt stress and the significantly or extremely significantly negative relationship between SII and morphological traits (PH, LL, LW, RHL), also between SII and physiological traits (gs, A, E, Ch) under stress. All these results provide the valuable base for the establishment of the reasonable salt-tolerance identification and classification standards and technical index, and further salt tolerance mechanism study.

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Conflict of interests. The authors declare no conflict of interests.

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APPENDIX

Table A1. *Germplasms origin and their classification on the base of salt tolerance*

Sr. No	Germplasms ID	SII	Group	Tolerance	Province/world
1.	8N327	16.51	1	HT	Yunnan Province
2.	8N825	17.59	1	HT	Tajikistan
3.	8N850	21.83	2	T	Washington, United States
4.	8N847	22.84	2	T	Washington, United States
5.	8N724	25.00	2	T	Pakistan
6.	8N869	25.28	2	T	Uzbekistan
7.	8N908	25.40	2	T	Washington, United States
8.	8N911	25.74	2	T	Washington, United States
9.	8N325	26.46	2	T	Yunnan Province
10.	8N360	28.70	2	T	Europe
11.	8N032	28.70	2	T	Shanghai
12.	8N128	29.31	2	T	Jiangsu Province
13.	T-167	29.38	2	T	Yunnan Province
14.	8N167	29.78	2	T	Yunnan Province
15.	8N364	29.89	2	T	Korea
16.	T-141	30.56	2	T	Shandong Province
17.	8N141A	30.89	2	T	Shandong Province
18.	8N141B	31.48	2	T	Shandong Province
19.	T-261	32.25	2	T	Jiangsu Province
20.	8N038A	32.30	2	T	Shandong Province
21.	8N719	33.33	2	T	Former Serbia and Montenegro
22.	8N261	34.04	2	T	Jiangsu Province
23.	T-17	34.12	2	T	Jiangsu Province
24.	T-258	34.14	2	T	Jiangsu Province
25.	8N026B	34.72	2	T	Washington United States
26.	8N748	34.72	2	T	Turkey
27.	8N715	35.45	3	MT	Illinois, United States
28.	8N195	35.66	3	MT	Jiangsu Province
29.	8N183	36.11	3	MT	Hubei Province
30.	8N899	36.11	3	MT	Washington United States
31.	8N655	36.11	3	MT	North Macedonia
32.	8N808	36.42	3	MT	Washington United States
33.	8N826B	36.90	3	MT	Washington, United State
34.	8N928	37.04	3	MT	Viet Nam
35.	8N234	37.17	3	MT	Hubei Province
36.	8N830	37.19	3	MT	Washington, United State
37.	8N714	37.30	3	MT	Washington, United States
38.	8N822	37.57	3	MT	Sachsen-Anhalt, Germany
39.	109Z	37.66	3	MT	Jiangsu Province
40.	8N777	37.70	3	MT	Andalucía, Spain
41.	8N817	37.70	3	MT	Slovenia
42.	8N170	37.74	3	MT	Yunnan Province

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43.	8N865	37.96	3	MT	Bulgaria
44.	8N145	38.10	3	MT	Sichuan Province
45.	8N845	38.40	3	MT	Washington, United State
46.	8N776	38.43	3	MT	Andalucía, Spain
47.	8N713	38.89	3	MT	California, United States
48.	8N1004	38.89	3	MT	Jiangsu Province
49.	8N992	39.05	3	MT	Jiangsu Province
50.	8N123A	39.15	3	MT	Jiangsu Province
51.	8N622	39.15	3	MT	Shandong Province
52.	8N257	39.29	3	MT	Jiangsu Province
53.	8N258	39.33	3	MT	Jiangsu Province
54.	8N862	39.35	3	MT	Jiangsu Province
55.	8N860	39.68	3	MT	Korea, South
56.	8N130A	39.81	3	MT	Anhui Province
57.	8N900	39.90	3	MT	Washington United States
58.	8N326	40.11	3	MT	Yunnan Province
59.	8N1011	40.21	3	MT	Jiangsu Province
60.	8N175	40.21	3	MT	Yunnan Province
61.	8N142	40.28	3	MT	Shandong Province
62.	8N224	40.33	3	MT	Shaanxi Province
63.	8N1005	40.74	3	MT	Jiangsu Province
64.	8N209	40.74	3	MT	Liaoning Province
65.	8N781	40.74	3	MT	Washington United States
66.	8N826	40.74	3	MT	Washington, United State
67.	8N846	40.74	3	MT	Washington, United State
68.	8N534	40.74	3	MT	Guizhou
69.	8N863	40.81	3	MT	Italy
70.	8N832	41.01	3	MT	Washington, United State
71.	T-36	41.08	3	MT	Shandong Province
72.	8N124	41.09	3	MT	Jiangsu Province
73.	8N096	41.14	3	MT	Gansu Province
74.	8N490	41.14	3	MT	United States
75.	8N922	41.42	3	MT	Yunnan Province
76.	8N864	41.62	3	MT	Bulgaria
77.	8N302	41.67	3	MT	Yunnan Province
78.	8N660	41.67	3	MT	Turkey
79.	8N722	41.67	3	MT	Chile
80.	8N753	41.67	3	MT	Washington United States
81.	8N773	41.67	3	MT	Andalucía, Spain
82.	8N890	41.67	3	MT	Washington United States
83.	8N501	41.85	3	MT	Egypt
84.	8N741	42.06	3	MT	Washington United States
85.	8N758	42.06	3	MT	Castilla-La Mancha, Spain
86.	8N154	42.15	3	MT	Shandong Province
87.	8N1022	42.20	3	MT	Shandong Province
88.	8N207	42.20	3	MT	Hebei Province

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89.	8N240A	42.33	3	MT	Jiangsu Province
90.	8N668	42.33	3	MT	Brazil
91.	8N365	42.55	3	MT	Korea
92.	8N118	42.59	3	MT	Shaanxi Province
93.	8N264	42.59	3	MT	Shandong Province
94.	8N206A	42.59	3	MT	Hebei Province
95.	8N903	42.59	3	MT	Washington United States
96.	8N172	42.68	3	MT	Yunnan Province
97.	8N745	42.72	3	MT	Albania
98.	JX-3	42.86	3	MT	Shandong Province
99.	8N898	43.06	3	MT	Washington, United States
100.	8N507	43.12	3	MT	Jiangsu
101.	8N734	43.12	3	MT	Andalucía, Spain
102.	8N789	43.21	3	MT	Varna, Bulgaria
103.	8N570	43.34	3	MT	Washington, United States
104.	8N104A	43.52	3	MT	Shaanxi Province
105.	8N366	43.52	3	MT	Korea
106.	8N737	43.52	3	MT	Shumen, Bulgaria
107.	8N799	43.52	3	MT	USA
108.	8N855	43.78	3	MT	Washington, United States
109.	8N246	43.92	3	MT	Yunnan Province
110.	8N434	43.98	3	MT	Henan
111.	8N155	44.09	3	MT	Shandong Province
112.	8N211	44.14	3	MT	Ningxia
113.	8N260	44.14	3	MT	Jiangsu Province
114.	8N642	44.44	3	MT	Guizhou
115.	8N402	44.44	3	MT	Hebei Province
116.	8N617	44.44	3	MT	Shandong Province
117.	8N252	44.84	3	MT	Yunnan Province
118.	8N436	44.91	3	MT	Henan
119.	8N772	44.97	3	MT	Andalucía, Spain
120.	8N1007	45.22	3	MT	Shandong Province
121.	8N069	45.24	3	MT	Shaanxi Province
122.	8N1019	45.37	3	MT	Shandong Province
123.	8N778	45.37	3	MT	Andalucía, Spain
124.	8N913	45.37	3	MT	lead the United States
125.	8N1016	45.37	3	MT	Shandong Province
126.	8N106	45.37	3	MT	Russia
127.	8N924	45.43	3	MT	Guizhou Province
128.	8N025	45.50	3	MT	Hubei Province
129.	8N371	45.74	3	MT	Sichuan Province
130.	8N779	45.83	3	MT	Andalucía, Spain
131.	8N358	45.86	3	MT	Europe
132.	8N139	45.90	3	MT	Anhui Province
133.	8N127	46.03	3	MT	Jiangsu Province
134.	8N723	46.03	3	MT	Washington, United States

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135.	8N505	46.03	3	MT	Jiangsu
136.	8N217	46.30	3	MT	Jiangsu Province
137.	8N425	46.30	3	MT	Henan
138.	8N762	46.30	3	MT	Former, Soviet Union
139.	8N771	46.30	3	MT	Andalucía, Spain
140.	8N797	46.30	3	MT	Washington, United States
141.	8N848	46.30	3	MT	Washington, United States
142.	8N220	46.43	3	MT	Shandong Province
143.	8N735	46.43	3	MT	Andalucía, Spain
144.	8N208	46.46	3	MT	Hebei Province
145.	8N768	46.50	3	MT	Andalucía, Spain
146.	8N102	46.63	3	MT	Heilongjiang Province
147.	8N413	46.69	3	MT	Henan
148.	8N835B	46.69	3	MT	Washington, United States
149.	8N836	46.69	3	MT	Washington, United States
150.	8N1009	46.76	3	MT	Shandong Province
151.	8N829	46.91	3	MT	Washington, United States
152.	8N750	46.96	3	MT	California, United States
153.	8N1010	47.09	3	MT	Shandong Province
154.	8N519	47.09	3	MT	Egypt
155.	8N404	47.12	3	MT	India
156.	8N508	47.22	3	MT	Jiangsu
157.	8N749	47.22	3	MT	Turkey
158.	ZS-5	47.27	3	MT	Shandong Province
159.	8N239A	47.31	3	MT	Jiangsu Province
160.	8N168B	47.53	3	MT	Yunnan Province
161.	8N678	47.55	3	MT	Washington, United States
162.	8N259	47.57	3	MT	Jiangsu Province
163.	8N250	47.62	3	MT	Yunnan Province
164.	8N496	47.62	3	MT	Egypt
165.	8N317	47.75	3	MT	Ningxia
166.	8N493	47.75	3	MT	Washington, United States
167.	8N643	47.80	3	MT	Guizhou
168.	8N233A	47.84	3	MT	Hubei Province
169.	8N268	47.90	3	MT	Jiangsu Province
170.	8N790	47.94	3	MT	California United States
171.	8N189	48.13	3	MT	Gansu Province
172.	8N245	48.15	3	MT	Yunnan Province
173.	8N031	48.15	3	MT	Shanghai
174.	8N151	48.15	3	MT	Inner Mongolia
175.	8N578	48.15	3	MT	Kazakhstan
176.	8N752	48.15	3	MT	California, United States
177.	8N876	48.15	3	MT	Washington, United States
178.	8N888	48.15	3	MT	Washington, United States
179.	8N755	48.22	3	MT	Uzbekistan
180.	8N429	48.28	3	MT	Henan

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181.	8N178	48.32	3	MT	Hebei Province
182.	8N1044	48.47	3	MT	Shandong Province
183.	8N362	48.54	3	MT	Korea
184.	8N420	48.54	3	MT	Henan
185.	MS No1	48.64	3	MT	Beijing Shi, China
186.	8N409	48.68	3	MT	Henan
187.	8N626	48.68	3	MT	Guizhou
188.	8N736	48.68	3	MT	Razgrad, Bulgaria
189.	8N763	48.68	3	MT	Former Soviet Union
190.	ZS-8	48.74	3	MT	Shandong Province
191.	8N498	48.77	3	MT	Egypt
192.	DX CK	48.93	3	MT	Shandong Province
193.	8N274	48.94	3	MT	Jiangsu Province
194.	8N401	49.07	3	MT	Hebei Province
195.	8N759	49.07	3	MT	Nepal
196.	8N782	49.07	3	MT	California, United States
197.	8N784	49.07	3	MT	California, United States
198.	8N740	49.21	3	MT	California, United States
199.	8N239	49.34	3	MT	Jiangsu Province
200.	8N821	49.34	3	MT	lead the United States
201.	8N535	49.52	3	MT	Guizhou
202.	8N821	49.56	3	MT	Kazakhstan
203.	8N043	49.60	3	MT	Shandong Province
204.	8N132	49.60	3	MT	Yunnan Province
205.	8N796	49.60	3	MT	California, United States
206.	8N126	49.60	3	MT	Jiangsu Province
207.	8N785	49.60	3	MT	Burgas, Bulgaria
208.	8N511	49.69	3	MT	Egypt
209.	8N200B	50.00	3	MT	Hebei Province
210.	8N411	50.00	3	MT	Henan
211.	8N921	50.00	3	MT	Yunnan Province
212.	8N191	50.10	3	MT	Gansu Province
213.	8N610	50.13	3	MT	Shandong Province
214.	8N614	50.26	3	MT	Shandong Province
215.	8N066	50.33	3	MT	Shaanxi Province
216.	8N072	50.40	3	MT	Shaanxi Province
217.	8N186	50.53	3	MT	Hebei Province
218.	8N762	50.53	3	MT	California, United States
219.	8N947	50.62	3	MT	Yunnan Province
220.	8N612	50.66	3	MT	Shandong Province
221.	8N868	50.66	3	MT	Uzbekistan
222.	8N1015	50.79	3	MT	Shandong Province
223.	8N766	50.79	3	MT	Andalucía, Spain
224.	8N870	50.79	3	MT	Uzbekistan
225.	8N623	50.90	3	MT	Guizhou
226.	8N725	50.93	3	MT	Pakistan

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227.	8N531	51.11	3	MT	Guizhou
228.	8N893	51.16	3	MT	Lead the United States
229.	8N620	51.23	3	MT	Shandong Province
230.	8N298	51.32	3	MT	Yunnan Province
231.	8N644	51.32	3	MT	California, United States
232.	8N412	51.46	3	MT	Henan
233.	8N1008	51.72	3	MT	Shandong Province
234.	8N783	51.72	3	MT	Montana, Bulgaria
235.	8N035	51.85	3	MT	Shandong Province
236.	8N769	51.85	3	MT	Andalucía, Spain
237.	8N788	51.85	3	MT	Yambol, Bulgaria
238.	8N542	52.03	3	MT	Guizhou
239.	8N641	52.03	3	MT	Guizhou
240.	8N653	52.20	3	MT	North Macedonia
241.	8N275	52.38	3	MT	Beijing Shi, China
242.	8N732	52.38	3	MT	Beijing Shi, China
243.	JX	52.46	3	MT	Shandong Province
244.	8N439	52.47	3	MT	Henan
245.	8N361	52.73	3	MT	Europe
246.	8N377	52.78	3	MT	Sichuan Province
247.	8N885	52.78	3	MT	lead the United States
248.	8N030B	52.78	3	MT	Heilongjiang Province
249.	8N613	52.78	3	MT	Shandong Province
250.	8N238	52.84	3	MT	Jiangsu Province
251.	8N754A	52.91	3	MT	California, United States
252.	8N647	53.04	3	MT	Serbia
253.	8N867	53.09	3	MT	Uzbekistan
254.	8N236	53.15	3	MT	Hubei Province
255.	8N637	53.23	3	MT	Guizhou
256.	8N1020	53.44	3	MT	Shandong Province
257.	8N021	53.57	3	MT	Hubei Province
258.	8N440B	53.62	3	MT	Xinjiang
259.	8N509	53.70	3	MT	Jiangsu
260.	8N527	53.70	3	MT	Guizhou
261.	8N706	53.70	3	MT	Vermont, United States
262.	8N254B	53.78	3	MT	Yunnan Province
263.	8N403	53.84	3	MT	Hebei Province
264.	8N787	53.86	3	MT	Pleven, Bulgaria
265.	8N413-1	53.97	3	MT	Henan
266.	8N222	54.01	3	MT	Shandong Province
267.	8N219	54.26	3	MT	Shandong Province
268.	8N036	54.50	3	MT	Shandong Province
269.	8N130B	54.50	3	MT	Anhui Province
270.	8N263	54.50	3	MT	Jiangsu Province
271.	8N060	54.59	3	MT	Shaanxi Province
272.	8N027	54.63	3	MT	Jiangxi Province

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273.	8N044	54.63	3	MT	Shandong Province
274.	8N078B	54.63	3	MT	Thailand
275.	8N100	54.63	3	MT	Shanghai
276.	ZS-6	54.76	3	MT	Shandong Province
277.	8N506	54.98	3	MT	Jiangsu
278.	8N586	55.03	3	MT	Guizhou
279.	8N047	55.29	3	MT	Shandong Province
280.	8N066B	55.42	3	MT	Shaanxi Province
281.	8N024	55.42	3	MT	Hubei Province
282.	8N039	55.56	3	MT	Shandong Province
283.	8N1038	55.56	3	MT	Shandong Province
284.	8N892	55.56	3	MT	Lead the United States
285.	8N414	55.69	3	MT	Henan
286.	8N621	55.93	3	MT	Shandong Province
287.	8N536	55.94	3	MT	Guizhou
288.	8N541	56.44	3	MT	Guizhou
289.	8N424	56.48	3	MT	Henan
290.	8N428	56.48	3	MT	Henan
291.	8N590	56.48	3	MT	China
292.	8N502	56.72	3	MT	Hubei Province
293.	8N125	56.79	3	MT	Jiangsu Province
294.	8N231	56.79	3	MT	Hubei Province
295.	8N738	57.01	3	MT	Plovdiv, Bulgaria
296.	8N422	57.41	4	S	Henan
297.	8N503	57.78	4	S	Jiangsu
298.	8N406	58.02	4	S	Gansu Province
299.	8N312	58.20	4	S	Xinjiang
300.	ZS-9	58.60	4	S	Shandong Province
301.	8N556	58.64	4	S	Guizhou
302.	8N423	58.86	4	S	Henan
303.	8N587	58.91	4	S	Shandong Province
304.	8N002	59.16	4	S	Sichuan Province
305.	8N764	59.26	4	S	Yunnan Sheng
306.	8N512	59.47	4	S	Egypt
307.	8N273	59.57	4	S	Jiangsu Province
308.	8N421	59.66	4	S	Henan
309.	8N560	59.92	4	S	Guizhou
310.	8N930	60.05	4	S	USA
311.	8N514	60.05	4	S	Egypt
312.	JX-1	60.74	4	S	Shandong Province
313.	8N372	60.85	4	S	Sichuan Province
314.	8N076	60.98	4	S	Xinjiang
315.	8N566	61.11	4	S	Germany
316.	8N654	61.42	4	S	North Macedonia
317.	8N786	62.33	4	S	Burgas, Bulgaria
318.	WQS	63.16	4	S	Shandong Province

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319.	8N780	63.89	4	S	Syria
320.	8N629	63.89	4	S	Guizhou
321.	8N037	64.01	4	S	Shandong Province
322.	JX-4	64.20	4	S	Shandong Province
323.	8N002B	64.35	4	S	Sichuan Province
324.	8N410	64.51	4	S	Henan
325.	8N728	64.81	4	S	Former Soviet Union
326.	8N249	65.50	4	S	Yunnan Province
327.	8N975	65.74	4	S	Guizhou Province
328.	8N254A	65.78	4	S	Yunnan Province
329.	8N761	65.87	4	S	Former, Soviet Union
330.	8N218	66.14	4	S	Jiangsu Province
331.	ZSS	67.20	4	S	Shandong Province
332.	8N324	68.31	4	S	Yunnan Province
333.	8N013	68.58	4	S	Sichuan Province
334.	8N529	68.77	4	S	Guizhou
335.	8N427	69.75	4	S	Henan
336.	8N332	70.49	4	S	Yunnan Province
337.	8N561	70.55	4	S	Guizhou
338.	8N675	72.09	4	S	Poland
339.	8N526	72.41	4	S	Guizhou
340.	8N950	73.15	4	S	USA
341.	8N030A	73.41	4	S	Heilongjiang Province
342.	8N1040	73.81	4	S	Shandong Province
343.	8N545	75.80	4	S	Guizhou
344.	8N760	76.54	4	S	Greece
345.	8N559B	77.65	4	S	Guizhou
346.	8N1046	79.63	4	S	Shandong Province
347.	8N970	79.63	4	S	USA
348.	8N676	80.56	4	S	Syria
349.	8N1042	81.48	4	S	Shandong Province
350.	8N952	91.53	5	HS	USA
351.	8N649	95.37	5	HS	North Macedonia
352.	8N953	96.30	5	HS	USA
353.	8N972	96.30	5	HS	USA
354.	8N954	98.15	5	HS	USA

HT, highly tolerant; T, tolerant; S, sensitive; HS, highly sensitive

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COMPARATIVE STUDY OF SIX MAIZE (*ZEA MAYS* L.) CULTIVARS CONCERNING CADMIUM UPTAKE, PARTITIONING AND TOLERANCE

AKRAM, M. A.[1,2] – WAHID, A.[2] – ABRAR, M.[1] – MANAN, A.[1] – NAEEM, S.[3] – ZAHID, M. A.[4] – GILANI, M. M.[5] – PAUDYAL, R.[6] – GONG, H. Y.[1] – RAN, J. Z.[1*] – DENG, J. M.[1*]

[1]*State Key Laboratory of Grassland Agro-Ecosystem, School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730000, China*

[2]*Department of Botany, University of Agriculture, Faisalabad-38040, Pakistan*

[3]*Gansu Key Laboratory of Biomonitoring and Bioremediation for Environmental Pollution, School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730000, China*

[4]*National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan*

[5]*Forestry College, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian Province, China*

[6]*State Key Laboratory of Cryospheric Science, Northwest Institute of Eco-Environment and Resources, Chinese Academy of Sciences, Lanzhou 730000, China*

[*]*Corresponding authors*
e-mail: ranjz@lzu.edu.cn; dengjm@lzu.edu.cn

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Abstract. This experiment was conducted to determine Cd accumulation, partitioning, translocation and their ultimate consequences on growth and biochemical traits of six maize cultivars to screen out Cd tolerant one. A significant decrease in growth and biochemical traits as well as in Cd tolerance index was found, especially at higher Cd level. Moreover, the varieties and Cd treatments were found significant for the growth and biochemical traits. Uptake of Cd, Ca, K and P in the different plant tissues were significantly affected by the varieties and Cd treatments. Translocation, bioconcentration and bioaccumulation factors were found non-significant for varieties and Cd treatments. A significant positive correlation was found between growth and biochemical traits while, among plant total Cd, Ca, K and P, total plant dry; and fresh weight and plant height were significantly negative. Furthermore, the PC1 was loaded by growth traits; Ca, K and P while PC2 by the Cd in different plant tissues. Hence, a strong negative correlation was observed between the PC1 and PC2 parameters. The maximum Cd accumulation was in the roots rather than the crown and leaf tissues of maize cultivars. The EV-1098 was to be more tolerant to Cd stress than other cultivars.
Keywords: *heavy metal toxicity, growth and biochemical traits, varietal differences, tolerance index, bioconcentration factor*

Introduction

Life and biodiversity are significantly affected by the accumulation of heavy metals in soils either by natural processes or by human activities. The toxicity of heavy metals is one of the major reasons of environmental and ecological issues (Roy et al., 2018). The rise of heavy metal contamination in agricultural fields and soils caused primarily by anthropogenic activities resulted in their incorporation into the grown crops and ultimately into the food chain (Siebers et al., 2014; Retamal-Salgado et al., 2017). A

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large number of industries produce wastewater containing significant concentrations of heavy metals which is discharged into drains and rivers. These wastewater effluents contain a large number of heavy metals such as Cr, Cd, Cu, Pb, Zn, and Ni that have a major impact on crops and their yields (Najam et al., 2015; Ali et al., 2020). The long-term irrigation with industrial wastewater allows heavy metals to persist in the soil, which increases their absorption and accumulation by the plants which ultimately enter into the food chain (Najam et al., 2015). Several non-essential heavy metals even in traces induce toxicity in plants and affect the growth of several crops (Marquez et al., 2018). Heavy metals such as Cd, Ni, Pb, Cu, Cr and Zn not only negatively affect the growth and development of plants but also disturb the micro-flora of the soils (Abdu et al., 2017). To achieve the goals of a sustainable environment and agriculture it is necessary to control heavy metal pollution on a priority basis (Naveed et al., 2020).

Cd is a poisonous, non-degradable and non-essential heavy metal that naturally exists in most of the soil types (Naveed et al., 2020). Its naturally occurring levels are usually < 1 mg/kg, but these levels have increased to 1000 mg/kg in some geographical regions because of haphazard urbanization/ industrialization in the last two centuries (Coakley et al., 2019). Cd exposures can be toxic or even lethal to plants in certain cases even at concentrations as low as 2.5 mg/kg (Henson et al., 2013). The anthropogenic activities increasing Cd concentration in the environment are manure (sewage sludge), combustion of fossil fuel, iron foundries, electroplating, smelting, waste disposal of water and usage of synthetic industrial products such as paints, pesticides and sludge (Anjum et al., 2015; Rizwan et al., 2017; Abedi and Mojiri, 2020). Interestingly, physiochemical properties of Cd are similar to other micronutrients such as Zn, which enable it to be taken up readily by plants and crops (Coakley et al., 2019). Cd salts are comparatively more soluble than others in the soil, consequently, Cd can readily be incorporated into the natural environment and easily accumulated in the roots and other edible parts of the plants (Naveed et al., 2020). Plants can easily transport Cd to different parts through the vascular tissues (xylem and phloem) (Ismael et al., 2019). The Cd has a greater absorption and accumulation rate than other heavy metals like Cu and Zn (Liao et al., 2019). It alters the physiological functions by disturbing many metabolic processes including nitrogen metabolism (Roy et al., 2016; Abedi and Mojiri, 2020). Cd absorption and accumulation depends upon the age of plants (Godinho et al., 2018) and its deposition gradually increases with time, affecting consumers directly or indirectly (Abedi and Mojiri, 2020). Daily consumption of 1 mg/kg (body weight) Cd is considered harmful to humans (Retamal-Salgado et al., 2017). The long-term exposure and ingestion of Cd-contaminated food is toxic to the food chain and poses a severe health risk to human. It is highly imperative that Cd-polluted soils be remedied to preserve and restore the ecological functionality of these soils (Naveed et al., 2020).

Accumulation of Cd in edible parts of the plant is more harmful than the overall plant intake. While certain techniques are being used to reduce the Cd in the food chain (Rai et al., 2019). Cd toxicity can negatively affect biomass, chlorophyll contents, number of leaves/flowers/fruits, leaf area, crop yield of plants and its ability to uptake essential nutrients (Ghani, 2010; Coakley et al., 2019). Cd accumulation is directly related to its applied concentration and exposure time (Rolli et al., 2010). Cd affects the lateral roots in maize, changes the root color and roots become stiff. It also decreases the coleoptiles size, causes chlorosis, and ultimately necrosis (Shafi et al., 2010; Kaznina and Titov, 2014). The increased Cd concentration amplified the Cd absorption in the shoots of maize (Nguyen et al., 2016) while roots accumulate more Cd than shoots (Stritsis and

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Claassen, 2013). Cd translocation from roots to shoots is limited by the plants of the Poaceae family in order to maintain the nutritional balance that is disturbed by the Cd induction, e.g., by the suberization of endoderm cells and lignification of root cortex cells (Kaznina and Titov, 2014). The main symptoms of Cd toxicity including stunted growth, enzyme activation or deactivation, photosynthetic activity and plant–water relationship disturbance (Raza et al., 2020). The plant species and varieties vary for Cd accumulation and translocation as well as Cd partitioning into their different tissues (Yang et al., 2014; Coakley et al., 2019), such as apple (Zhou et al., 2017), maize (Shah et al., 2016; Rizwan et al., 2017), mungbean (Ghani, 2010), mustard and oats (Boros-Lajszner et al., 2020), pea (Naveed et al., 2020), rice (Fahad et al., 2015; Marquez et al., 2018), sorghum (Roy et al., 2016) and wheat (Shafi et al., 2010; Abedi and Mojiri, 2020). The varied distribution of Cd at the cellular/subcellular level in different organs was attributed to the high accumulation capacity of plants (Coakley et al., 2019). Plants have adapted different mechanisms for Cd tolerance and to mitigate its toxic effects, such as maize compartmentalizes Cd as a mechanism of tolerance in the cell wall of stems (Akhter et al., 2014).

Maize (*Zea mays* L.) is a cereal crop grown worldwide belonging to grasses (Poaceae). It is known as a heavy metal tolerant and accumulator because of its survival ability in the metal-polluted soils and metal accumulation capacity. Some maize genotypes show tolerance to heavy metal toxicity (Rizwan et al., 2017) and Cd contaminated soils (Van Slycken et al., 2013). Due to this property maize is potentially used plant for phytoremediation, especially in Cd-contaminated soils (Huang et al., 2020; Raza et al., 2020). Plant tolerance to heavy metals largely depends on the efficiency of uptake, translocation, and sequestration in specialized tissues and cell organelles (Boros-Lajszner et al., 2020). Maize is commonly used for glucose preparation, edible oil production and several other products (Shah et al., 2016). The maize is also one of the highest growing crops and has the survival ability in the metal-polluted soils and metal accumulation capacity concerning other crops (Wuana and Okieimen, 2010). Maize is considered among 400 plants that accumulate heavy metals and survive in Cd-polluted soils but maybe with low biomass production (Waseem et al., 2014). The maize production is decreasing due to the heavy metal accumulation in soils. Therefore, to find the resistant maize varieties are necessary to fulfill the food requirement of the worldwide increasing population.

Pakistan is one among the agricultural countries in the world and its large population (> 70%) living in the villages directly or indirectly depends on agriculture (Bashir et al., 2012). Pakistan is the 20th largest producer of maize in the world. However, rapid urbanization and an increase in population demand healthier and higher crop yields of rice, sugarcane, wheat and maize (Ali et al., 2017). In Pakistan, the vegetables and crops are commonly irrigated with canal water that gets polluted by industrial wastes when passing by industrial areas, which is commonly contaminated with heavy metals (Naveed et al., 2020). Among these heavy metals, Cd is of major concern. Maize fabrication is significantly reduced in Pakistan owing to the use of sewage water (Waseem et al., 2014). Industrial wastewater is extensively used for irrigation in the agricultural areas of Pakistan. The wide distribution of Cd in wastewater has been reported in the different areas of Pakistan (Waseem et al., 2014; Najam et al., 2015). The maximum amount of Cd (5.35 mg/L) in wastewater was recorded in the Korangi region of Karachi (Amin et al., 2014), which exceeded the 0.10 mg/L permissible threshold set by NEQS-Pak (Waseem et al., 2014). In addition, the Cd concentration in

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wastewater was also found above the said threshold limit in the Punjab province (0.18 to 0.37 mg/L) (Mahmood and Malik, 2014) and the Khyber Pakhtunkhwa (KPK) province (0.19 to 0.62 mg/L) (Rehman et al., 2008).

Thus, in this context, it is urgent to investigate the strategies to reduce the bioavailability of these heavy metals for plants, particularly crops and vegetables. Moreover, it is also required to find out the Cd-tolerant varieties. There are few reports on the varietal differences for accumulation, translocation and partitioning of Cd in different plant tissues (DPT) under induced Cd toxicity. So, the present study was conducted: 1) To screen out the Cd tolerant variety and to investigate the varietal performance of maize to induced Cd toxicity; 2) To determine the extent of the accumulation, partitioning and translocation of Cd in different parts (crown tissue, leaves and roots) of maize varieties, and 3) To estimate the consequence of Cd appliance on the growth and biochemical traits of various maize varieties.

Materials and Methods

Study location

The experiment was conducted in the rain protected warehouse of the Old Botanical Garden, University of Agriculture, Faisalabad-Pakistan under natural conditions. The geographical location of the experimental site is 31˚45′ N, 73˚14′ E at 180 m above the sea level with a semi-arid climate. The mean annual precipitation and evaporation are 375 mm and 1600 mm, respectively. The mean annual temperature is 24.8°C and the temperature normally ranges from 50°C to 4°C in the summer and winter season, respectively (Akram, 2020).

Experimental details

The experiment was designed to assess the Cd-accumulation in different maize parts: crown tissue (CT), leaves (L) and roots (R) and to check the effect of Cd-toxicity on growth and biochemical traits as well as the growth response of maize cultivars. The seeds of six maize cultivars (EV-1098, Sahiwal-2002/SA-2002/Sahiwal, EV-5098, Agati-2002/AG-2002/Agaiti, EV-6098, and Sadaf) were obtained from the Maize and Millets Research Institute (MMRI), Yusafwala, Sahiwal District, Pakistan. The 20 seeds per pot were sown in the plastic pots (20 cm diameter × 28 cm height) that filled with 10 kg of river sand. The sand was thoroughly washed with tap water and then thrice with distilled water before filling in the pots. The pots were placed in the warehouse under natural conditions of light and temperature. The experiment was arranged in a Completely Randomized Design (CRD) with a factorial arrangement.

Treatment application

Three different Cd-concentrations (0, 500 and 1000 µM) were made by dissolving cadmium chloride ($CdCl_2.2.5H_2O$) of Merck Company in distilled water. After seeds germination (three days), the seedlings were thinned to keep 5 uniform and healthy seeds per pot. Then, the plants were irrigated with Hoagland's nutrient containing solution (Hoagland and Arnon, 1950; Zhang et al., 2020) for a regular period of three days, until the completion of the experiment. After that, the 28-days old plants of all the varieties were treated with enhanced Cd-concentrations (0, 500 and 1000 µM solution) after every three-day interval seven times. Two factors (there different Cd-

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concentrations and six maize cultivars) were combined to make 18-treatment and each treatment was replicated thrice (54-pots). Only the water was supplied to the control plants. The Cd-treated and control plants were grown for another 21-day.

Growth traits

After 21 days of Cd-treatment, the morphological parameters such as the number of leaves, shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW) and stem diameter (SD) was recorded. Before uprooting, the shoot length (SL), the number of green leaves per plant (NOL) and stem diameter (SD) of each plant was measured. The SL was measured from the stem base to the top of the plant. The NOL for each treatment was counted and means values were calculated from three replicates. The SD was measured with Vernier Calliper. The RL was measured from the bottom of the plant to the stem base. After that, all the plants were harvested. The SFW and RFW of three plants per replicate were recorded immediately after uprooting the plants and the mean value per plant was calculated. Then, washed with distilled water twice, blotted dry and transferred to paper bags and stored for 24-48 hours in an oven set at 65°C and used for subsequent analysis.

Digestion of plant material for chemical analysis and determination of K^+, Ca^{2+}, P and Cd^{2+}

The plant parts including crown tissue (CT), leaves (L) and roots (R) were separated and shade dried to determine the K^+, Ca^{2+}, phosphorus (P) and cadmium (Cd^{2+}) contents. 0.5 g dried well ground plant material and 5 mL of Conc. HNO_3 was taken in digestion flasks and incubated overnight at room temperature. Then, 0.5 mL of HNO_3 was added into each digestion flask to boost the reaction and the flasks were placed on the hot plate. The temperature was gradually increased up to 200°C for 30-45 minutes until the complete transparent digestion. After cooling the flasks, the extract was filtered by filter paper and the volume was raised to 50 mL by adding distilled H_2O in the volumetric flask and used for the determination of K^+, Ca^{2+}, P and Cd^{2+}.

The potassium (K^+) and calcium (Ca^{2+}) contents were recorded using Flame Photometer (Jenway PFP7). Phosphorus (P) was determined by the ammonium molybdate method (Sparks et al., 1996; Akram et al., 2020). The Cd^{2+} amount in different plant parts was determined by the atomic absorption spectrophotometer (AAS) (Perkin Elmer Analyst-100).

Data analysis

The following indices were determined.

Bioconcentration factor (BCF) and transportation index (Ti)

Cadmium uptake was assessed by the bioconcentration factor (BCF) and bioaccumulation factor (BAF). The concentrations of plant and soil/substrate Cd was determined according to dry weight. BCF and BAF indicate the plant's ability to accumulate a specific metal regarding its concentration in the growing medium/substrate. They were calculated by the following formulas (Retamal-Salgado et al., 2017):

$$BAF_{CT} = \frac{C_{CdCT}}{C_{Substrate}} \quad \text{(Eq.1)}$$

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$$BAF_L = \frac{C_{CdL}}{C_{Substrate}} \quad \text{(Eq.2)}$$

$$BCF_R = \frac{C_{CdR}}{C_{Substrate}} \quad \text{(Eq.3)}$$

where C_{CdCT}, C_{CdL}, and C_{CdR} are the concentrations of Cd in the crown tissue, leaves, and roots, respectively; and $C_{Substrate}$ in the growing medium/substrate.

Translocation factor (TF)

The translocation factor (TF) gives the aerial parts/root concentration of metal and shows the plant's ability to transfer the metal from plant roots to the aerial parts (crown tissue and leaves). TF was calculated by the following formulas (Retamal-Salgado et al., 2017):

$$TF_{CT} = \frac{C_{CdCT}}{C_{CdR}} \quad \text{(Eq.4)}$$

$$TF_L = \frac{C_{CdL}}{C_{CdR}} \quad \text{(Eq.5)}$$

where C_{CdCT}, C_{CdL}, and C_{CdR} are the concentrations of Cd in the crown tissue, leaves, and roots, respectively.

Tolerance index (TI)

The tolerance index (TI) shows the plant's ability to tolerate Cd toxicity. TI was calculated using the following equation (Retamal-Salgado et al., 2017):

$$TI = \frac{DBM_{Cd-treatments}}{DBM_{Control}} \quad \text{(Eq.6)}$$

where $DBM_{Cd\text{-}treatments}$ and $DBM_{Control}$ are the dry biomass (DBM) of each Cd treatment and DBM of the control treatment (no added Cd), respectively.

Statistical analysis

To confirm the data variability and result's validity, all the data were analyzed at two levels. Firstly, all the data were used to treat all observations at the same time by principal component analysis (PCA) to assess the correlation among all the plant traits, Cd-treatments and growth parameters. Secondly, the data were analyzed at the variety level for each plant trait, Cd-treatments and plant parts. Tukey's test was conducted to observe the significant differences within-and-between Cd-treatment means. The Pearson correlations were conducted using the "lm" function in the R package (Akram et al., 2020). Mean values of the Ca^{2+}, K^{+1}, P and Cd^{2+} in different plant parts (crown tissue, leaves and roots) were mapped against each cultivar to observe the whole data patterns. The linear regressions were constructed to assess the bivariate relationship between different plant traits and parts of all the cultivars. All the values in figures and tables expressed as mean (triplicate) ± standard error (SE) and the values were considered significant at $p < 0.05$. All the statistical data were arranged in Excel sheets and analyzed using SPSS 21.0 and R software (version 3.6.0, R Development Core Team 2018).

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Results

Effect of Cd on the plant biomass and related growth traits

Varieties and Cd treatments were found to be significant for all the growth traits, such as shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW), shoot length (SL), root length (RL), stem diameter (SD) and the number of leaves plant^{-1} (NOL) (*Table 1*). The SFW, RFW, SDW, RDW, SL, RL, SD and NOL interacted and significantly changed with the Cd treatments (*Figs. S1, S2*). Under control conditions, the varietal effect of all the growth traits was noted significantly different for all the six varieties, which exhibited EV-1098 as the highest biomass yielding and SA-2002 lowest biomass yielding variety among all other varieties (*Table 1; Figs. S1, S2*). The EV-1098 performed better for all traits than other varieties. However, the biomass and all the other growth-related traits showed a significant decreasing trend for all the six varieties with an increased level of Cd. The reduction in the SFW, RFW, SDW, RDW, SL, RL, SD and NOL was recorded 56%, 45%, 34%, 30%, 61%, 45%, 35% and 63% (from control level), respectively. Maximum reduction was found in the NOL and minimum in the RDW, 63% and 30% reduction from the control level, respectively. All the varieties and treatments were found significant, while variety (V) × treatment (T) interactions were noted non-significant for all the growth traits, except stem diameter (SD) (*Table 1*). Significant positive correlations were observed between the different growth traits (*Fig. S3*).

Table 1. *Shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW), shoot length (SL), root length (RL), stem diameter (SD) and the number of leaves per plant (NOL) for the different Cd treatments*

Traits		SFW	RFW	SDW	RDW	SL	RL	SD	NOL
Maize varieties (V)	EV-1098	32.95 a	24.04 a	9.19 a	5.63 a	67.87 a	45.67 a	0.54 a	4.56 a
	SA-2002	23.45 d	20.00 b	6.38 c	3.70 c	54.25 d	30.83 e	0.36 d	4.22ab
	EV-5098	25.89bcd	16.53 c	6.93 c	3.90 c	57.93 cd	33.69 de	0.39 cd	3.78ab
	AG-2002	29.89ab	22.43ab	8.41ab	5.12ab	63.77ab	42.70ab	0.49 b	3.44 b
	EV-6098	24.52 cd	19.84 b	7.58bc	4.49bc	59.76bc	39.91bc	0.42 c	3.89ab
	Sadaf	28.51abc	21.95ab	7.30bc	4.12 c	61.19bc	37.14 cd	0.43 c	4.11ab
Treatments (T)	To (Control)	34.71 a	26.95 a	10.98 a	6.41 a	73.33 a	52.49 a	0.63 a	5.00 a
	T1 (500 µM)	28.34 b	20.24 b	8.20 b	5.11 b	64.12 b	39.00 b	0.46 b	3.83 b
	T2 (1000 µM)	19.56 c	15.20 c	3.71 c	1.95 c	44.95 c	23.48 c	0.22 c	3.17 c
ANOVA	V	***	***	***	***	***	***	***	*
	T	***	***	***	***	***	***	***	***
	V × T	ns	ns	ns	ns	ns	ns	*	ns

Different letters in the same column indicate the significant differences compared with Cd treatments and maize varieties according to Tukey's test ($p < 0.05$)

Cd uptake, translocation, partitioning and its effect on the different biochemical traits

The uptake of cadmium in the crown tissue (CT) was significantly affected by the varieties (V), Cd treatments (T) and interaction between them (V × T) (*Table 2; Fig. S4*). The contrast to different Cd levels (*Table 2*), the maximum Cd accumulation was

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noted with 1000 μM $CdCl_2$. While the contrast between different varieties (*Table 2*), the highest uptake of Cd in the crown tissue was recorded in two varieties, such as SA-2002 and EV-6098 (0.10 mg g^{-1}) the same amount of Cd in both varieties; and the lowest Cd uptake was found in the EV-1098 (0.03 mg g^{-1}) variety with significant differences (*Table 2, Fig. 1*).

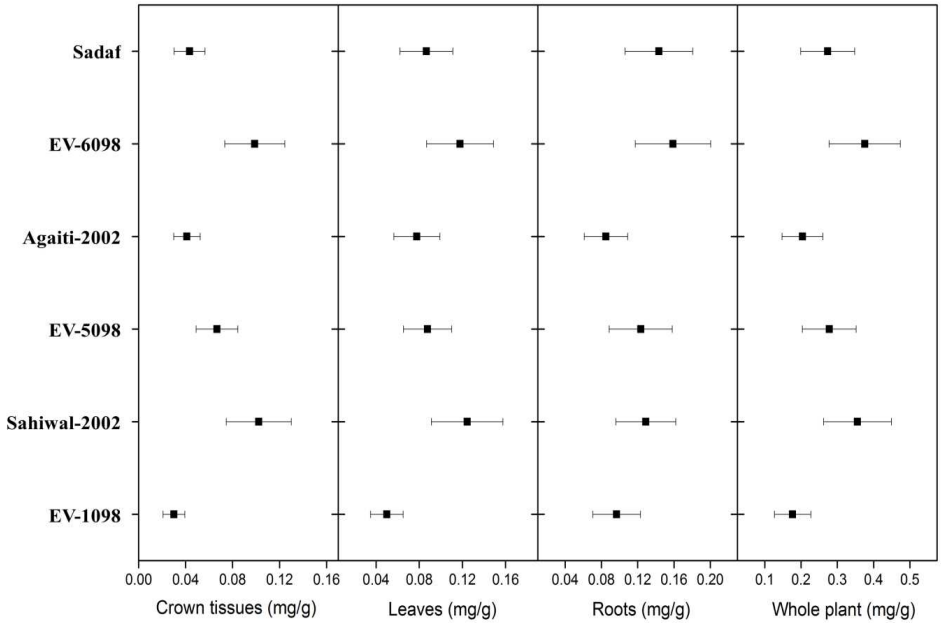

Figure 1. *The concentration of Cd (mean ± SE) in the different plant parts of the six maize varieties*

The Cd uptake in the leaves (L) was substantially influenced by the varieties (V), Cd treatments (T) and interaction between them (V × T) (*Table 2*). In comparison to $CdCl_2$ levels (*Table 2; Fig. S4*), the significant higher uptake of Cd was observed with 1000 μM $CdCl_2$ (0.17 mg g^{-1}) followed by 500 μM $CdCl_2$ (0.11 mg g^{-1}). The contrasting between different varieties (*Table 2*), it was noted that the significantly higher amount of Cd has accumulated in the leaves of the SA-2002 (0.12 mg g^{-1}) variety and the lowest in the EV-1098 variety (*Table 2, Fig. 1*).

As for the roots (R), the Cd uptake was significantly affected by the varieties (V), Cd treatments (T) and interaction between variety (V) × treatments (T) (*Table 2*). In the comparison between the Cd levels (*Table 2*), the highest uptake of Cd was recorded with 1000 μM $CdCl_2$ (0.22 mg g^{-1}) followed by 500 μM $CdCl_2$ (0.15 mg g^{-1}) with significant differences. In the case of varieties (*Table 2*), the higher Cd absorption in the roots of EV-6098 and Sadaf varieties with the values 0.16 mg g^{-1} and 0.14 mg g^{-1}, respectively. The significant lower Cd uptake was found in the EV-1098 (0.10 mg g^{-1}) variety (*Table 2, Fig. 1*). The mean values (mean ± standard error) for Cd uptake in the different plant parts and whole plant by all the studied varieties were shown in *Fig. 1*. The highest amount of Cd for the whole plant was uptake by two varieties: SA-2002 and EV-6098. The lowest Cd was uptake by the EV-1098 variety (*Fig. 1*).

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Table 2. *The concentration of Cd in crown tissue (Cd-CT), leaves (Cd-L) and roots (Cd-R); Ca concentration in crown tissue (Ca-CT), leaves (Ca-L), roots (Ca-R); K concentration in crown tissue (K-CT), leaves (K-L), roots (K-R); and P concentration in crown tissue (P-CT), leaves (P-L), roots (P-R)*

Traits		Cd-CT	Cd-L	Cd-R	Ca-CT	Ca-L	Ca-R	K-CT	K-L	K-R	P-CT	P-L	P-R
Maize varieties (V)	EV-1098	0.03 d	0.05 e	0.10 d	4.12 a	4.40 b	4.53 b	8.05 a	9.17 a	10.85 a	0.25 a	0.27 a	0.23 a
	SA-2002	0.10 a	0.12 a	0.13 c	3.55 b	3.70 c	3.77 c	3.73 d	4.27 e	5.37 e	0.17 e	0.18 d	0.16 d
	EV-5098	0.07 b	0.09 c	0.12 c	3.75 b	4.48ab	4.77 a	5.22 c	6.17 c	7.48 c	0.21bc	0.22 b	0.21 b
	AG-2002	0.04 c	0.08 d	0.09 e	3.57 b	4.61 a	4.67ab	5.86 b	7.21 b	8.83 b	0.22 b	0.20 c	0.22 a
	EV-6098	0.10 a	0.12 b	0.16 a	3.74 b	4.33 b	4.48 b	5.00 c	5.38 d	6.66 d	0.19 d	0.20 c	0.21 b
	Sadaf	0.04 c	0.09 c	0.14 b	3.69 b	3.76 c	3.89 c	3.27 e	5.02 d	6.61 d	0.21 c	0.19 cd	0.19 c
Treatments (T)	To (Control)	0.00 c	0.00 c	0.00 c	4.10 a	4.52 a	4.68 a	6.58 a	7.87 a	9.52 a	0.22 a	0.23 a	0.22 a
	T1 (500 µM)	0.07 b	0.11 b	0.15 b	3.74 b	4.24 b	4.34 b	5.25 b	6.01 b	7.47 b	0.21 b	0.21 b	0.21 b
	T2 (1000 µM)	0.12 a	0.17 a	0.22 a	3.37 c	3.88 c	4.03 c	3.74 b	4.73 c	5.92 c	0.19 c	0.19 c	0.19 c
ANOVA	V	***	***	***	***	***	***	***	***	***	***	***	***
	T	***	***	***	***	***	***	***	***	***	***	***	***
	V × T	***	***	***	**	**	**	***	***	***	ns	ns	ns

Means sharing different letters in the same column indicate the significant differences compared with Cd treatments and maize varieties according to Tukey's test ($p < 0.05$)

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The Cd, Ca, K and P contents in the different plant tissues (such as crown tissues, leaves and roots) were significantly affected by varieties, Cd levels and interaction between them (*Table 2; Figs. 1,2*). The maximum Cd was accumulated in the roots and minimum in the crown tissues of all varieties (*Fig. S4*). Additionally, the roots retained maximum Cd rather than crown tissues and leaves. In general, the EV-1098 variety showed a minimum uptake of Cd compared with other varieties (*Fig. 1*). However, the same variety performed better in terms of Ca, K and P contents in different plant tissues as compared with other varieties (*Fig. 2*). In short, Cd contents in all plant tissues tended to increase with increased Cd level.

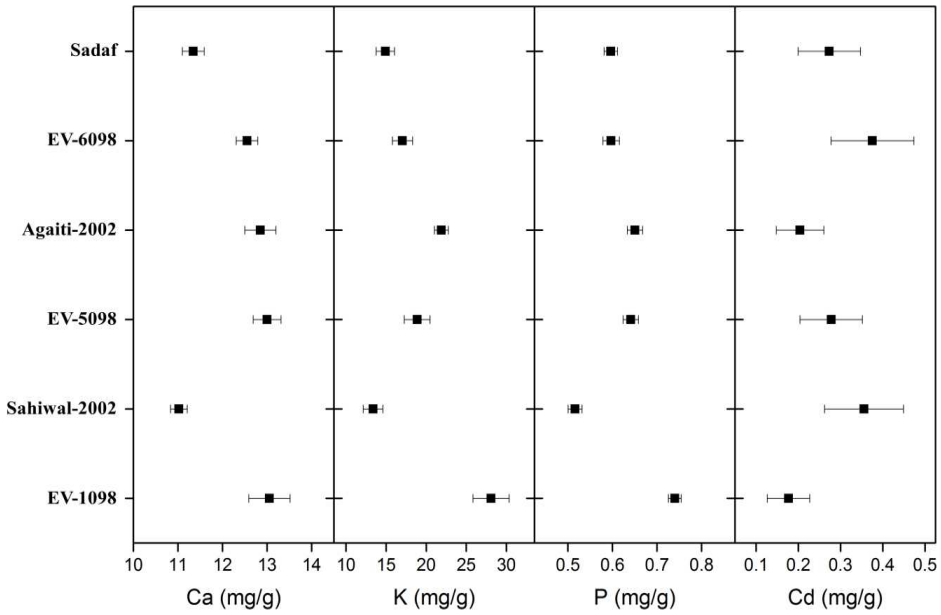

Figure 2. *The concentration of Ca, K, P and Cd (mean ± SE) in the six maize varieties*

The Ca, K and P contents interacted and significantly changed with the increasing Cd treatments (*Table 2*). In control conditions, the varietal effect of Ca, K and P contents were observed different significantly. However, an opposite trend was followed by Ca, K and P contents in all plant parts under Cd treatments i.e. an increase in the Cd level resulted in a decrease in maize plant biochemical traits (Ca, K and P contents) in the crown tissues, leaves and roots (*Table 2*).

The reduction in the plant total Ca, K and P contents from the control level were recorded 85%, 60% and 85%, respectively. All the varieties and treatments were found highly significant for the Cd, Ca, K and P contents in all plant parts (CT, L and R), while variety (V) × treatment (T) interactions were recorded non-significant only for the P contents in all plant parts (*Table 2*). The Ca, K and P contents exhibited significant positive correlations in three different plant parts (*Fig. S5*).

Correlations between Cd, plant morphological and biochemical traits

The regression lines were drawn to determine the correlation among different studied traits, such as plant total dry weight; and total Ca, K and P (*Fig. 3*). Results revealed that there existed a significantly positive correlation among plant total dry weight (PTDW); and total Ca (PT-Ca), K (PT-K) and P (PT-P). The linear regression

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coefficient reached to significant level for total Ca, K and P, respectively (*Fig. 3*). However, a significant negative correlation was observed among plant total Cd (PT-Cd), PT-Ca, PT-K and PT-P, total plant dry (PTDW) and fresh weight (PTFW) and plant height as shown in *Fig. 3*. The negative significant linear regression coefficient was recorded for total Ca, K, P, total dry weight, total fresh weight and plant height (*Fig. 3*).

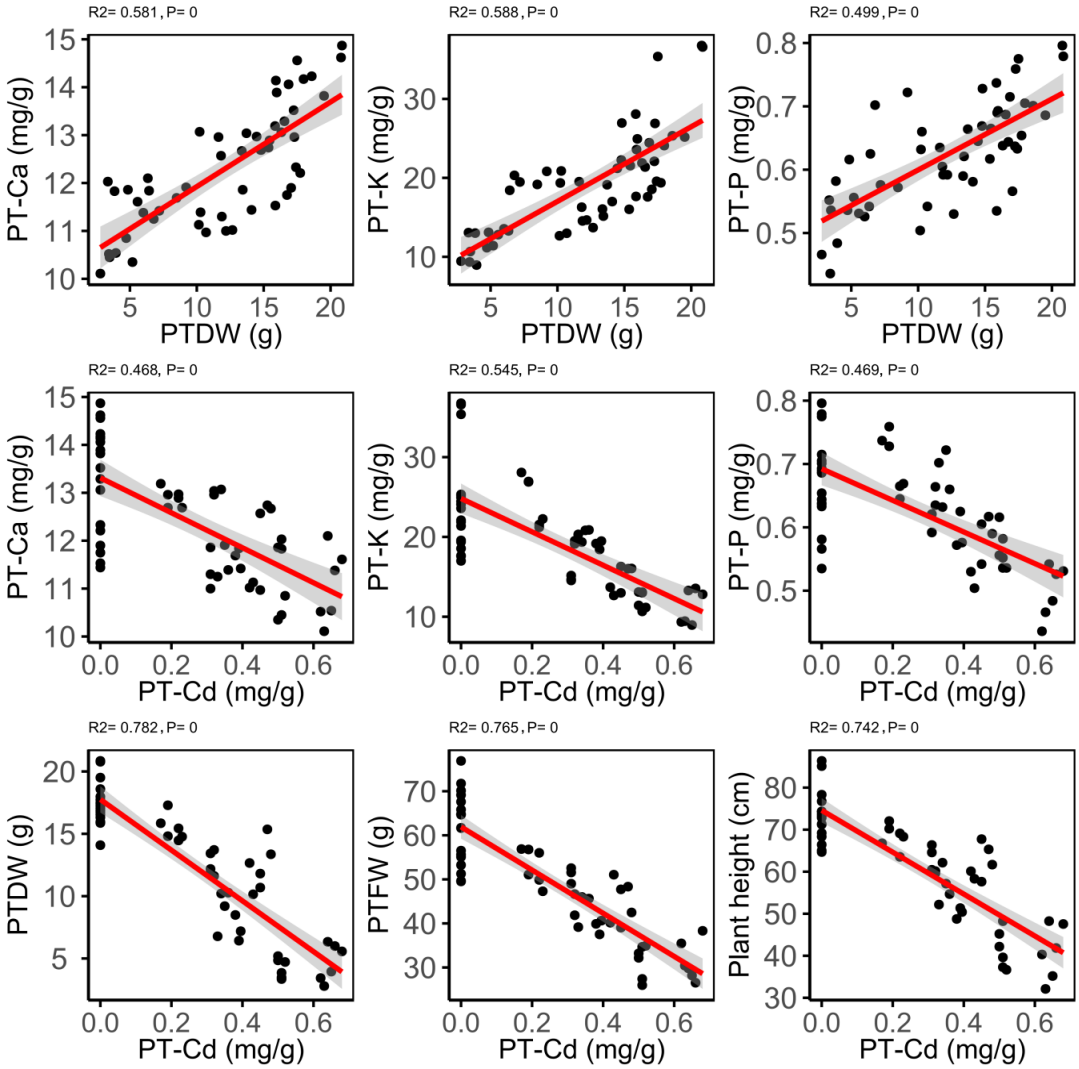

Figure 3. *The relationship between plant total dry weight (PTDW) and total Ca, K and P contents (PT-Ca, PT-K and PT-P, respectively) (in panel a, b, c); plant total Cd (PT-Cd) and total Ca, K and P contents (PT-Ca, PT-K and PT-P, respectively) (in panel d, e, f); and plant total Cd (PT-Cd) and total plant dry weight (PTDW), fresh weight (PTFW) and plant height (in panel g, h, i)*

Pearson correlation and principal component analysis (PCA)

Pearson correlation for plant different traits was shown in *Table 3*. The different significant correlations (both positive and negative) were found among maize plant growth traits (shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW), shoot length (SL), root length (RL), stem diameter

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(SD) and the number of leaves per plant (NOL); biochemical traits (Ca concentration in crown tissue (Ca-CT), leaves (Ca-L), roots (Ca-R), K concentration in crown tissue (K-CT), leaves (K-L), roots (K-R), P concentration in crown tissue (P-CT), leaves (P-L), roots (P-R); and Cd related traits, such as Cd concentration in crown tissue (Cd-CT), leaves (Cd-L) and roots (Cd-R) (*Table 3*). Particularly, the Cd in all the plant parts (CT, L and R) showed significant negative correlations with all the growth and biochemical traits of plants. Conversely, all growth traits exhibited significant positive correlations with all the biochemical traits (*Table 3*).

The principal component analysis (PCA) for this study is shown in *Fig. 4*. The first two PCA components exhibited maximum variation (84.1%) for all the parameters tested within the dataset. The PC1 and PC2 explained 74.6% and 9.5% variations, respectively. Moreover, the PC1 was positively loaded by the variables SDW, RFW, SDW, RDW, SL, RL, SD, NOL, CaCT, CaL, CaR, KCT, KL, KR, PCT, PL and PR; and PC2 was positively loaded by the variables CdCT, CdL and CdR. In comparison, a strong negative correlation was observed between the PC1 and PC2 parameters (*Fig. 4*).

Effect of Cd on the plant biomass and related growth traits, different factors/indices for Cd translocation, bioaccumulation, bioconcentration and tolerance

Translocation factors (TF)

The translocation factors (TF) was used to determine the plant's ability to translocate Cd from the lower part (roots) to the aerial parts (crown tissue and leaves) of the plant, which are the mean values of different Cd treatments and maize varieties shown in *Table 4*. The variations in TF_{CT} and TF_{L} showed accordingly with the different treatments and varieties. TF_{CT} and TF_{L} values observed at 1000 µM (0.54 and 0.76) were similar and not statistically different from those measured at 500 µM (0.51 and 0.74) (*Table 4*).

Bioconcentration factors (BCF)

For BCF, neither significant differences were noted among Cd treatments ($p > 0.05$) and nor for the different maize varieties (*Table 4*). The BCF showed an inverse relationship with Cd treatments, therefore with the increasing Cd supply, the BCF decreased (*Table 4*).

Bioaccumulation factors (BAF)

Conversely, BAF indicates the plant's capacity to accumulate Cd in the aerial part (crown tissue and leaves) of the plant, concerning the concentration of Cd in the substrate/soil. The variations in BAF_{CT} and BAF_{L} showed accordingly in *Table 4*. Maximum BAF_{CT} and BAF_{L} measured at 500 µM (1.29 and 1.87) and 1000 µM (1.05 and 1.49) were not statistically different (*Table 4*).

Tolerance index (TI)

The significant differences ($p< 0.05$) for TI were found at different treatments (Control, 500 µM and 1000 µM) with TI values 1.01, 0.77, and 0.33, respectively (*Table 4*). While, no significant differences ($p> 0.05$) were observed between the varieties (EV-1098, SA-2002, EV-5098, AG-2002, EV-6098 and Sadaf) with TI values 0.75, 0.64, 0.65, 0.74, 0.72, and 0.66, respectively. TI was decreased with the increasing Cd supply (*Table 4*).

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Table 3. *Pearson correlation for plant growth traits, biochemical traits and Cd related traits (Cd concentration in different plant tissues)*

Traits	SFW	RFW	SDW	RDW	SL	RL	SD	NOL	CdCT	CdL	CdR	CaCT	CaL	CaR	KCT	KL	KR	PCT	PL	PR
SFW	1																			
RFW	0.818**	1																		
SDW	0.865**	0.861**	1																	
RDW	0.831**	0.823**	0.932**	1																
SL	0.863**	0.821**	0.933**	0.937**	1															
RL	0.867**	0.841**	0.930**	0.925**	0.937**	1														
SD	0.877**	0.884**	0.960**	0.936**	0.949**	0.937**	1													
NOL	0.584**	0.714**	0.682**	0.636**	0.625**	0.631**	0.676**	1												
CdCT	-0.804**	-0.777**	-0.822**	-0.797**	-0.819**	-0.834**	-0.837**	-0.629**	1											
CdL	-0.832**	-0.828**	-0.889**	-0.852**	-0.871**	-0.887**	-0.906**	-0.712**	0.959**	1										
CdR	-0.811**	-0.840**	-0.873**	-0.839**	-0.840**	-0.863**	-0.892**	-0.689**	0.879**	0.939**	1									
CaCT	0.748**	0.740**	0.789**	0.771**	0.765**	0.774**	0.789**	0.658**	-0.670**	-0.760**	-0.704**	1								
CaL	0.592**	0.462**	0.651**	0.649**	0.630**	0.669**	0.666**	0.257	-0.510**	-0.574**	-0.590**	0.551**	1							
CaR	0.572**	0.397**	0.600**	0.602**	0.570**	0.625**	0.619**	0.285*	-0.486**	-0.559**	-0.565**	0.523**	0.917**	1						
KCT	0.709**	0.629**	0.725**	0.749**	0.709**	0.734**	0.741**	0.472**	-0.622**	-0.670**	-0.668**	0.718**	0.781**	0.756**	1					
KL	0.780**	0.683**	0.750**	0.749**	0.749**	0.783**	0.776**	0.518**	-0.751**	-0.755**	-0.719**	0.731**	0.769**	0.737**	0.945**	1				
KR	0.787**	0.712**	0.752**	0.761**	0.756**	0.781**	0.792**	0.520**	-0.750**	-0.759**	-0.714**	0.735**	0.771**	0.735**	0.923**	0.983**	1			
PCT	0.719**	0.613**	0.686**	0.680**	0.692**	0.708**	0.713**	0.404**	-0.745**	-0.710**	-0.587**	0.661**	0.679**	0.642**	0.784**	0.871**	0.884**	1		
PL	0.610**	0.499**	0.589**	0.611**	0.613**	0.611**	0.616**	0.440**	-0.615**	-0.643**	-0.534**	0.744**	0.583**	0.572**	0.832**	0.852**	0.841**	0.829**	1	
PR	0.676**	0.579**	0.700**	0.711**	0.701**	0.729**	0.724**	0.319*	-0.671**	-0.683**	-0.602**	0.660**	0.838**	0.801**	0.812**	0.838**	0.848**	0.892**	0.742**	1

SFW: shoot fresh weight; RFW: root fresh weight; SDW: shoot dry weight; RDW: root dry weight; SL: shoot length; RL: root length; SD: stem diameter; NOL: number of leaves; CdCT: Cd concentration in crown tissue; CdL: Cd concentration in leaves; CdR: Cd concentration in roots; CaCT: Ca concentration in crown tissue; CaL: Ca concentration in leaves; CaR: Ca concentration in roots; KCT: K concentration in crown tissue; KL: K concentration in leaves; KR: K concentration in roots; PCT: P concentration in crown tissue; PL: P concentration in leaves; PR: P concentration in roots. Correlation is significant at ** $p \leq 0.01$and * $p \leq 0.05$

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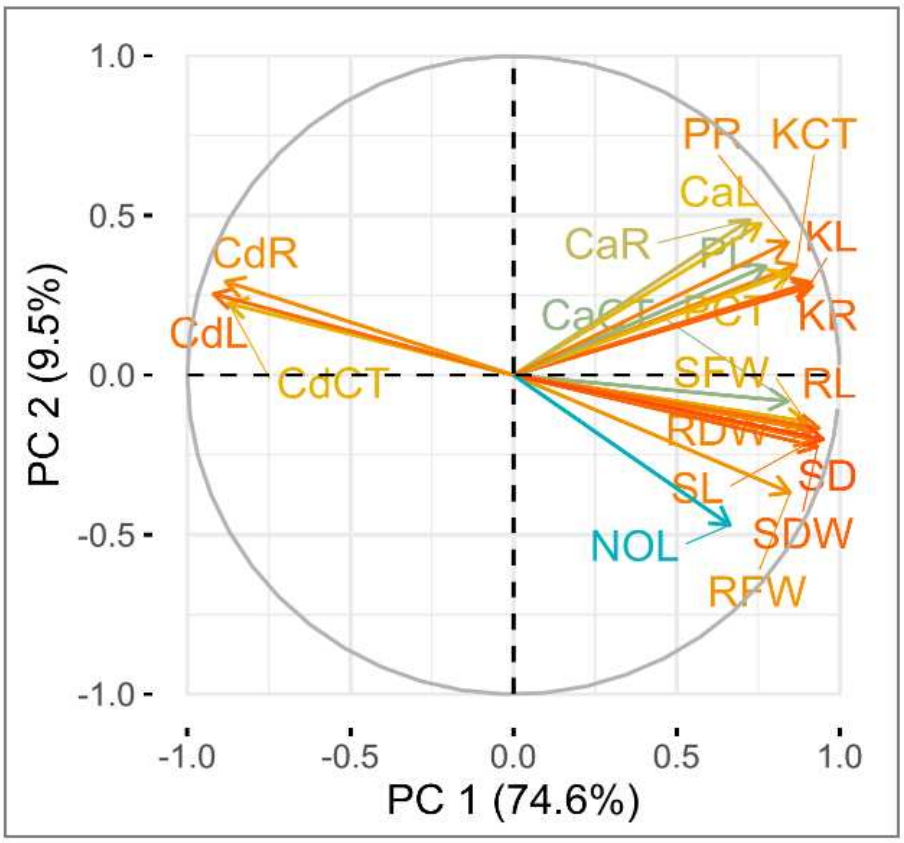

Figure 4. *The expression of growth traits, biochemical traits and Cd (in the different plant parts) on the two principal components analysis (PCA) axes. Loading values for PC axis 1 and 2; and color's difference show the contribution of each trait/variable*

Table 4. *Bioaccumulation factor (BAF), bioconcentration factor (BCF), translocation factor (TF) and tolerance index (TI) values for cadmium in the crown tissue (CT), leaves (L) and roots (R) according to different Cd treatments and maize varieties*

Factors		BAF		BCF	TF		TI
		BAF_{CT}	BAF_{L}		TF_{CT}	TF_{L}	
Treatments (T)	To (Control)	0.00	0.00	0.00	0.00	0.00	1.01a
	T1 (500µM)	1.29	1.87	2.60	0.51	0.74	0.77b
	T2 (1000µM)	1.05	1.49	1.97	0.54	0.76	0.33c
Maize varieties (V)	EV-1098	0.35	0.58	1.19	0.20	0.33	0.75
	SA-2002	1.26	1.54	1.65	0.52	0.64	0.64
	EV-5098	0.83	1.13	1.48	0.37	0.50	0.65
	AG-2002	0.50	0.95	1.02	0.33	0.62	0.74
	EV-6098	1.26	1.47	2.00	0.42	0.49	0.72
	Sadaf	0.50	1.04	1.81	0.19	0.39	0.66

Different letters in the same column indicate the significant differences compared with Cd treatments and maize varieties according to Tukey's test ($p < 0.05$)

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Discussion

Effect of Cd on the plant biomass and related growth traits

The growth traits such as SFW, RFW, SDW, RDW, SL, RL, SD and NOL interacted and significantly changed with the Cd treatments (*Table 1*). The growth traits of all varieties were reduced at applied Cd treatments and the highest reduction in these traits was pragmatic at 1000 μM $CdCl_2$ treatment level (*Table 1*). The growth traits decreased with the increasing Cd supply in the substrate/soil (Rascio et al., 1993; Klaus et al., 2013; Alia et al., 2015; Anjum et al., 2015). The fresh and dry weights of shoot and root; and shoot and root length reduced with increasing Cd level (*Table 1*). This reduction in the fresh and dry biomass as well as the length of maize plants may be due to Cd toxicity as well as nutrient disparity, lower water rate and nutrient uptake in plants (Ghani, 2010; Faizan et al., 2011; Alia et al., 2015; Rizwan et al., 2017; Abedi and Mojiri, 2020). Moreover, this could be due to a reduction in xylem transport caused by the interruption of the transpiration process in metal hassle, especially Cd (Hayat et al., 2020). The Cd toxicity also decreased the SD and NOL of maize plants i.e. the decreased trend with the exceeding Cd supply was observed (*Table 1*) in previous studies (Aslam et al., 2015; Figlioli et al., 2019). Generally speaking, the EV-1098 and AG-2002 variety performed better for all the growth traits than other varieties. However, the growth traits showed a significant decreasing trend for all the six varieties with an increased level of Cd (*Table 1*).

Cd uptake, translocation, partitioning and its effect on the different biochemical traits

The Cd uptake, translocation, partitioning and its effect on the different biochemical traits were observed in the different plant parts (leaves, crown tissue, and roots) of all varieties of maize at different Cd levels (*Table 2*). The Cd partitioning in the crown tissue, leaves and roots was substantially affected by the varieties, Cd levels and their interactions; particularly at higher Cd levels. The Cd accumulation gradually increased with increasing applied Cd levels in all the studied plant parts (*Table 2*), as observed in the former study (Nguyen et al., 2016). The Cd accumulation trend was recorded as: crown tissue < leaves < roots, 58%, 65% and 68%, respectively. The maize extraction capacity exhibit that the maize has the potential to accumulate heavy metals especially Cd and can be used for phytoextraction purposes (Retamal-Salgado et al., 2017). The maximum Cd was accumulated in the roots of all the six maize varieties rather than aerial plant parts (*Table 2*), similar results were found in previos studies (Zhao, 2011; Stritsis and Claassen, 2013; Singh and Srivastava, 2016; Ling et al., 2017; Boros-Lajszner et al., 2020), these results are in contrast to that the maximum Cd was accumulated in the plant aerial parts of maize rather than roots (Retamal-Salgado et al., 2017). It may be due to that the root first comes into contact with Cd and due to its immobility, more Cd retained in the roots (Anjum et al., 2015). Moreover, the Cd translocation from roots to aerial plant parts also limited by the suberization of endoderm and lignification of root cortex cells (Kaznina and Titov, 2014). For varieties, the minimum Cd concentration was noted for the EV-1098 variety (*Table 2*, *Fig. 1*), this is because the higher dry mass production was recorded in the same variety (*Table 1*) than other varieties (Trejo et al., 2016; Retamal-Salgado et al., 2017). The limitations of Cd accumulation in the roots and translocation from roots to other plant parts and several other factors could be involved to deal with Cd toxicity in maize (Yang et al., 2014). It may suggest that maize has effective and strong defense mechanisms to reduce

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Cd toxic effect than all other crops, including the Cd accumulation in the roots (Retamal-Salgado et al., 2017). Secondly, the Zn availability in the soils plays an important antagonistic role for Cd accumulation in the maize plants (Rizwan et al., 2019). Finally, the lower Cd levels possibly attributed to the higher efficiency of growth traits (*Table 1*) and biochemical traits/nutrients (*Table 2*), which result in the dilution of Cd toxic effect on maize plant (Retamal-Salgado et al., 2017; Rizwan et al., 2019). Overall, the total Cd extraction is also found significantly higher in the roots than the crown tissue and leaves (*Table 2*) (Singh and Srivastava, 2016; Boros-Lajszner et al., 2020). The same pattern was found in the other plants like wheat (Abedi and Mojiri, 2020) and mustard and oats (Boros-Lajszner et al., 2020).

The micro- and macro, both types of nutrients are required to perform the normal functioning, growth, and development in the plants. The deficiency of these nutrients, especially of macronutrients such as potassium, phosphorus, or calcium significantly affects the plant's metabolic processes (Sitko et al., 2019). The process of photosynthesis can't occur without the nutrients supply and directly dependent on mineral nutrition in plants (Engels et al., 2012). The uptake of heavy metals occurs in competition with other metals/elements such as Zn, Cu, Mn, Cd and Fe and may reduce the Fe, K, P, Zn and Ca uptake in the plants (Engels et al., 2012; Alia et al., 2015). The Ca, K and P are among essential nutrients that are required for plant growth (Engels et al., 2012). The Cd uptake, accumulation and partitioning adversely affect the nutrients uptake and distribution in plants (Alia et al., 2015). The Ca, K and P contents in the different plant tissues were significantly affected by varieties, Cd levels and interaction between them (*Fig. 3, Table 2*). In short, the Ca, K and P contents interacted and significantly decreased with the increasing applied Cd levels in all the studied plant parts of all the six maize varieties (*Table 2*) (Alia et al., 2015; Nguyen et al., 2016). The higher Cd concentration interrupts the ATPase and other enzymes functioning which uptake the K and as a consequence reduced K availability for plants (Erel et al., 2015). The K deficiency indirectly influences the photosynthetic activity and stomatal conductance of plants (Akram et al., 2020). Thus, a sufficient amount of K contents is necessary for the higher rate of plant water contents, transpiration, and stomatal conductance (Jin et al., 2011; Sitko et al., 2019). Ca plays an active role in stress signals transduction and works as an intracellular messenger (Engels et al., 2012; Hochmal et al., 2015). The distribution and uptake of Ca also considerably reduced because of the Cd treatment (*Table 2*), which may be due to the disruption of essential nutrient uptake supply like K, Ca and Zn by Cd (Alia et al., 2015). Moreover, the increase in Cd concentration also decreased the phosphorus contents in all parts of the maize plant (*Table 2*) (Shareef et al., 2018). Phosphorus is one of the main macronutrients that are necessary for nucleic acids, membrane lipids and the synthesis of ATP in addition to other metabolites (Akram et al., 2020). Moreover, P deficiency can disturb the regulation of stomata and transpiration (Singh et al., 2017; Sitko et al., 2019). In varieties, the EV-1098 variety performed better in terms of Ca, K and P contents in different plant parts and uptake maximum Ca, K and P as compared with other varieties (*Table 2, Fig. 2*) because minimum Cd uptake was noted for the same variety (*Table 2*). It also supports that increasing Cd levels decrease the minerals uptake and vice versa (*Table 2*) (Alia et al., 2015; Nguyen et al., 2016).

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Pearson correlation and principal component analysis (PCA)

The positive correlations between growth traits (SFW, RFW, SDW, RDW, SL, RL, SD and NOL) with biochemical traits (CaCT, CaL, CaR, KCT, KL, KR, PCT, PL and PR) (*Table 3*) exhibit the contribution of biochemical traits in the growth and biomass production of maize varieties. The adverse effects of Cd on the plant growth and biochemical traits in all plant parts (*Tables 1-3*) may be attributed to the disruption of essential plant nutrients absorption pattern by decreasing the efficacy of root proliferation. Moreover, the distribution and uptake of Cd associated with metal transporters (divalent cations), as the nutrients uptake higher, may result in the form of direct conflict with Cd for transportation in the plants (Naveed et al., 2020). Further, all the Cd treatments and studied plant traits were effectively displaced along with the first two PC axes (*Fig. 4*), which suggests the applied Cd treatments had a deteriorative effect on the studied traits of maize plants to the control (Kaznina and Titov, 2014; Nguyen et al., 2016).

Different factors/indices for Cd translocation, bioaccumulation, bioconcentration and tolerance

The plant's phytoextraction capacity is defined as TF, which is the measure of Cd concentration in the aerial parts and the roots of the plant (Retamal-Salgado et al., 2017). For all the treatments, TF_{CT} and TF_{L} values were found <1 (*Table 4*), which meant that the Cd concentration in the roots was higher than other plant tissues (crown tissue and leaves). As for 0 and 1000 μM, the big difference between the concentrations of roots and other plant parts suggests that there is an internal restriction for the Cd transport from roots to other plant tissues, resulting in higher Cd concentrations in roots rather than crown tissue and leaves (*Table 2*). The apoplastic barriers in the roots of maize played a significant role under Cd stress. Higher Cd concentrations may result in harm to root apoplastic barriers, which would be helpful in retarding Cd ion's transport from roots to other plant tissues (Ling et al., 2017). The TF values in this study (*Table 4*) don't coincide with the findings of the earlier researchers and TF values found lower than the reported values (Liu et al., 2013; Azzi et al., 2017; Retamal-Salgado et al., 2017), which may be affected by soil pH and differences of Cd treatment that result in the reduction of Cd supply from roots to the plant aerial parts (Liu et al., 2013). This result indicates the low Cd translocation efficiency of maize varieties used in this study.

The Cd absorption capacity of plants from substrate/soil is defined as BCF, which is the relationship of Cd concentration in the plant roots and the substrate/soil. The BCF values vary from 1.02 to 2.60 (*Table 4*) and found higher than the results of other authors (Retamal-Salgado et al., 2017), which may be due to difference in the Cd treatments and maize cultivars/varieties response to Cd stress (Ghani, 2010; Nguyen et al., 2016; Shah et al., 2016; Rizwan et al., 2017). The BCF values >1 (*Table 4*) exhibit the high Cd bioaccumulation capacity of maize at the root level (*Table 2*) (Singh and Srivastava, 2016). However, most of the BAF values found comparatively lower than BCF values (*Table 4*). It is an indication of an intrinsic limitation on Cd transport from roots to the aerial parts (crown tissue and leaves), resulting in higher Cd concentrations retains in the roots rather than other plant parts (*Table 2*) (Ling et al., 2017). Consequently, high Cd concentration interrupts the apoplastic pathway and retard the Cd transport from roots to aerial parts (Ling et al., 2017; Retamal-Salgado et al., 2017).

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The dry mass production in the different parts (crown tissue, leaves and roots) of maize plants were impaired by the different Cd levels (*Tables 1, 4*); whereas no significant differences were noted between the varieties for TI values (*Table 4*). It may be due to the higher Cd concentration that damaged the plant roots, affect nutrients uptake in the roots and subsequently reduce/inhibit the plant growth (Klaus et al., 2013; Kaznina and Titov, 2014; Yang et al., 2014). The Cd levels (Yang et al., 2014) were lower than those used in this study, it suggests that Cd levels applied in substrate/soil are higher than the threat threshold (3.5 mg kg^{-1}) (Yang et al., 2014). The decreased TI value with the increasing Cd supply (*Table 4*) further strengthens that the high Cd concentrations reduce the plant growth (Ghani, 2010).

Conclusions

The growth (plant biomass, height, stem diameter and the number of leaves) and biochemical traits (Ca, K and P) are found sensitive to Cd stress and negatively influenced by different Cd levels. The Cd toxic effect directly depended on the applied Cd stress, an increase in the Cd level resulted in a decrease in maize plant growth and biochemical traits. A considerable positive correlation was noted between the growth and biochemical traits. The uptake, translocation and partitioning of Cd were varied between the different plant parts and varieties, while, the Cd contents in all plant parts tended to increase with increased Cd level. The translocation factor (TF < 1) values emphasize that maize roots have some internal restriction, so the low Cd is translocated from the substrate to roots. Moreover, the maximum Cd accumulated and retained in the roots rather than crown tissues and leaves of studied maize varieties. The EV-1098 variety performed better for all growth and biochemical traits than other varieties. Further, the EV-1098 variety was found tolerant to Cd stress even at higher Cd levels (1000 μM); while SA-2002 and EV-6098 sensitive. The results revealed the diverse varietal performance/response of studied maize varieties to induced Cd toxicity. Consequently, the EV-1098 variety could be good in terms of the growth in Cd-polluted soils. In future, molecular studies are suggested at gene level to better understand the Cd interaction with the plant's physiological and metabolic processes.

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APPENDIX

Supplementary information

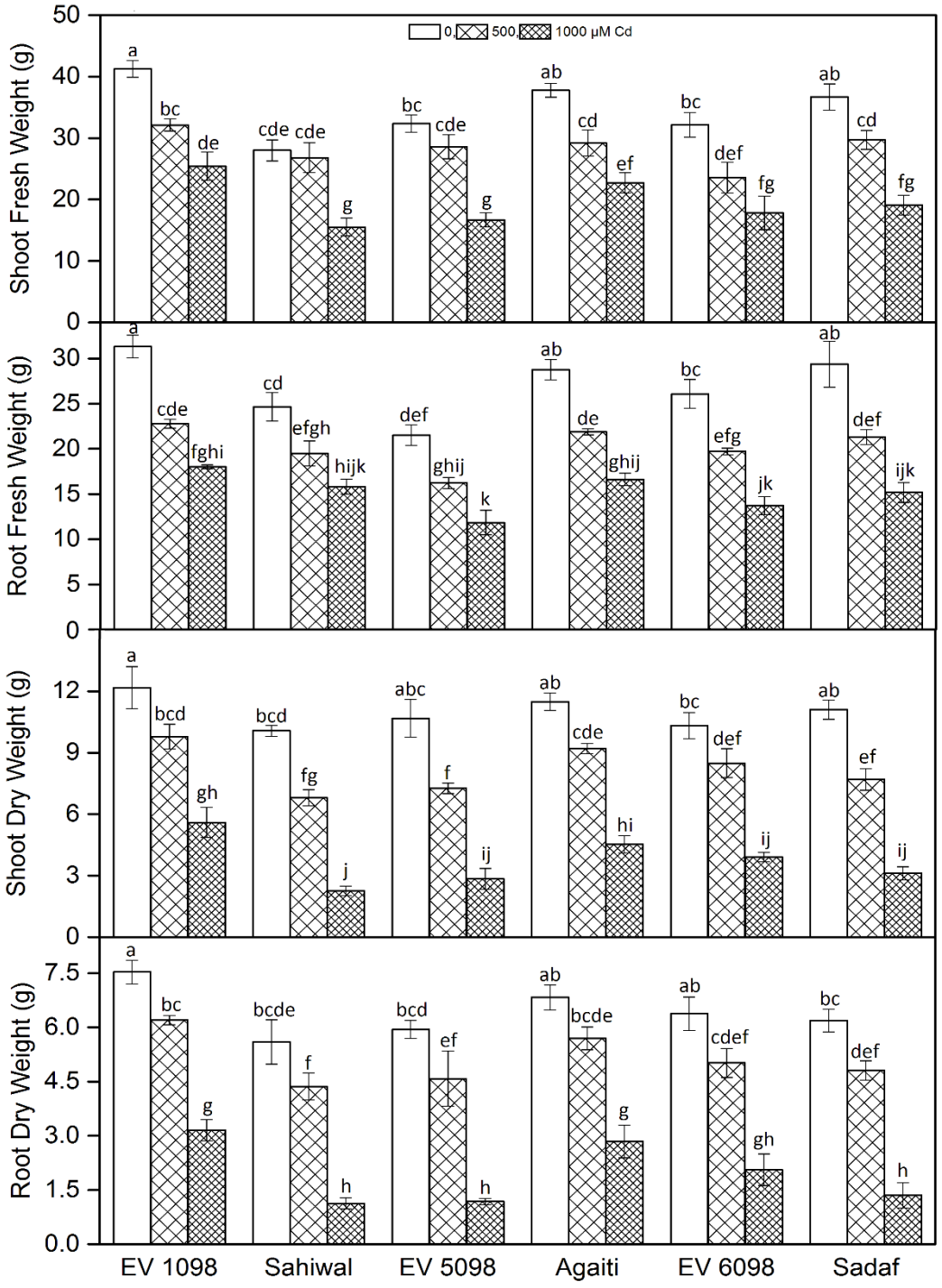

Figure S1. *Effects of Cadmium (Cd) treatments on shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW) and root dry weight (RDW) in the six maize cultivars. Bars (mean values ± SE) having different letters show mean values that are significantly different ($p<0.05$)*

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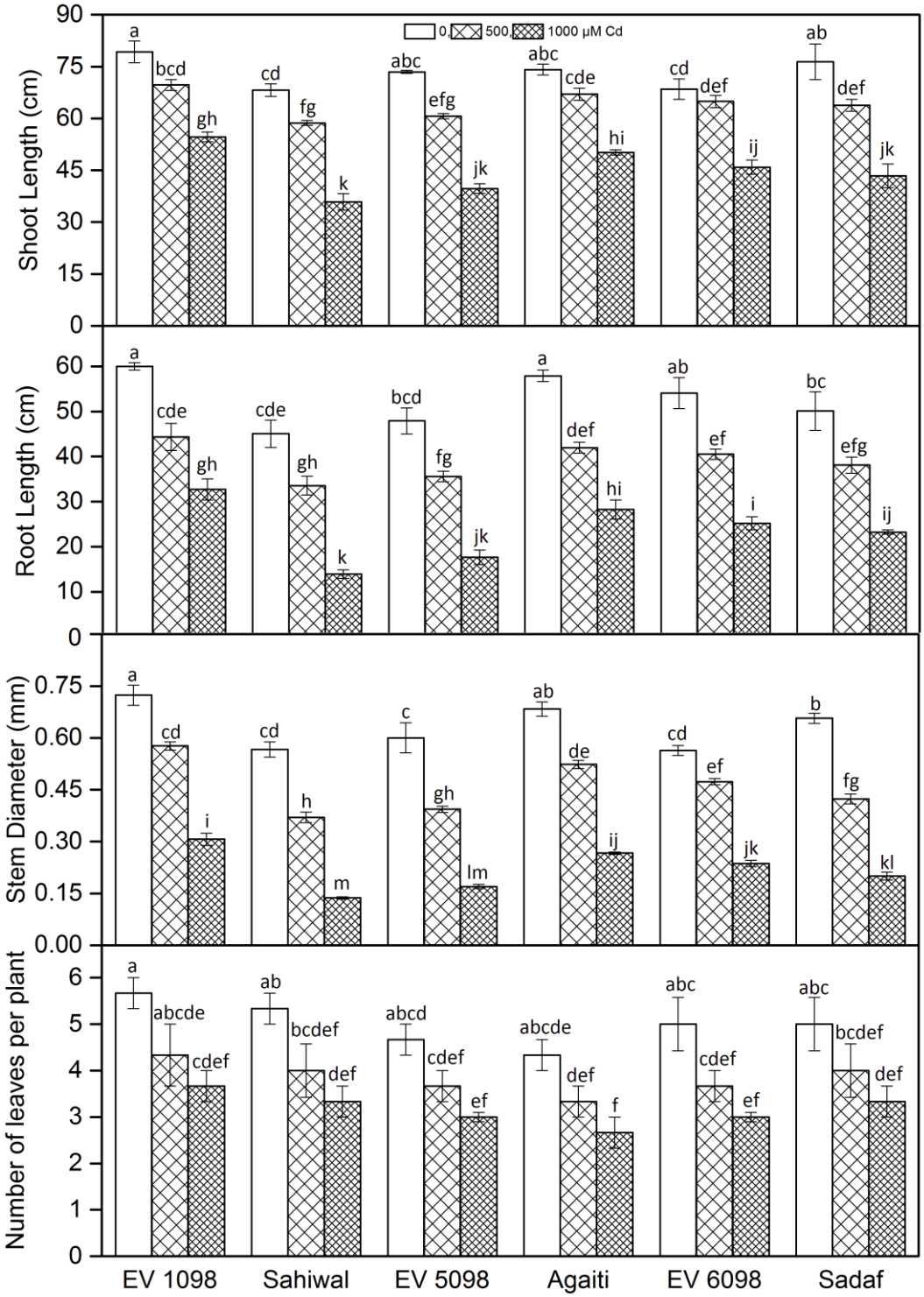

Figure S2. *Effects of Cadmium (Cd) treatments on shoot length (SL), root length (RL), stem diameter (SD) and number of leaves per plant (NOL) in the six maize cultivars. Bars (mean values ± SE) having different letters show mean values that are significantly different ($p<0.05$)*

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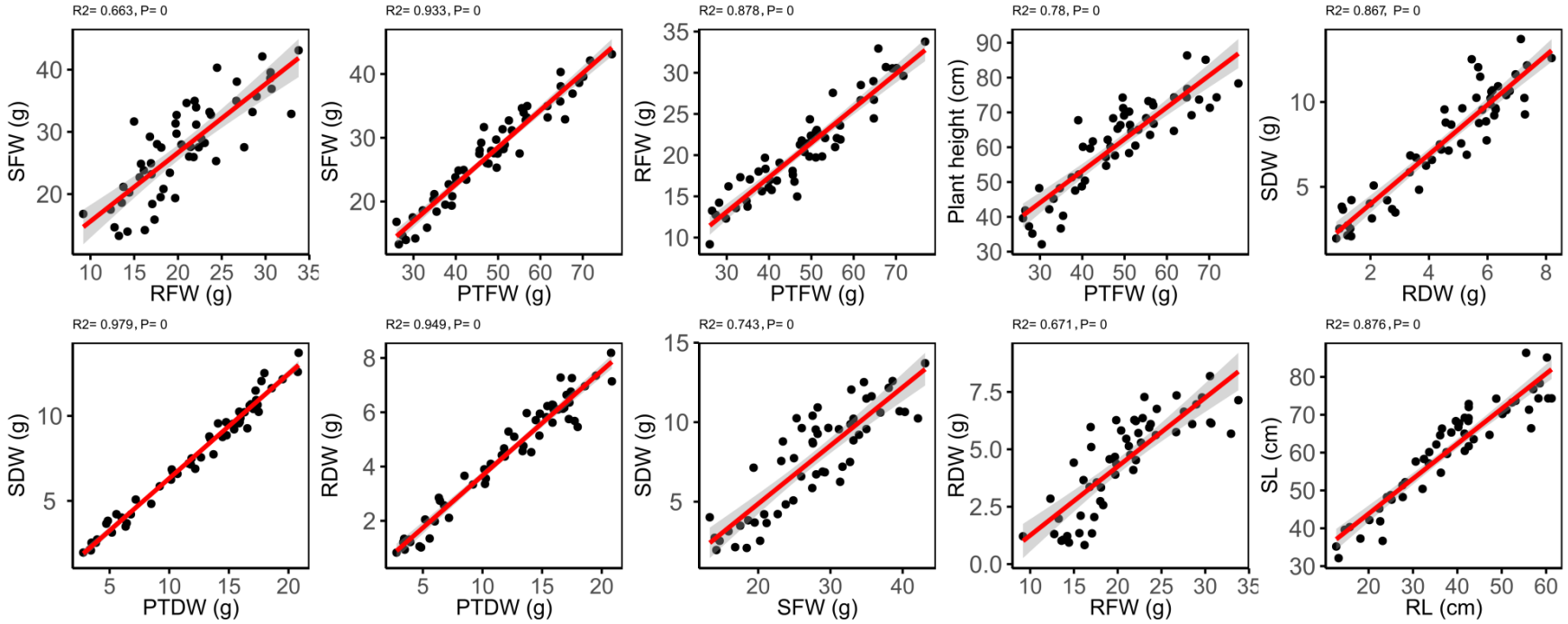

Figure S3. *Relationship between shoot fresh weight (SFW), root fresh weight (RFW), plant total fresh weight (PTFW), shoot dry weight (SDW), root dry weight (RDW), plant total dry weight (PTDW), shoot length (SL) and root length (RL)*

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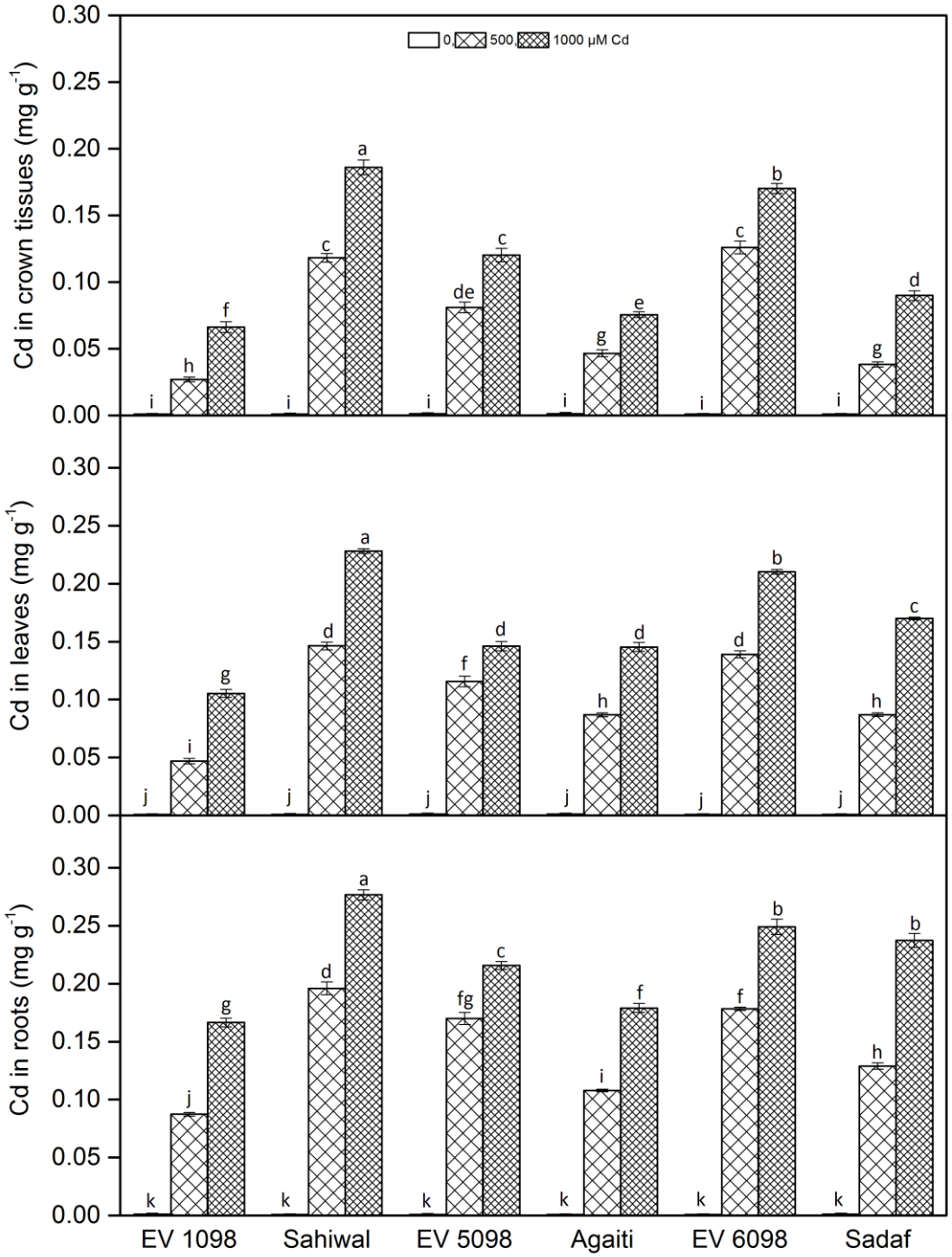

Figure S4. *Cadmium (Cd) partitioning in the crown tissues, leaves and roots of six maize cultivars at three different Cd treatments (Mean ± SE). Bars (mean values ± SE) having different letters show mean values that are significantly different ($p<0.05$)*

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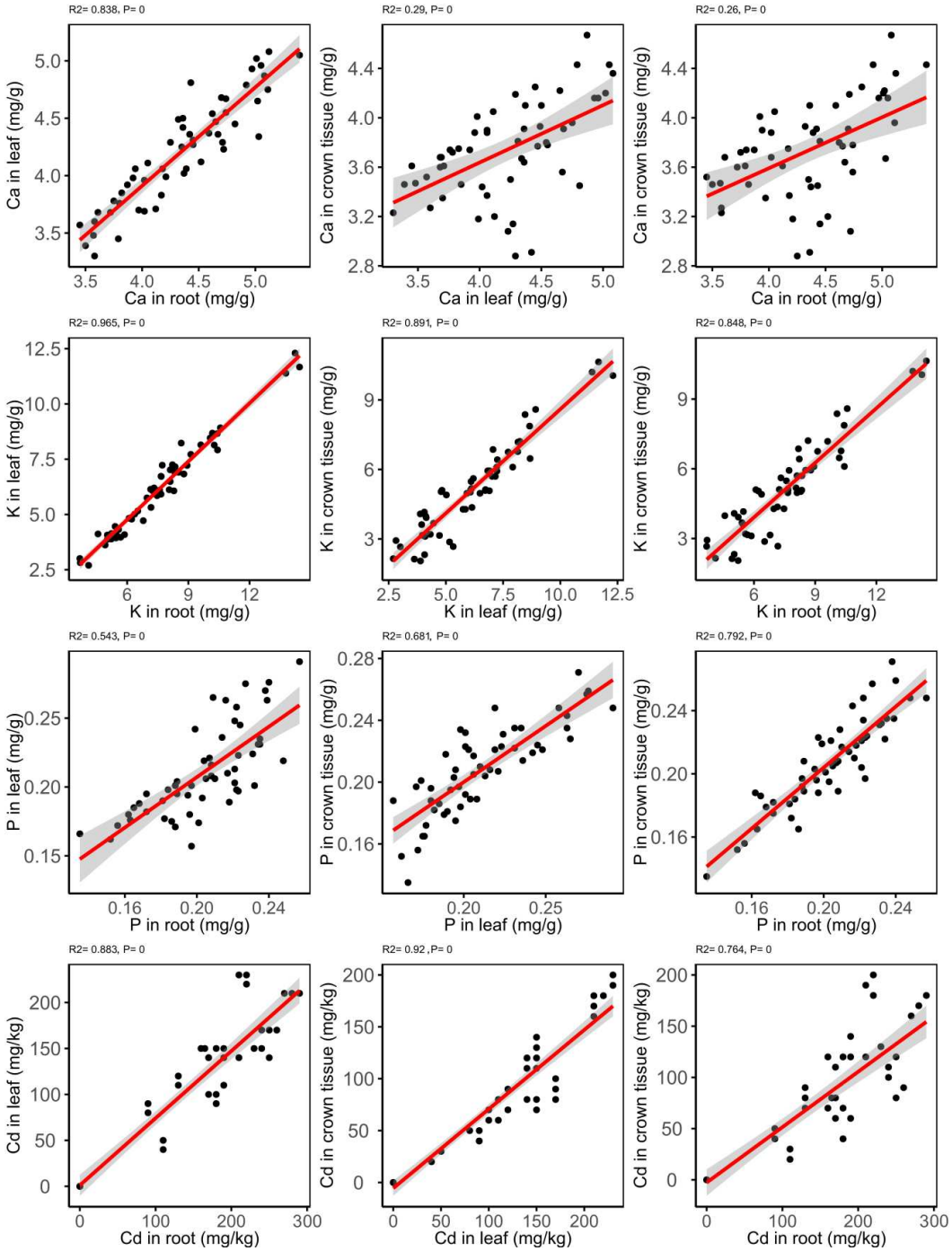

Figure S5. *The relationships between Calcium (Ca), Potassium (K), Phosphorus (P) and Cadmium (Cd) in the three different plant parts*

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EFFECTS OF N, P AND K FERTILIZERS ON EDIBLE AMARANTH (*Amaranthus* spp.) GROWN ON THE RED SOIL OF OKINAWA

AKAMINE, H. – OHSHIRO, M. – HOSSAIN, M. A.*

Faculty of Agriculture, University of the Ryukyus, Okinawa 903-0213, Japan

**Corresponding author*
e-mail: amzad@agr.u-ryukyu.ac.jp; phone: +81-98-895-8824

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Abstract. Fertilizer regimes were evaluated on edible amaranths to understand fertilizer management on the red soil (pH 5.1) of Okinawa. Effects of fertilizers 0 (Control), N, P, K, N+P (NP), N+K (NK), P+K (PK) and N+P+K (NPK) were evaluated on red leaf amaranth in two experiments. Each of the N, P and K fertilizers was applied at 50 g m^{-2}. In addition, the effects of NPK (N:P:K=1:1:1) fertilizer at 0, 10, 20, 30 and 40 g m^{-2} were evaluated on red stem amaranth and red leaf amaranth. Growth and yield of amaranth cultivated under N, P, K, NK and PK treatments were very poor, but significantly higher with the NPK followed by NP. Growth parameters and yield greatly increased with the NPK fertilizer at 30-40 g m^{-2} for red stem amaranth and 20-30 g m^{-2} for red leaf amaranth. Mineral contents in the amaranths did not clearly differ with the different fertilizers. Mineral contents were higher or same in the amaranths cultivated with the fertilizer NPK at 30-40 g m^{-2}, compared to those under control treatments. The results indicate that combined fertilizer NPK at 30-40 g m^{-2} is effective for higher yield and quality of amaranth in the red soil.
Keywords: *acidic soil, growth characteristics, minerals, nutritive-value, tropical vegetable*

Introduction

Different plant species respond differently to soil nutrient status, and fertilizer rates and combination (Hossain and Ishimine, 2005; Akamine et al., 2007; Chowdhury et al., 2008; Hossain et al., 2011). Balanced fertilizer is effective to sustain soil fertility, and growth, yield and quality of a plant species (Hossain et al., 2004; Akamine et al., 2007; Chowdhury et al., 2008; Shimray et al., 2019). The major nutrients N, P and K individually or together maintain growth, yield and quality of plants (Ivonyi et al., 1997; Nakano and Morita, 2009; Skwaryo-Bednarz et al., 2011; Hossain et al., 2012). Nitrogen, the principal element of chlorophyll, influences photosynthetic efficiency, which contributes to 26-41% of crop yield (Maier et al., 1994; Ivonyi et al., 1997). Potassium regulates activities of minerals and promotes N uptake efficiency of plants. Insufficient K causes shoot yellowing and low resistance to cold and drought in plants (Oya, 1972). Phosphorus enhances absorption of other nutrients and promotes plant growth when applied with other fertilizers (Akamine et al., 2007).

Amaranthus is a promising food crop for its resistance to heat, drought, diseases and pests, as well as high nutritional value (Sreelathakumary and Peter, 1993; Rastogi and Shukla, 2013; Longato et al., 2017; Maurya and Arya, 2018; Soriano-García et al., 2018). Several *Amaranthus* species are popularly cultivated as vegetable and grain in Africa, Bangladesh, Caribbean, China, Greece, India, Nepal and South Pacific Islands (Prakash and Pal, 1991; Stallknecht and Schulz-Schaeffer, 1993; Svirskis, 2003; Dewan et al., 2017). Vegetable amaranth, superior in taste to spinach (*Spinacia oleracea*), possesses higher carotenoids (90-200 mg kg^{-1}), protein (14-30%), carbohydrate

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(5.0 g 100 g^{-1}), fat (0.1 g 100 g^{-1}), calories (43 Kcal 100 g^{-1}) and ascorbic acid (28 mg 100 g^{-1}) (Abbott and Campbell, 1982; Prakash and Pal, 1991; Shittu et al., 2006; Dewan et al., 2017). *Amartanthus* possesses antioxidant, antimalarial and antiviral properties, which prevent cancer, cardiovascular diseases, diabetes, etc. (Dasgupta and De, 2007; Khandaker et al., 2008; Shukla et al., 2010; Adegbola et al., 2020).

Amaranthus grows very fast in tropical and subtropical regions under different agroclimatic and edaphic conditions (Singh and Whitehead, 1996; Dewan et al., 2017). In Okinawa, some amaranth species are found as weed in different soils (Hossain and Ishimine, 2005; Ohshiro et al., 2015). Some edible amaranth lines have been selected as summer vegetables in Okinawa (Ohshiro et al., 2015). Shittu et al. (2006) reported that balanced fertilizer is required to sustain higher yield and nutrients of amaranth in a specific soil.

Soil types and fertilizer regimes were evaluated on growth, yield, and quality of some *Amaranthus* lines in our previous study (Ohshiro et al., 2016). We have also evaluated fertilizer N levels and combined fertilizer NPK on amaranth in three major soil types, gray soil, dark-red soil and red soil of Okinawa. Gray soil was best for higher growth and yield of amaranths, and N fertilizer applied alone increased growth and yield in gray soil but not in dark red soil and red soil. On the other hand, combined fertilizer NPK resulted in the highest growth parameters and yield of amaranths in all soils. Previous studies evaluated fertilizer management strategies for amaranth cultivation on gray soil and dark-red soil (Ohshiro et al., 2016; Akamine et al., 2020). Gray soil covers only 5% of land, whereas dark-red soil covers 35% and red soil covers 60% in Okinawa (Hossain and Ishimine, 2005). In addition, we did not evaluate the effects of separate and combined application of N, P and K on the amaranths in red soil. Therefore, the objectives of this study were to (i) identify the effect of different fertilizer elements and (ii) evaluate rates of combined fertilizer on growth, yield and quality of edible amaranth lines to understand fertilizer management practices in red soil of Okinawa.

Materials and methods

Soil collection

Red soil (Ultisol, Kunigami mahji) was collected from the top 50 cm layer of a field in Nago city, Okinawa. The soil pH was 5.1, and the soil contained 0.06% total N and 0.20% total C. Sodium (Na), K, Ca, Mg, Al, Fe, P and Mn contents in soil were 0.69, 0.93, 14.51, 1.64, 0.04, 0.25, 0.28 and 0.02 mg kg^{-1}, respectively. Coarse sand, fine sand, silt, clay, and apparent density were 16.92%, 20.44%, 26.62%, 30.92%, and 0.92 g cm^{-3}, respectively.

Amaranth lines

Edible red stem amaranth (BB line) and red leaf amaranth (BC line) of *Amaranthus tricolor* selected as higher yield and quality in our previous studies (experiments conducted from April, 2010 to May, 2011) were evaluated in this study (Ohshiro et al., 2015).

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Experiment 1: Effects of N, P and K fertilizers applied alone and in combination on amaranth cultivated during November 5 to December 15, 2014

A glasshouse experiment was conducted at the Subtropical Field Science Center of the University of the Ryukyus, from November 5 to December 15, 2014. The experiment was consisted of eight treatments with five replications (planters). The fertilizer treatments were control (Cont), N, P, K, N plus P (NP), N plus K (NK), P plus K (PK) and N plus P plus K (NPK). Each of the N, P and K fertilizers at 50 g m^{-2} (5.0 g per planter) was mixed with 13 kg of air dried soil per planter (size 65E; 0.1 m^2) prior to the seed sowing according to the treatment design. Seed of red leaf amaranth was sown on the soil surface and covered with 0.5 cm soil layer. The planters were placed randomly, and the plants were thinned to the 8 healthiest stands per planter at 2- to 3-leaf stage. Water was applied as required (considering soil moisture checked by squeezing the soil sample firmly in hand to form an irregularly shaped "ball", plant size and growth stage, daily weather condition, etc.) every day for proper seedling emergence and plant growth. All the windows were kept open to maintain outdoor conditions (light, temperature, humidity) in the glasshouse during the experiment, except typhoon and rainy days.

Experiment 2: Effects of N, P and K fertilizers applied alone and in combination on amaranth cultivated during February 19 to April 4, 2015

Experiment 1 was repeated to reconfirm the effects of the fertilizers on the amaranth in the same glasshouse from February 19 to April 19, 2015. The experiment was consisted of eight treatments with five replications (planters). The same amaranth line, planter, treatments, fertilizer rates, seed sowing and management practices applied in the experiment 1 were taken in this experiment.

Experiment 3: Effects of NPK fertilizer rates on amaranth cultivated during June 20 to July 24, 2014

This experiment was conducted in the same glasshouse from June 20 to July 24, 2014. Each experiment was consisted of five treatments with four replications (planters). The fertilizer treatments of 0 g m^{-2} (Control, 0 g $planter^{-1}$), 10 g m^{-2} (1 g $planter^{-1}$), 20 g m^{-2} (2 g $planter^{-1}$), 30 g m^{-2} (3 g $planter^{-1}$) and 40 g m^{-2} (4 g $planter^{-1}$) were taken. The fertilizers of N ($CO(NH_2)_2$), P_2O_5 ($CaH_4(PO_4)_2H_2O$) and K_2O (KCl) were applied at the ratio of N:P:K=1:1:1. The fertilizers were mixed with 13 kg air dried soil per planter (size 65E) prior to the seed sowing according to the treatments. Seeds of red stem amaranth (BB line) and red leaf amaranth (BC line) were sown on the soil surface and covered with 0.5 cm soil layer. The planters were placed randomly, and the plants were thinned to the 10 healthiest stands per planter at 2- to 3-leaf stage. Water was applied as required every day for proper seedling emergence and plant growth.

Data collection

In the experiment 1, five plants were harvested at 40-day after seed sowing (DAS) from each planter, and plant height, stem diameter, leaf number, largest leaf area, total leaf area, and fresh and dry weights of leaf, stem and shoot (leaf+stem is called yield) were determined. In the experiment 2, plant height and leaf number were measured 6 times at a five-day interval strating from 23 DAS, and five plants were harvested from

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each planter at 44 DAS and similar data parameters were measured. In the experiment 3, five plants were harvested from each planter at 35 DAS, and similar data parameters were measured. Stem diameter was measured at 5 cm from the soil surface.

Determination of leaf area, dry weight, mineral, nitrogen, carbon, soil pH and nutrients

Leaf area was measured with an automatic area meter (AAM-8, Hayashi Denkoh Co. Ltd.). Various parts of amaranth plants were dried at 60 ℃ for 48 h for chemical analysis, and at 80 ℃ for dry weight measurement using forced convection oven (DRLF23WA, Advantec). Soil samples were dried at room temperature of 25-28 ℃ for 5 days. The plant parts and soil were ground finely for chemical analysis. Mineral contents of soil and nutrients of amaranth were determined with Inductively Coupled Plasma Spectrometer (ICPS-8100, Shimadzu Co. Ltd.). Total C and N were determined with Gas Chromatograph (Soil GS-8A, Shimadzu Co. Ltd., NC-220F Juka analysis center) and Sumigraph (NC-90A, Shimadzu Co. Ltd.). Soil pH was determined with TOA pH meter (HM-20S, Toa Electronic Ltd.).

Statistical analysis

Average data for each replication was calculated, and then mean and standard deviation (SD) of the replications were determined using analysis of variance. Fishers protected least significant difference (LSD) test at the 5 % level was used to compare treatment means. The amaranth lines were analysed separately.

Results

Effects of fertilizer N, P and K applied alone and in combination on growth and yield of amaranth line BC

Effects of fertilizer N, P and K applied alone and in combination on growth, plant height and leaf number of amaranth line BC cultivated from November 5 to December 15, 2014 are shown in the *Figs. 1 and 2*. The plants with the fertilizer N and NK grew for some days but did not survive finally. Growth of the plants with the fertilizer K, P and PK was very poor, and many of plants died. The growth of amaranth was best with the fertilizer NPK followed by NP.

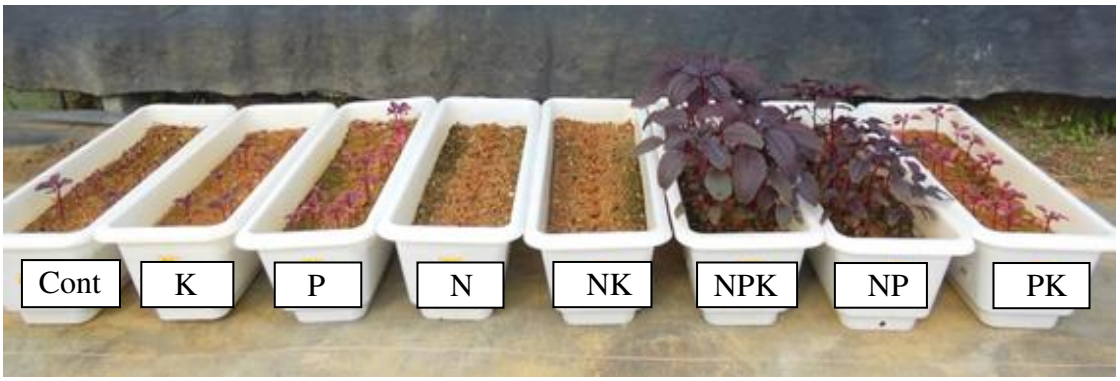

Figure 1. *Effects of fertilizer N, P and K applied alone or in combination on growth of amaranth line BC cultivated from November 5 to December 15, 2014*

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Plant height was the highest with the combined fertilizer of NPK, which was 3.6 times higher than that with the control treatment (*Fig. 2*). The fertilizer N and NK showed adverse effect on plant height. The other fertilizer treatments resulted in increased plant height. The fertilizer NP resulted in the second highest plant height. Plant height with the NPK was about two times higher than the plant with NP (*Fig. 2*).

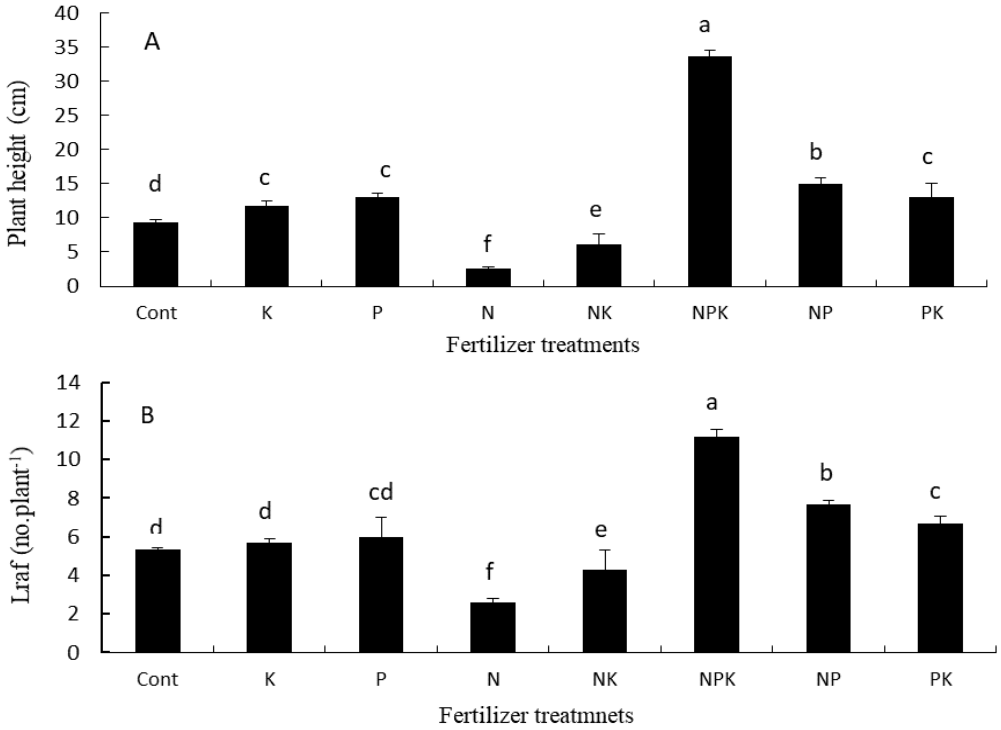

Figure 2. *Effects of fertilizer N, P and K applied alone or in combination on plant height (A) and leaf number (B) of amaranth line BC cultivated from November to December, 2014. Bars with the same letter are not significantly different at the 5% level, as determined by LSD test. Error bars represent standard deviation of the data*

The plant with the fertilizer NPK had highest leaf number (11) followed by the fertilizer NP (8) (*Fig. 2*). The fertilizer K and P applied alone did not result increased leaves per plant. The plant cultivated with the fertilizer N and NK resulted in the decreased number of leaves as compared with the control plant.

Data were not recorded for the fertilizers N and NK due to very poor plant growth. Stem diameter, largest leaf area, total leaf area, fresh leaf and dry leaf of amaranth were increased with the fertilizers NPK and NP (*Table 1*). Fresh and dry weight of stem and shoot (yield) were highest with the fertilizers NPK followed by NP. The fertilizers K, P and PK did not increase the growth parameters and yield of the amaranth. All the growth parameters and yield were highest with the fertilizer NPK followed by NP.

Plant height and leaf number of amaranth BC line cultivated from February 19 to April 4, 2015 under different fertilizers are shown in the *Fig. 3*. Plant height and leaf number (*Fig. 3*) were highest with the fertilizer NPK followed by NP. The leaf number was 11, 9 and 6 with the fertilizer NPK, NP and NK, respectively. The other fertilizer treatments did not show positive effect on plant height. The fertilizers P and PK showed somewhat positive effect on leaf number.

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Table 1. *Effects of fertilizer N, P and K applied alone or in combination on growth parameters and yield of amaranth cultivated from November to December, 2014*

Fertilizer treatment	Stem diameter (mm)	Largest leaf area (cm^2)	Total leaf area (cm^2)	Fresh leaf weight (g /plant)	Dry leaf weight (g /plant)	Fresh stem weight (g /plant)	Dry stem weight (g /plant)	Fresh shoot weight (g /plant)	Dry shoot weight (g /plant)
Cont	1.698c	2.325c	6.535c	1.456c	0.120c	0.602c	0.066c	2.058c	0.186c
K	1.750c	1.993c	6.675c	1.496c	0.210c	0.734c	0.048c	2.230c	0.258c
P	2.025c	2.743c	7.873c	1.887c	0.287c	1.065b	0.085c	2.952c	0.372c
N	-	-	-	-	-	-	-	-	-
NK	-	-	-	-	-	-	-	-	-
NPK	6.593a	26.760a	130.238a	15.561a	1.775a	17.566a	0.907a	33.127a	2.682a
NP	3.330b	11.250b	45.983b	4.998b	0.685b	2.659b	0.240b	7.657b	0.925b
PK	2.393c	5.486c	13.515c	1.660c	0.229c	1.689b	0.211b	3.349bc	0.440c

Data with the same letter within each column for each applied fertilizer are not significantly different at the 5% level, as determined by LSD test. - data not recorded due to poor growth

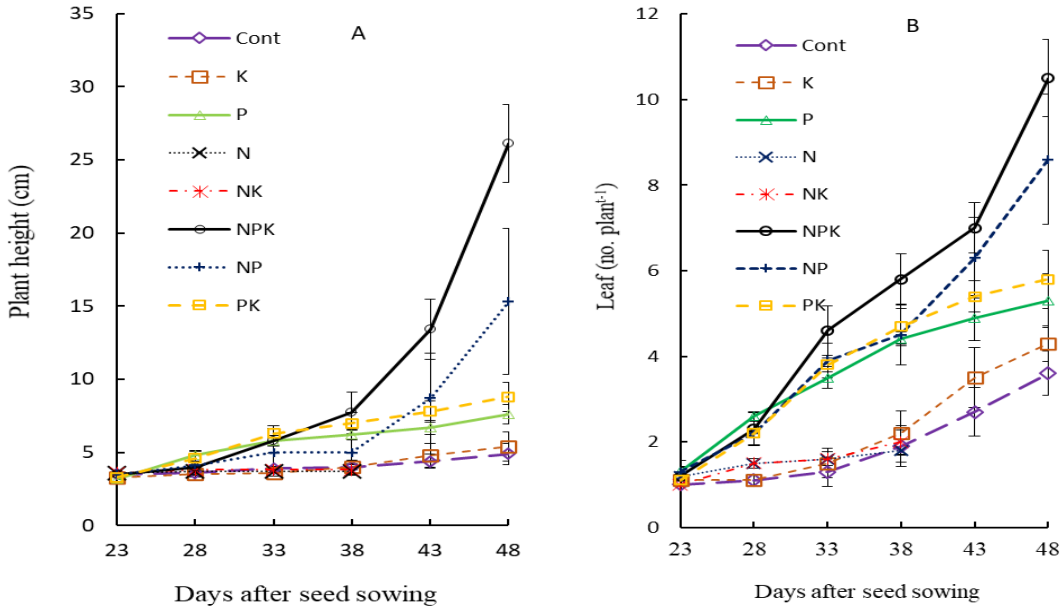

Figure 3. *Effects of fertilizer N, P and K applied alone or in combination on plant height and leaf number of amaranth line BC cultivated from February to April, 2015. Error bars represent standard deviation of the data*

Growth parameters and yield of amaranth line BC were not measured due to very poor growth (*Table 2*). Stem diameter and largest leaf area were highest with the fertilizer NPK followed by NP (*Table 2*). Total leaf area, fresh leaf and dry leaf increased with only NPK. Fresh and dry weights of stem and shoot were highest with the fertilizer NPK followed by NP (*Table 2*). Most of the growth parameters and yield (shoot) were highest with the fertilizer NPK followed by NP (*Table 2*).

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Table 2. *Effects of fertilizer N, P and K applied alone or in combination on growth parameters and shoot (yield) of amaranth cultivated from February to April, 2015*

Fertilizer treatment	Stem diameter (mm)	Largest leaf area (cm^2)	Total leaf area (cm^2)	Fresh leaf weight (g /plant)	Dry leaf weight (g /plant)	Fresh stem weight (g /plant)	Dry stem weight (g /plant)	Fresh shoot weight (g /plant)	Dry shoot weight (g /plant)
Cont	1.880c	6.807b	19.350b	1.456b	0.233b	0.602c	0.066c	2.058b	0.299c
K	1.946c	3.973c	13.680b	1.496b	0.210c	0.734c	0.058c	2.230b	0.268c
P	2.120b	6.073b	17.893b	1.887b	0.287b	1.065b	0.148b	2.952b	0.436b
N	-	-	-	-	-	-	-	-	-
NK	-	-	-	-	-	-	-	-	-
NPK	6.748a	34.893a	147.510a	15.561a	1.773a	17.566a	0.907a	33.127a	2.682a
NP	2.705b	7.518b	32.610b	3.066b	0.403b	1.689b	0.211b	4.755b	0.615b
PK	2.331b	4.711c	8.953c	0.413c	0.201c	0.202d	0.120b	0.615c	0.321c

Data with the same letter within each column for each applied fertilizer are not significantly different at the 5% level, as determined by LSD test. - data not recorded due to poor growth

Effects of NPK fertilizer rates on growth and yield of amaranth BB and BC lines

Growth of amaranth BB and BC lines cultivated in red soil is shown in the *Fig. 4*. The growth of amaranth BB line was better with the increasing fertilizer rates. However, the growth was similarly higher with the fertilizers 30 and 40 g m^{-2}. The growth of amaranth BC line increased with the increasing fertilizer rates up to 30 g m^{-2}, and the growth was found to be lower with the fertilizer 40 g m^{-2} than with the 30 g m^{-2}. The plant grown without fertilizer was very poor in both the amaranth lines.

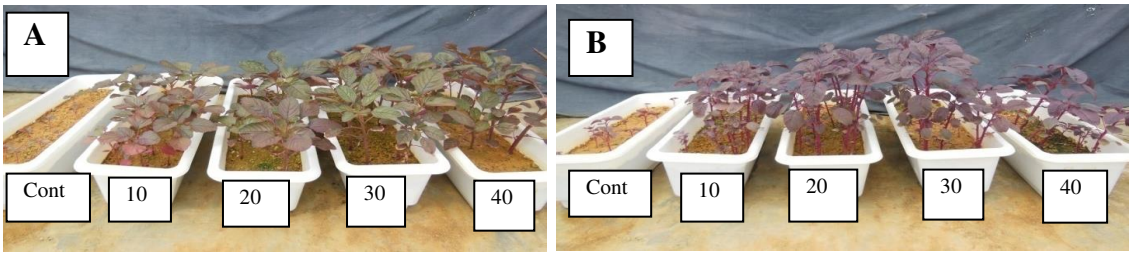

Figure 4. *Effects of combined fertilizer NPK rates on growth of amaranth lines BB (A) and BC (B) cultivated from June to July, 2014 in red soil. Cont (0 g), 10 (10 g m^{-2}), 20 (20 g m^{-2}), 30 (30 g m^{-2}), 40 (40 g m^{-2})*

Plants height of the amaranth BB line increased similarly with all the fertilizer rates, however the plant height was highest with the 40 g m^{-2} followed by 30 g m^{-2} (*Fig. 5*). On the other hand, plants height of the amaranth line BC increased with all the fertilizer rates, and the plant height was similarly highest with the 30 and 40 g m^{-2} (*Fig. 5*).

The leaf number of BB line increased with all the fertilizer rates, which was similarly highest with the fertilizer rate of 30 and 40 g m^{-2} (*Fig. 6*). The BC line obtained similarly higher leaf number with all the fertilizer rates, however the leaf number was found to be highest with the fertilizer 20 and 30 g m^{-2} (*Fig. 6*).

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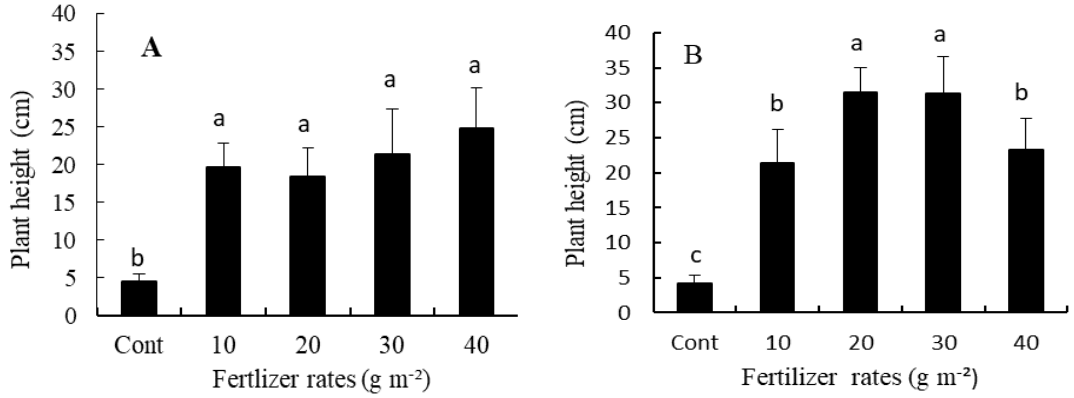

Figure 5. *Effects of combined fertilizer NPK rates on plant height of amaranth lines BB (A) and BC (B) cultivated from June to July, 2014. Bars with the same letter are not significantly different at the 5% level, as determined by LSD test. Error bars represent standard deviation of the data*

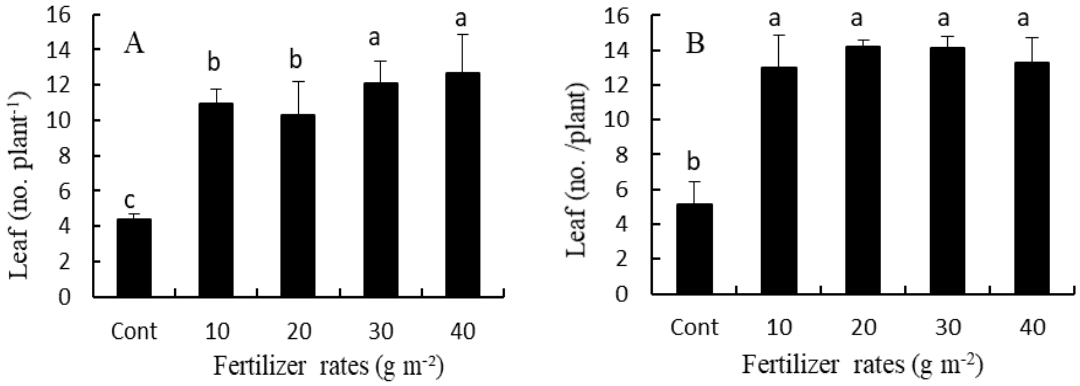

Figure 6. *Effects of combined fertilizer NPK rates on leaf number of amaranth lines BB (A) and BC (B) cultivated from June to July, 2014. Bars with the same letter are not significantly different at the 5% level, as determined by LSD test. Error bars represent standard deviation of the data*

Stem diameter of amaranth BB line was similarly higher with the fertilizer rate of 20-40 g m^{-2} (*Table 3*). Largest leaf and total leaf area were similarly highest with the 30-40 g m^{-2}. Fresh and dry weight of leaf and stem was similarly higher with the fertilizer rate of 20-40 g m^{-2}. Fresh and dry shoot weight was highest with the 40 g m^{-2} followed by 30 g m^{-2} (*Table 3*).

Amaranth BC line had increased growth parameters and yield (shoot) with all the fertilizer rates (*Table 3*), and the growth parameters and yield were similarly higher with the fertilizer rates of 20-30 g m^{-2}. The growth parameters and yield were found to be decreased with the fertilizer of 40 g m^{-2} (*Table 3*).

Effects of fertilizer N, P and K applied alone and in combination on minerals, N and C content of amaranths

The Na content increased with the fertilizer P and NP and decreased with the NPK and PK (*Table 2*). The K content increased with all the fertilizer treatments except NP,

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which was highest with the fertilizer NPK followed by P. The Ca content increased with the fertilizer K but decreased with the fertilizer NPK and NP. The content of Mg was not influenced with the fertilizers. The P content increased with the fertilizer NPK but decreased with the P and NP. The N content was highest with the fertilizer NPK followed by NP, and C content was lower with all the fertilizers (*Table 4*). Data was not recorded for the fertilizer treatments of N and NK due to very poor plant growth.

Table 3. *Effects of combined fertilizer NPK rates on plant growth parameter and yield (shoot) of amaranth lines BB and BC cultivated in red soil from June to July, 2014*

Fertilizer rates	Stem diameter	Largest leaf area	Total leaf area	Fresh leaf weight	Dry leaf weight	Fresh stem weight	Dry stem weight	Fresh shoot weight	Dry shoot weight
Lines (g m^{-2})	(mm)	($cm^2 leaf^{-1}$)	($cm^2 plant^{-1}$)	($cm^2 plant^{-1}$)	($cm^2 plant^{-1}$)	($cm^2 plant^{-1}$)	($cm^2 plant^{-1}$)	($cm^2 plant^{-1}$)	($cm^2 plant^{-1}$)
BB Cont	1.28c	2.08c	5.61d	0.15c	0.03c	0.16c	0.01c	0.31e	0.04d
BB10	4.16b	22.69b	101.60c	4.62b	0.61b	2.30b	0.21b	6.92d	0.82c
BB20	5.40ab	22.56b	131.39b	5.51ab	0.78ab	3.39ab	0.35a	8.90c	1.13b
BB30	5.61ab	24.37ab	138.75ab	6.73a	0.84a	3.55ab	0.38a	10.28b	1.22ab
BB40	6.01a	26.70a	153.19a	7.58a	0.97a	4.54a	0.41a	12.12a	1.38a
BC Cont	1.57c	1.71d	7.54d	0.25d	0.14c	0.13c	0.05c	0.38c	0.19c
BC10	4.88b	16.76c	75.74c	2.96c	0.41b	3.48b	0.30b	6.44bc	0.72b
BC20	7.06a	27.75a	121.82ab	6.55a	0.58a	9.22a	0.50a	15.77a	1.07a
BC30	6.69ab	27.56a	134.95a	6.25a	0.57a	9.51a	0.47a	15.76a	1.04a
BC40	5.85b	24.44b	105.91b	4.72b	0.39b	5.36b	0.23b	10.08b	0.62b

Data with the same letter within each column for each applied fertilizer are not significantly different at the 5% level, as determined by LSD test

Table 4. *Effects of fertilizer N, P and K applied alone or in combination on mineral, total nitrogen and total carbon content of amaranth cultivated from November to December, 2014*

Fertilizer Treatment	Na (mg g^{-2})	K (mg g^{-2})	Ca (mg g^{-2})	Mg (mg g^{-2})	Fe (mg g^{-2})	P (mg g^{-2})	TN (%)	TC (%)
Cont	6.1bc	61.6d	29.0b	20.5a	0.70ab	14.3b	2.43b	39.27a
K	6.9b	74.3c	46.6a	23.9a	1.01a	15.2b	2.49b	37.88c
P	10.4a	95.6b	27.7b	25.5a	0.53b	10.9c	1.64c	37.85c
N	–	–	–	–	–	–	–	–
NK	–	–	–	–	–	–	–	–
NPK	4.5c	118.2a	13.7d	11.5b	0.69ab	29.5a	6.49a	38.69b
NP	10.6a	43.8e	18.1cd	19.1a	0.48b	3.9e	3.87ab	37.87c
PK	4.3c	76.3c	25.6bc	19.1a	0.22c	6.7d	1.73c	37.22d

Data with the same letter within each column for each applied fertilizer are not significantly different at the 5% level, as determined by LSD test. - data not recorded due to poor growth

Effects of fertilizer NPK rates on mineral, N and C content of amaranths

In the amaranth BB line, Na and Ca content increased with the fertilizer rate of 10-20 g m^{-2} and 10 g m^{-2}, respectively (*Table 5*). The content of K and Mg was similarly higher with the 20-40 g m^{-2} and 10-30 g m^{-2}, respectively. The content of Al,

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Fe and P increased with all the fertilizer rates., and N and C contents were highest with the 20 g m^{-2}. In the BC line, the content of Na, K, Fe and P increased with all the fertilizer rates, and Ca, Mg and Al increased with the fertilizer at 10-30 g m^{-2}, 20-40 g m^{-2} and 40 g m^{-2}, respectively (*Table 5*). The K, Mg and P contents were highest with the 30 g m^{-2} followed by 40 g m^{-2}, and Ca was highest with the 20 g m^{-2} followed by 30 g m^{-2}. The Al content was highest with the 40 g m^{-2} followed by 30 g m^{-2} and Fe was highest with the 10 g m^{-2} followed by 20 g m^{-2}. The N content was highest with the 40 g m^{-2} followed by 30 g m^{-2} and C content was highest with the 10 g m^{-2} followed by 20 g m^{-2}.

Table. 5. *Effects of combined fertilizer NPK rates on mineral, nitrogen and carbon content of amaranth lines BB and BC cultivated in red soil from June to July, 2014*

Line	Fertilizer rates (gm^{-2})	Na (mg g^{-2})	K (mg g^{-2})	Ca (mg g^{-2})	Mg (mg g^{-2})	Al (mg g^{-2})	Fe (mg g^{-2})	P (mg g^{-2})	TN (%)	TC (%)
BB	Cont	6.73b	80.16c	52.48b	35.93b	0.45c	0.79c	22.63d	–	–
	10	7.63ab	112.00b	70.53a	44.36a	0.53b	0.83b	31.23c	2.75d	36.99b
	20	8.30a	135.66a	56.02b	44.25a	0.56a	0.81bc	30.53c	3.78a	37.21a
	30	6.83b	142.83a	55.73b	41.65a	0.57a	0.91a	44.00b	3.40b	36.93b
	40	6.86b	151.66a	55.60b	36.06b	0.58a	0.92a	49.26a	3.06c	37.00b
BC	Cont	6.23c	73.03d	36.40c	36.36bc	0.51b	0.68b	13.63d	–	–
	10	8.86b	117.66c	43.70b	32.53c	0.53b	0.90a	22.13c	2.23d	38.03a
	20	10.90a	144.33b	53.43a	38.16b	0.54ab	0.82a	23.03c	2.98c	37.40a
	30	10.93a	188.66a	48.53ab	44.93a	0.56ab	0.74b	31.60a	3.99b	37.07a
	40	9.56ab	175.66a	37.40c	44.10a	0.60a	0.72b	28.06b	5.07a	35.73b

Data with the same letter within each column for each applied fertilizer are not significantly different at the % level, as determined by LSD test. - data not recorded due to insufficient sample

Discussion

The growth of amaranth was best with the fertilizer NPK followed by NP, and plant growth was very poor with the fertilizer N, P, K, NK and PK and many of the plants died (*Figs. 1, 2 and 3*). The fertilizer N and NK showed adverse effect on plant height and leaf. All the growth parameters and yield (shoot) of amaranth were highest with the fertilizer NPK followed by NP (*Table 1*). The fertilizer N and NK showed adverse effect on all the growth parameters and yield of the amaranth, which indicate that P level is not available and N is not effective without P in the red soil. The fertilizers K, P and PK did not increase the growth parameters and yield, which indicates that amaranth plant cannot grow without combined fertilizers NP or NPK in the red soil. Similarly, several studies reported that shoot and root growth is reduced by P deficiency, N fertilizer contributes to 26-41% of crop yield, and P and K promote absorption of other nutrients and plant growth (Oya, 1972; Maier et al., 1994; Ivonyi et al., 1997; Sarker et al., 2002; Akamine et al., 2007).

Plants height of the BB line increased with all the fertilizer rates, however the fertilizer rates did not differ from each other, but they differed from the control. Plant height of the BC line did not increase with all the fertilizer rates, while it seems that a plateau was reached at 20-30 g m^{-2} rates, then the height decreased (*Fig. 5*). Similarly, the leaf number of the BC line increased only compared to control, therefore

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determining the highest value at a certain fertilizer rate is not relevant (*Fig. 6*). The stem diameter and leaf area of BB line were similarly highest with the 30-40 g m^{-2} (*Table 4*). Weight of leaf and stem was similarly higher with fertilizer rates of 20-40 g m^{-2}. Fresh and dry shoot weight was highest with the 40 g m^{-2} followed by 30 g m^{-2}. All the growth parameters and yield of amaranth BC line were similarly higher with the fertilizer rates of 20-30 g m^{-2} and found to be decreased with the 40 g m^{-2}. Similarly, Hossain et al. (2004) reported that too much fertilizer has a negative impact on *Panicum repens*. The amaranth BB line obtained 29% higher shoot (yield) biomass which required higher rate of fertilizer NPK, compared to line BC (the value was calculated from the best data of each amaranth line, *Table 3*). Similarly, *Panicum repens* required increasing rate of fertilizer with the increasing shoot biomass (Hossain et al., 2004).

The Na content was lower but K content was highest with the fertilizer NPK (*Table 2*). The Ca content increased with the fertilizer K but decreased with the NPK and NP. The content of Mg was not influenced with the fertilizers. The P and N content in amaranth increased with the fertilizer NPK. The results indicate that a specific fertilizer does not show the same trend in the accumulation of all the minerals and N; some minerals increased but other minerals decreased with a fertilizer element. Similar results were reported in several plants (Hossain et al., 2011; Gruber et al., 2013). It is difficult to clarify the fertilizer effects on the mineral accumulation in amaranth plants cultivated on red soil, which supported the results reported by Akamine et al. (2020).

The content of Na and Ca in amaranth BB increased with the fertilizer rate of 10-20 g m^{-2} and 10 g m^{-2}, respectively (*Table 5*). The content of K, Mg, Al and Fe was similarly increased with the fertilizer at 20-40 g m^{-2}, 10-30 g m^{-2}, 20-40 g m^{-2} and 30-40 g m^{-2}, respectively. The content of N was higher with the 20 g m^{-2}. The Na content of amaranth BC line was similarly higher with the 20-40 g m^{-2}. The K content was highest with the 30 g m^{-2} followed by 40 g m^{-2}, and Ca was highest with the 20 g m^{-2} followed by 30 g m^{-2}. The Mg and P content was highest with the 30 g m^{-2} followed by 40 g m^{-2}, Al was highest with the 40 g m^{-2} followed by 30 g m^{-2}, and Fe was highest with the 10 g m^{-2} followed by 20 g m^{-2}. The N content was highest with the 40 g m^{-2} followed by 30 g m^{-2}. These results indicate that mineral accumulation influenced by fertilizer rates differ with the amaranth lines, and a certain level of fertilizer NPK may be required to accumulate a particular mineral, which is similar to the results in other study (Akamine et al., 2020). In addition, it is thought that positive or negative interactions occur among the minerals and N existed in the soil and supplied fertilizers, which influence differently in accumulation of mineral and N in the amaranth plants. However, the major minerals were increased with the fertilizer rates of 20-40 g m^{-2} in both the amaranth lines.

Conclusion

The plant cultivated with the fertilizer N, P, K, NK and PK was very poor in growth, and N and NK showed adverse effect. All the growth parameters and yield of amaranth were highest with the fertilizer NPK followed by NP. Growth parameters and yield were significantly higher with the fertilizer 30-40 g m^{-2} for the BB line, and with the 20-30 g m^{-2} for the BC line. The fertilizer NPK resulted higher content of K, P and N, and lower content of Na and Ca in the amaranths. The content of Na and Ca was not influenced by the fertilizer NPK rates, whereas the content of K, Fe and Mg was higher with the fertilizer 30-40 g m^{-2} in the amaranth BB line. The content of Na, K, Ca, Mg, P

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and N was higher with the fertilizer 30 g m^{-2} in the BC line. The results indicate that mineral accumulation differed with the fertilizer rates and amaranth lines, however major minerals increased with the fertilizer NPK rates of 20-40 g m^{-2}. The results indicate that P level was not available, N was not effective without P, amaranth plant could not grow without combined fertilizers NP or NPK, and mineral contents in the amaranths did not differ clearly with the individual or combined fertilizers in the red soil. Above results suggest that fertilizer NPK at 30-40 g m^{-2} is effective for higher yield and quality of amaranth in the red soil of Okinawa. However, it was not possible to evaluate clear effects of fertilizer N, P and K on yield and quality of amaranth in this soil. Therefore, more detailed experiments should be conducted considering the physical and chemical properties of the red soil to clarify the actual effects of individual and combined fertilizers of N, P and K on yield and mineral content of different amaranth cultivars.

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Conflicts of interests. The authors declare no conflict of interests.

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MODELS FOR ESTIMATING THE ABOVEGROUND BIOMASS OF HALOXYLON AMMODENDRON IN MINQIN, CHINA

TANG, C.-W.* – WANG, R. – LI, C.-L. – LU, G.-C.

School of Environmental and Municipal Engineering, Lanzhou Jiaotong University, Lanzhou 730070, Gansu, China
(phone: +86-139-1906-5396)

**Corresponding author*
e-mail: cuiwent73@mail.lzjtu.cn

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Abstract. To improve the natural environment of arid and desert areas and to protect sand vegetation, we take estimation of the aboveground biomass of *Haloxylon ammodendron* in the Liangucheng National Reserve as our research object in this study, using SPSS and Excel software for data analysis and processing. Models for estimating aboveground biomass in sandy and gravel soil were established, and the results showed that this value for *H. ammodendron* was significantly correlated with basal diameter and plant height, as well as their complex variables. The best estimation models in sandy and gravel soil are $W = 0.138\ (DH)^{1.397}$ and $W = 0.189\ (DH)^{1.433}$, which have prediction accuracies of 82.825% and 83.688% and average relative errors of 14.392% and 13.455%, respectively. These models have high fitting precisions and can be used to estimate the biomass of *H. ammodendron*.
Keywords: *desert vegetation; estimation models; Liangucheng National Reserve; sandy soil; gravel soil*

Introduction

Land desertification is one of the ten global environmental problems (Temidayo, 2015; Edward et al., 1998). Desert ecosystems are distributed in arid regions, with few species of animals and plants and a fragile ecological environment. The total area of arid and semi-arid land in the world is 5.17×10^7 km^2, about 70% of which is threatened by desertification (Cheng et al., 2013; Kosmas et al., 2014; Wang and Zhou, 2018). In China, desertified land is mainly distributed in the northwestern part of the country, having an area of 2.61×10^6 km^2. This comprises about 27.2% of the total national land. The degraded ecosystem in desert regions, which has become one of the major ecological/environmental problems in western China, exerts a negative impact upon economic and social development (Xu et al., 2019; Bian, 2011; Han et al., 2013; Hao, 2017).

Plants are important in desert ecosystems. We can learn about the productivity and environmental quality of such ecosystems by assessing plant biomass, which reflects the total amount of organic matter accumulated by the plant community within a certain time. Research on biomass can help us better understand the landscape structure and function of the vegetation community and the current situations of desert communities as well as the ecological environment (González-Paleo and Ravetta, 2018; Yin et al., 2018). Constructing a biomass-estimation model to reduce field work and avoid environmental damage has become a major method for estimating biomass (Zhang et al., 2014; Fan et al., 2011). Models for estimating the aboveground biomass of desert plants have been extensively studied in recent years. The aboveground biomass and biomass of stems of *Haloxylon ammodendron* in 3 ecotypes (sand, salt, and gravel) of the Gurbantongut Desert were investigated, and fitted estimation models were

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established (Song and Hu, 2011). Alberto Búrquez and Angelina Martínez-Yrízar established a biomass-estimation model for plant communities in three habitats (plain, dry valley, and hillside) of the Sonoran desert (Búrquez and Martínez-Yrízar, 2011). There have been some studies of the biomass-allocation patterns and estimation models of 5 desert-dominant shrubs in Western Ordos, Inner Mongolia (Dang et al., 2017).

Haloxylon ammodendron, which has good drought resistance, is a desert plant adapted to the mid-temperate desert climate. It is also an important tree species for windbreaking and sand fixation in arid regions because of its developed roots. It belongs to the category of secondary protected plants. Minqin, located in the blown-sand area of northwestern China, is a source of sandstorms. The ecological environment of this region is currently facing a grim situation due to its location and climate. Although environmental conditions are suitable for *H. ammodendron*, there are few natural plants in Minqin. In order to combat desertification and sandstorms, Haloxylon was introduced from Xinjiang by the government of Minqin beginning in the mid-1960s. People have paid greater attention to the cultivation and protection of Haloxylon following the establishment of the Liangucheng National Reserve; it also plays an important role in improving the fragile ecological environment of Minqin and its surrounding areas, reducing natural disasters, and maintaining ecological balance (Li and Liu, 2018; Chang et al.,2008; Ma and Wei, 2003).

Many scholars have studied the situation of *H. ammodendron* in Minqin in terms of such features as population characteristics and soil properties (Zhang et al., 2009, 2018; Chang et al., 2012; Wang et al., 2019); however, little has been written about the estimation of its biomass in this area. In this paper, we take *Haloxylon ammodendron* as our research object and Liangucheng National Reserve in Minqin, Gansu as our research area. *H. ammodendron* growing on sandy and gravel soils was investigated. SPSS and Excel software were used to analyze and process the data, and models for estimating the biomass of *H. ammodendron* in different habitats were established. These will be helpful in the further study of desert vegetation in arid areas. The purpose of this article is to protect the desert-vegetation community and ecosystem, observe the growth of *H. ammodendron*, and provide basic information for desertification control, ecological restoration, and sustainable development of degraded deserts in the reserve.

General situation in the study region

Liangucheng National Reserve in Minqin is the largest desert nature reserve in China and the only one in Gansu Province. It is located in the middle of Badain Jaran and the Tengger Desert, around the Minqin Oasis in the northeast of Hexi Corridor, downstream from Shiyang River (102°30′–103°57′E, 38°10′–39°9′N). The reserve is divided into three parts: a core region (1,210.6 km^2), a buffer region (1,516.6 km^2), and an experimental region (1171.6 km^2). The total land area is 3,898.8 km^2, accounting for a quarter of the area of Minqin (*Fig. 1*).

The region has an extremely arid continental climate with low rainfall, high evaporation, significant temperature variation, strong winds, and frequent sandstorms. Evaporation exceeds precipitation by 20 times with the annual mean precipitation being 110 mm. Summer and autumn rainfall account for about 80% of annual rainfall. The annual mean temperature here is 7.7 ℃, the extreme low temperature is −27.3 ℃, the extreme high temperature is 39.5 ℃, and the average diurnal variation is 14 ℃. The

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relative humidity is 45%, there are 137 frost-free days per year, and the maximum depth of frozen soil is 105 cm. The annual average wind speed is 2.4 $m \cdot s^{-1}$ and the average annual days of strong wind and sandstorms are 27.4 and 25.9, respectively. The soil texture is generally aridisols which is based on Soil Taxonomy (ST).

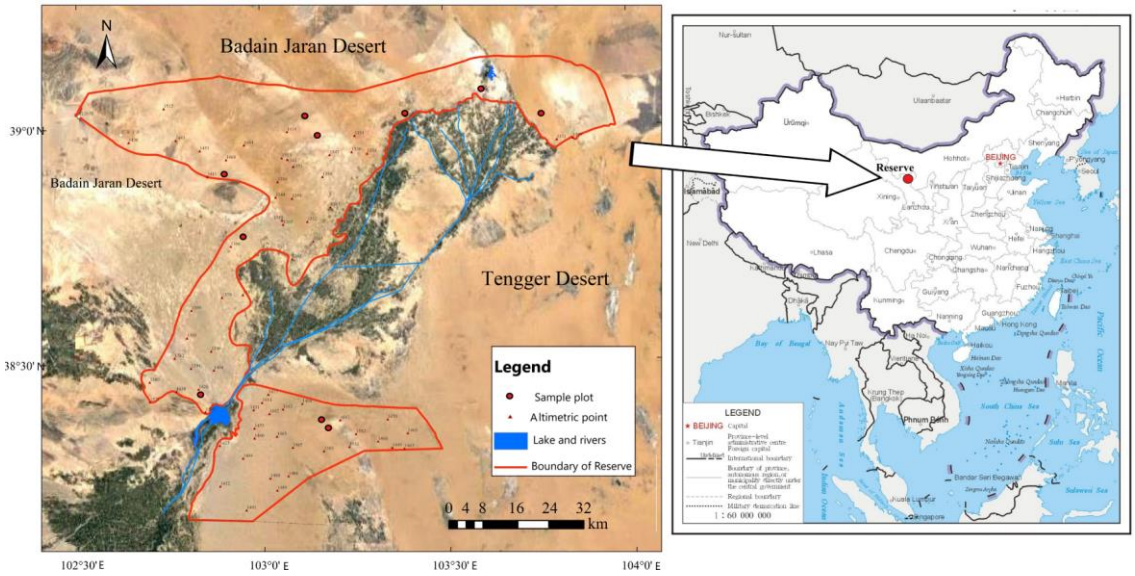

Figure 1. *Geographical location map of study area*

The vegetation community in the reserve mainly consists of super-xerophytes with obvious zonal characteristics. The major plant types in this area are xerophytes, psammophytes, and halophytes. And the vegetation is mainly composed of perennial herbs, subshrubs, and shrubs. There are two types of vegetation: natural and artificial. The main plant species of local natural vegetation are *Calligonum mongolicum*, *Nitraria tangutorum*, *Nitraria sphaerocarpa*, *Artemisia desertorum*, *Reaumuria songarica*, and others. The artificial vegetation includes the forest of *Haloxylon ammodendron* and *Calligonum mongolicum*, with *Haloxylon ammodendron*, *Caragana korshinskii*, *Tamarix chinensis*, *Hedysarum scoparium*, and *Calligonum mongolicum* as the major dominant species composing the community (Wang, 2003; Song et al., 2003; Ma et al., 2019).

Methods

Data source

Field investigation is adopted for this study. Plant samples were collected from sample plots of the reserve in September 2019, and *H. ammodendron* growing on sandy and gravel soils were investigated. Haloxylon in the reserve is mostly planted artificially. According to the opinions of local researchers concerning the relationship between Haloxylon and the environment, 10 typical vegetation regions (100 m × 100 m) of *Haloxylon ammodendron* are randomly selected in the reserve, including 6 regions of sandy-soil habitat and 4 regions of gravel-soil habitat. 5 plots (20 m × 20 m) were established in each region by five-spot-sampling method and 3–4 plants were randomly selected for investigation in each plot. The height (cm) and basal diameter (cm) of *H.*

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ammodendron were measured, and the plants were harvested and weighed on the spot to obtain the fresh weight (g). The plants were taken back to the laboratory and dried to a constant weight (80 ℃ for 48 h), and the dry weight (g) was obtained.

Selecting models

There are many models for estimating the biomass of trees, and some commonly used variables include basal diameter, plant height, and crown diameter. As a desert plant, *H. ammodendron* grows in sandy areas with strong winds for a long time. Because of the weather and other external conditions, it has a variety of tree shapes, and the crown diameter can easily change greatly. Hence, crown diameter is not suitable for use as an estimation factor in this study. In this paper, SPSS 19.0 and Microsoft Excel 2010 were used for data processing, and information from 7 plants was randomly selected from the data of each habitat for verification of accuracy. Other information was used to establish the estimation models. The independent variables of the models were selected based on statistical analysis of the dry weight (DW), basal diameter (D), plant height (H), and other complex variables (D^2, D^2H, DH, etc.).

To ensure the accuracy of the estimation, three different kinds of equation (linear, linear in two variables, and power function) were used to establish the biomass-estimation models of *H. ammodendron*. Such models are commonly used for the biomass estimation of shrubs and small trees (Zhao et al., 2004; Tao and Zhang, 2013). The basic forms of these equations are as follows:

$$Y = ax + b \quad \text{(Eq.1)}$$

$$Y = a_1x_1 + a_2x_2 + \ldots + a_nx_n + b \quad \text{(Eq.2)}$$

$$Y = ax^b \quad \text{(Eq.3)}$$

In the formulas, y is biomass, $x_1, x_2 \ldots x_n$ are the biomass-related factors, and a and b are the undetermined coefficients.

Model evaluation

After the establishment of the models, they must be evaluated to check whether they meet accuracy requirements and to select the best estimation model. There are many evaluation methods and indicators of the model, including the goodness-of-fit test, residual analysis, and determinant-coefficients analysis. The evaluation indices used in this paper are the correlation coefficient (r), residual sum of squares (RSS), adjusted R^2, average absolute value of relative error (RMA), root-mean-square error (RMSE), and prediction precision (P). The calculating formulas are as follows:

$$RSS = \sum_{i=1}^{n}(y_i - \hat{y}_i)^2 \quad \text{(Eq.4)}$$

$$TSS = \sum_{i=1}^{n}(y_i - \hat{y}_i)^2 + \sum_{i=1}^{n}(\hat{y}_i - \bar{y}_i)^2 \quad \text{(Eq.5)}$$

$$\text{Adjusted } R^2 = 1 - [RSS/(n - k - 1)] / [TSS/(n - 1)] \quad \text{(Eq.6)}$$

$$RMA = \frac{1}{n}\sum_{i=1}^{n}|(y_i - \hat{y}_i)/\hat{y}_i| \times 100\% \quad \text{(Eq.7)}$$

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$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}} \quad \text{(Eq.8)}$$

$$P = \left(1 - \frac{t_\alpha \sqrt{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}}{\hat{y}_i \sqrt{n(n-m)}}\right) \times 100\% \quad \text{(Eq.9)}$$

Here, y_i is the measured value, $\hat{y}_i$ is the estimated value, $\bar{y}_i$ is the average of the measured values, n is the number of samples, (n – k − 1) is the df (degree of freedom) of the residual sum of squares, (n − 1) is the df of the total sum of squares, m is the number of parameters in the regression model, ($t\alpha$) is the t-distribution value for a confidence level of α (α = 0.05).

RSS indicates how the degree of the measured values differs from that of the model; the adjusted R^2 can reflect the goodness of fit of the regression model and remove the influence of the number of variables. RMA and RMSE can measure the deviation between the estimated value and the measured value; P can test the estimation effect of the model (Song and Hu, 2011; Xu and Zou, 2004).

Results and analysis

Selecting variables

In this paper, correlation analysis of biomass and the indices of *Haloxylon ammodendron* in different habitats is performed (*Table 1*). The results show that the aboveground biomass of *H. ammodendron* is significantly correlated with these indices. The correlation coefficient between DW and D of *H. ammodendron* in sandy soil is lower than that in gravel soil; that between DW and H,DH is slightly lower than that in gravel soil (0.003 and 0.011 respectively); the correlation coefficients between DW and D^2, D^2H, and $(D^2H)^2$ are higher than those in gravel soil. In general, the correlation degrees of biomass and other indices of *H. ammodendron* in sandy soil are higher than those in gravel soil.

The correlation coefficients between biomass and the indices of Haloxylon in sandy soil range from 0.748 to 0.976 (D^2H > D^2 > DH > $(D^2H)^2$ > D > H), and those in gravel soil range from 0.751 to 0.964 (DH > D > D^2H > D^2 > $(D^2H)^2$ > H). In these two habitats, the highest degrees of interrelation are D^2H and DH, and their correlation coefficients are also in the forefront in the other habitat. For an integrative consideration, D^2H and DH are selected as independent variables of the biomass-estimation models.

Establishing biomass-estimation models

There are the biomass-estimation models for *H. ammodendron* in the form of linear, multiple-linear-regression, and power-function equations (*Table 2*). The correlation coefficients of the models are all higher than 0.9. We therefore offer our preliminary judgment that the models can be used to estimate Haloxylon biomass. The accuracies for all the models are validated and compared to each other. And the evaluation indices are calculated according to *Eq.4–Eq.9*. The RSS of the model is all high; this may be due to the large number and values of samples.

The correlation coefficients of the models of Haloxylon in sandy soil are between 0.947 and 0.978. Among the models, the prediction precision (P) and adjusted R^2 of

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Model 2 are highest (87.348%, 0.953) and the RSS and RMSE are smallest. *Model 3* has the highest correlation coefficient (0.978) and the smallest RMA. Putting all of this together, *Model 2* is the best model for estimating the aboveground biomass of *H. ammodendron* in sandy soil.

Table 1. *Correlation analysis of biomass and the indices of Haloxylon ammodendron in different habitats*

	D	H	DH	D^2	D^2H	$(D^2H)^2$
Sandy soil	0.900**	0.748**	0.953**	0.963**	0.976**	0.926**
Gravel soil	0.931**	0.751**	0.964**	0.861**	0.919**	0.766**

Table 2. *Estimation models of the aboveground biomass of H. ammodendron*

Habitat	Estimation model	r	Adjusted R^2	RSS	RMA (%)	RMSE	P (%)
Sandy soil	W = 1.211 (DH) −23.716 (Model 1)	0.959	0.915	8508.349	45.786	18.829	82.979
	W = 0.633 (D^2H) + 7.635 (Model 2)	0.955	0.953	4700.012	27.901	13.994	87.348
	W = 0.191 (D^2H) + 0.538 (DH) + 2.367 (Model 3)	0.978	0.952	7481.456	26.216	17.656	82.771
	W = 0.146 $(DH)^{1.372}$ (Model 4)	0.955	0.904	7434.171	29.327	17.600	82.825
	W = 1.895 $(D^2H)^{0.791}$ (Model 5)	0.947	0.893	12700.418	27.019	23.004	77.987
Gravel soil	W = 1.598(DH) −18.536 (Model 6)	0.964	0.922	3639.759	26.832	16.124	84.931
	W = 0.826 (D^2H) + 16.432 (Model 7)	0.919	0.831	7866.363	33.900	23.704	77.846
	W = 0.108 (D^2H) + 1.410 (DH) − 15.157 (Model 8)	0.964	0.916	3553.483	28.203	15.932	84.375
	W = 0.189 $(DH)^{1.433}$ (Model 9)	0.986	0.969	3987.974	17.605	16.878	83.688
	W = 2.139 $(D^2H)^{0.847}$ (Model 10)	0.981	0.959	6568.847	19.430	21.661	79.258

The correlation coefficients of the models for estimating *H. ammodendron* biomass in gravel soil are between 0.919 and 0.986. *Model 6* has the highest prediction accuracy (84.931%), but other indices are in the middle of the five models; the r and adjusted R^2 values of *Model 9* are the highest (0.986, 0.969), the RMA is the lowest, and its prediction accuracy is only 1.243% lower than *Model 6*. By synthesizing these factors, we find that *Model 9* is the best biomass-estimation model for *H. ammodendron* in gravel soil.

Verification and analysis of accuracy

The relative error reflects the reliability of measurement. The randomly selected Haloxylon data are used for verification and analysis of accuracy and the estimated values of Haloxylon biomass are calculated according to the regression equations. The correlation coefficient and relative error between the estimated and measured values are calculated (*Table 3*).

The results show that the values estimated by the models were significantly correlated with the measured values, with correlation coefficients between 0.889 and 0.969. The average relative errors of *Model 4–Model 10* are small, the model-fitting accuracies are high, and these models can be used to estimate the aboveground biomass of *H. ammodendron*. The average relative errors of *Model 1–Model 3* are too large and the estimation accuracies of the results are too low, making them unsuitable for biomass

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estimation. This may be due to the large variation in the water content of *H. ammodendron* sampled from sandy soil, as well as the influence of sampling time, weather, and plant-preservation mode. The accuracies of these models may reach an acceptable limit if the circumstances of the sampling are standardized.

Table 3. *Correlation coefficients and relative errors between the estimated and measured values of H. ammodendron biomass*

Habitat	Estimation model	Correlation coefficients	Average relative error (%)	Relative error range (%)	Outlier of relative errors (%)
Sandy soil	W = 1.211 (DH) −23.716 (Model 1)	0.889**	79.217	2.660–40.999	347.377 106.492
	W = 0.633 (D^2H) + 7.635 (Model 2)	0.938**	30.550	9.665–43.962	
	W = 0.191 (D^2H) + 0.538 (DH) + 2.367 (Model 3)	0.912**	43.057	1.004–42.090	99.767 97.814
	W = 0.146 $(DH)^{1.372}$ (Model 4)	0.926**	14.392	0.255–20.025	
	W = 1.895 $(D^2H)^{0.791}$ (Model 5)	0.914**	17.114	1.218–35.144	
Gravel soil	W = 1.598(DH) −18.536 (Model 6)	0.962**	15.474	1.831–31.716	
	W = 0.826 (D^2H) + 16.432 (Model 7)	0.926**	9.533	4.873–22.046	
	W = 0.108 (D^2H) + 1.410 (DH) − 15.157 (Model 8)	0.960**	15.049	3.678–28.413	
	W = 0.189 $(DH)^{1.433}$ (Model 9)	0.969**	13.455	4.156–22.735	
	W = 2.139 $(D^2H)^{0.847}$ (Model 10)	0.926**	15.216	2.795–28.463	

Ps: The relative error range do not conclude outliers
** Significant correlation at 0.01 level

In the two habitats, the correlation coefficients between the estimated and measured values of sandy soil are between 0.889 and 0.938, and those of gravel soil are between 0.926 and 0.969. The correlation coefficient and average relative error of the sandy-soil habitat are higher than those of the gravel soil, the relative error range is larger, and there are outliers in the sandy-soil habitat. The relative errors of some plants are far higher than the average level. From the above analysis, we conclude that the fitting accuracy of the biomass-estimation model of the gravel soil is higher than that of the sandy soil.

Analysis and reselection of best-estimate models

The average relative error of the best biomass-estimation model of *H. ammodendron* in gravel soil is low (13.455%), making it feasible for use in estimating Haloxylon biomass. However, due to the low fitting accuracies of linear Equations (*Models 1–3*), *Model 4* is chosen as the best biomass-estimation model of *H. ammodendron* in a sandy-soil habitat, rather the two other power-function models. The indices of *Model 4* are all better than those of *Model 5*, except for RMA. In conclusion, the best estimation models of sandy soil and gravel-soil habitats are W = 0.146 $(DH)^{1.372}$ and W = 0.189 $(DH)^{1.433}$, which are both power-function models with DH as an independent variable.

Discussion and conclusion

The aboveground biomass of *Haloxylon ammodendron* was very significantly correlated with basal diameter (D), plant height (H), and their complex variables (DH, D^2H, etc.). In this paper, models for estimating the aboveground biomass of *Haloxylon*

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ammodendron in sandy and gravel-soil habitats were established with DH and D^2H as independent variables. These models included a linear equation, a multiple-linear-regression equation, and a power-function equation. The prediction accuracy was between 77.987% and 87.348%. The power-function equations with DH as an independent variable had the best fitting effect. The best fitting model of the sandy-soil habitat was $W = 0.146\ (DH)^{1.372}$, with an r value of 0.955, adjusted R^2 of 0.904, prediction precision (P) of 82.825%, correlation coefficient between the measured and estimated values of 0.926, and average relative error of 14.392%. The best fitting model for the gravel-soil habitat was $W = 0.189(DH)^{1.433}$, with an r value of 0.986, adjusted R^2 of 0.969, and prediction precision of 83.688%. The correlation coefficient between the measured and estimated values of this model is 0.969, and the average relative error is 13.455%.

Models for estimating the aboveground biomass of *Haloxylon ammodendron* in northern China have been studied in recent years. Zhang Hua estimated the aboveground biomass of *H. ammodendron* in the Qingtu Lake at Minqin Qasis, and the best fitting model is $W = 0.8276\ (D^2H)^{0.9185}$ ($R^2 = 0.77$) (Zhang et al., 2020). In the Gurbantongut Desert, Song Yuyang established the biomass-estimation models for *H. ammodendron* in three ecotypes (sand, salt, and gravel), the best estimation models are $W = 65.421\ (D^2H)^{0.8748}$, $W = 57.754\ (D^2H)^{0.8499}$ and $W = 84.409\ (D^2H)^{0.7416}$, which have adjusted R^2 between 0.9782 and 0.9836 (Song and Hu, 2011); the fitted estimation model for *H. ammodendron* of the Gurbantongut Desert established by Tao Ye is $W = 0.3628\ (CH)^{0.9605}$($R^2 = 0.959$), C is the crown area (Tao and Zhang, 2013). Dang Xiaohong established the biomass estimation model for *H. ammodendron* in northern edge of the Hobq Desert, the model is $W = 5.27\ (CH)^{0.794}$ ($R^2 = 0.923$) (Dang et al., 2016).

The power-function equations have the best fitting effect in these studies. This is consistent with the results in this paper, but different areas have different estimation models, which may be due to the vegetation growth condition in different study areas. The variable used in the model of this article was slightly different from that of other studies. Some scholars established models with D^2H as an independent variable. The model with D^2H as an independent variable in this paper also achieved a high precision, only slightly lower than that with DH as an independent variable. Based on the field investigation, there was little difference in the basal diameter of *H. ammodendron*, which made the biomass less affected by basal diameter. It could result from the age of *H. ammodendron* (mostly less than 15 years). Some scholars selected CH as a variable to build models. This is due to different vegetation growth conditions and the difficulty in obtaining data. According to the research, the basal diameter of *H. ammodendron* in Gurbantunggut Desert is mostly very small, and the crown area has higher utility. The base of plant in northern edge of the Hobq Desert is easy to be buried by sand, which makes it difficult to measure the shrub base diameter. In contrast, the plant height and crown width of the shrub are easy to obtain.

Compared with the biomass models obtained by other scholars, R^2 of the best fitting model in this study is higher than that of most other models, the accuracy for this model is high. It can be seen from the model-accuracy verification that the correlations between the estimated and measured values of the estimation models were all extremely significant. However, the relative errors fluctuated greatly, except for the models which had excluded, the relative errors of the models for the sandy-soil and gravel-soil habitat were between 0.255%–35.144% and 1.831%–31.716%, respectively. The models have limitations in practical applications. To solve this problem, we should obtain data from more sampling points and during different seasons and years. Biomass-estimation

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models of different soil moistures, Haloxylon ages, basal diameters, and the like can also be established to improve the models' prediction ability.

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ASSESSING THE EFFECT OF NATURAL ATTENUATION ON SEWAGE SLUDGE USING FABA BEAN (*VICIA FABA*) TEST AND GC-MS ANALYSIS

EL-SHABOURY, G. A.[1*] – GHRAMH, H. A.[1,2,3] – IBRAHIM, E. H.[1,2,4] – HAROUN, S. A.[5]

[1]*Biology Department, Faculty of Science, King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*

[2]*Research Center for Advanced Materials Science (RCAMS), King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*

[3]*Unit of Bee Research and Honey Production, Faculty of Science, King Khalid University, P.O. Box 9004, Abha 61413, Saudi Arabia*

[4]*Blood Products Quality Control and Research Department, National Organization for Research and Control of Biologicals, Cairo, Egypt*

[5]*Botany Department, College of Science, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt*

**Corresponding author*
e-mail: Gamal3003@yahoo.com; phone: +966-56-276-2992; fax: +966-17-241-8205
(ORCID: http://orcid.org/0000-0003-1040-2049)

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Abstract. Sewage sludge (SS) is the final product of wastewater treatment its accumulation can cause serious environmental problems; however, it may be used as agricultural fertilizer. Our objective was to evaluate the effect of natural attenuation on SS by studying the cytotoxicity and genotoxicity potential of different concentrations of SS extract and soil mixtures using the *Vicia faba* L. test, and to study the microbiological biodiversity and presence of toxic furans and dioxins during natural attenuation. Cytological investigation of treated *V. faba* roots after 12 months of natural attenuation revealed that the percentage of different types of mitotic abnormalities increased with the concentration of raw SS from induced DNA damage in *V. faba* roots. However, this effect declined during the attenuation periods. Although after the natural attenuation period, significant effects were detected for the highest tested concentration (100% SS). Nine different bacterial colonies were observed in SS, but only three colonies remained after 12 months. Moreover, Gas Chromatography-Mass Spectrometry analysis showed that the concentrations of toxins were reduced during the natural attenuation periods. Therefore, we concluded that raw SS should not be used as soil fertilizer without detoxification, and its toxic potential must be formally assessed.
Keywords: *biosolids, detoxification, chromosomal aberration, cytotoxicity, genotoxicity*

Introduction

Sewage sludge refers to the residual, semi-solid material produced as a secondary product during sewage treatment of municipal or industrial wastewater. It is mainly composed of organic matter; nutrients (nitrogen and phosphorus) and toxic contaminants (Gray, 2010). Due to its high organic and nutrients contents that are necessary for the plant growth, SS is considered a good fertilizer in agricultural areas. However, raw sewage sludge may contain toxic contaminants and pathogenic microorganisms; thus, specific treatment is required for raw SS before use in agriculture to neutralize toxins and avoid direct exposure to human pathogenic microorganisms

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(Clarke and Smith, 2011). Natural attenuation depends on natural processes that decrease or "attenuate" toxicity and concentrations of contaminants in soil. To ensure the effectiveness of natural attenuation, the conditions are monitored. Natural attenuation involves chemical, physical and biological processes that normally occur in the environment (Mazzeo et al., 2015)

Allium cepa is considered a biological marker for cellular and DNA damages because it assesses the efficiency of environmental decontamination developments (Mazzeo et al., 2010; Souza et al., 2013). Also, Fels et al. (2015) employed *Vicia faba* micronucleus (MN) test to assess the mutagenicity of the raw sewage sludge polluted with hexavalent chromium and composts of this residue mixed with palm waste (mixture A: 1/3 sludgeþ2/3 palm waste and mixture B: ½ sludgeþ½ palm waste).

Vicia faba micronucleus (MN) test assesses genotoxicity and chromosomal aberrations. It is commonly used to assess the genotoxicity of organic and inorganic compounds from soils (Marcato-Romain et al., 2009), manufacturing sewages and wastewater (Shukla et al., 2007), organic substances such as SS or composts (Kapanen et al., 2013), and from water (Monarca et al., 2003). Furans and dioxins are toxic organic components of SS (Mazzeo et al., 2015). Since furan is highly volatile in nature, the most common technique for the analysis of this substance is headspace sampling (Crews and Castle, 2007). Furans have also been investigated using a solid-phase microextraction (SPME) technique in combination with GC–MS (Gas Chromatography-Mass Spectrometry) (Crews and Castle, 2007; Mesias and Morales, 2014).

In this study, the *V. faba* root-micronucleus test was used to evaluate cytotoxicity and genotoxicity induced by raw SS and composts of SS mixed with soil for different time periods; furthermore, microorganisms biodiversity was studied through different natural attenuation periods, and toxins were detected using GC-MS analysis, in order to assess the co-composting effect to remove the toxicity.

Materials and methods

Sample preparation

Samples of anaerobic SS were collected from a wastewater treatment plant (WWTP), located in the municipality of Abha (Saudi Arabia), that receives only effluents from domestic sewage, three samples were taken over three weeks 20 kg each week and were dried under shade for 10-15 days in the greenhouse of King Khalid University, Abha (Saudi Arabia). The SS samples were mixed with a reference soil (collected from a depth of 0-20 cm from the adjacent agricultural fields) in proportions of 10%, 25% and 50% SS.

Detoxification of SS through monitored natural attenuation

Samples containing 10%, 25%, 50% and 100% raw SS were located in micro-perforated bags with openings that were 0.5 mm in diameter and 1 cm apart. Each bag was filled with 4 kg of each sample. The bags were buried in the area surrounding the greenhouse in holes with a maximum depth of 30 cm to permit interactions between the samples and the external environment and to avoid significant temperature fluctuations for 6 and 12 months. The greenhouse is an ideal location for this kind of experiment because it is free of waste products, which could contaminate the samples. Moreover, it prevente the distribution of contaminants.

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Preparation of SS aqueous extract and treatment

For each sample, 125 g (equivalent dry weight) of each sample were dissolved in 500 ml distilled water with steering for 10 min and incubated with shaking at 22 °C for 3 days as described by ABNT (2004). Then, the solutions were filtered through a 0.45-mm pore Whatman filter papers to obtain extracts containing water-soluble substances; the supernatant was collected and stored at 4 °C until use.

Vicia faba seedlings were prepared according to Marcato-Romain et al. (2009). Dry *V. faba* seeds were soaked for 6 h in ultrapure water. Seed coats were removed, and seeds were placed on layers of wet cotton to germinate. After 3 days, germinated seeds with primary roots that were approximately 2 cm in length were selected for the treatment assay. The primary root tips were removed to stimulate the growth of secondary roots. Five independent replicates were made for each test. These germinated seedlings were treated for 3 days with the aqueous extract of the soil only, ultrapure water (negative control), 10 mg/L methyl methanesulfonate (Sigma-Aldrich, mutagenic agent) (positive control), SS 100%, SS 50%, SS 25% and SS 10% SS mixed with soil for natural attenuation periods 0, 6, 12 months, all tests were performed in duplicate.

The germinated treated *V. faba* secondary roots were collected and fixed in a mixture of ethanol and acetic acid (3:1 v/v) as a fixative for 6 h at room temperature. Next, the roots were washed with distilled water, and then kept in 70% ethanol at 4 °C until use. The fixed roots were stained using the Fulgen squash technique, and then mitotic division slides were prepared. Genotoxic effects were estimated in meristematic cells of *V. faba* which presented different types of chromosomal aberrations (e.g., bridges, losses, breakage, and adherence) and nuclear abnormalities (e.g., budding, lobulated nuclei, and binucleated cells). Cytotoxicity was evaluated through recording the changes in the mitotic index, and the mutagenic potential was evaluated through micronuclei recorded of F1 cells and chromosomal breaks in meristematic cells. These parameters were observed under a light microscope, and10 slides per treatment were assessed with 500 cells were examined per slide. The efficiency of the natural attenuation was estimated by comparing the recorded results in 5 individuals for each treatment along with the negative control using a two-way ANOVA.

Microbiological biodiversity

To evaluate the diversity of the microorganisms existing in the SS and the soil, 0.5 g of each sample (100% SS and soil as control) was dissolved in 100 ml of sterilized saline solution after 0, 6, and 12 months of monitored natural attenuation; 20 ul of this solution was spread with an inoculation loop, onto Petri dishes in duplicated containing blood agar, SS agar, and MacConkey agar in duplicate. The Petri dishes were incubated at 35 °C. The plates were observed after 4 days. The colony-forming units (CFUs) was used to record the germinated microorganisms colonies found in each sample.

Detection of furans and dioxins using GC-MS analysis

Furans and dioxins, as example toxins, in the SS and the soil (100% SS and soil as controls) were detected using GC-MS as described by El-Shaboury et al. (2017). The GC-MS analyses were performed using the GC Shimadzu system QP2010 with a gas chromatograph interfaced with a mass spectrometer with a fused Elite-1 silica capillary

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column. The GC-MS conditions were as follows: carrier gas: Helium with constant flow (1.0 ml/min), Injector temperature of 250 °C, Split Ratio = 2. The over temperature programme was as follows: Initial temperature of 40 °C (with a 1 min delay), increase temperature from 40 °C to 150 °C at a rate of 10 °C/min (with no delay), then increase temperature from 150 °C to 280 °C at a rate of 5 °C/min for 5 min (with a delay); the total runtime = 30 min. The Injected Volume of the aqueous extract of SS, which contain the water-soluble substances, was 1 µL, and the Interface Temperature = 280 °C. Interpretation of the mass spectrum GC-MS was performed with the NIST Ver. 2.1 MS data library (Joulain and Koenig, 1998). Finally, dioxins and furans were quantified at different retention times for each sample.

Data analyses

The data were examined for their homogeneity of variance and normality of distribution, and when necessary, the data were log-transformed before a two-way analysis of variance (ANOVA) was performed. Significant differences in the Mitotic Index, Mutagenic Alterations, Genotoxic Alterations, Micronuclei in F1 cells of *Vicia faba* cells treated with different concentrations of raw sewage sludge among different months were evaluated using a two-way ANOVA. Statistica 7.1 was used to process all the statistical analyses (Statsoft, 2007).

Results

Cytotoxicity and mutagenic alteration of treated V. faba seedlings

For evaluating the recommended natural attenuation period for SS-soil mixture to be used safely as soil fertilizer; the mitotic index, genotoxicity, cytotoxicity, and F1 cells micronuclei were recorded in *V. faba* roots treated with extracts of different concentration of SS mixed with soil. Sewage sludge different concentrations were appointed to significantly ($P < 0.001$) change all the parameters (*Table 1*). The application of SS significantly ($P < 0.001$) increased the mitotic index.

Generally, the mitotic index was increased with time prolonged throw natural attenuation periods in all examined concentrations of SS. There was complete inhibition for seed germination for seeds treated with aqueous extract of 100% SS at 0 months (raw SS); however, for the same concentration of SS, a low mitotic index was recorded (34.3%) after 6 months of natural attenuation. The highest mitotic index was recorded for 50% SS concentration after 12 months (50.17%) comparing with the positive control.

Genotoxicity, cytotoxicity and micronuclei in F1 cells of *V. faba* were induced owing to SS treatment for different natural attenuation periods, including different types of chromosomal abnormalities (e.g., delays, chromosomal bridges, chromosomal breaks, and lagging and unoriented chromosomes) as shown in *Figure 1* and *Table 1*. The highest genotoxicity (11.0 and 9.95) were found for 100% and 25% SS concentrations, respectively, after a 6-month natural attenuation period, but the lowest genotoxicity (2.06) was found for 50% SS after 12 months. Owing to the high toxicity of raw SS (0-month treatment), high percentages of stickiness as chromosomal abnormalities across mitotic division were recorded as severe to moderate stickiness (*Fig. 1*). Genotoxicity was significantly reduced with different concentrations of SS-soil mixture after 12 months than other treatments.

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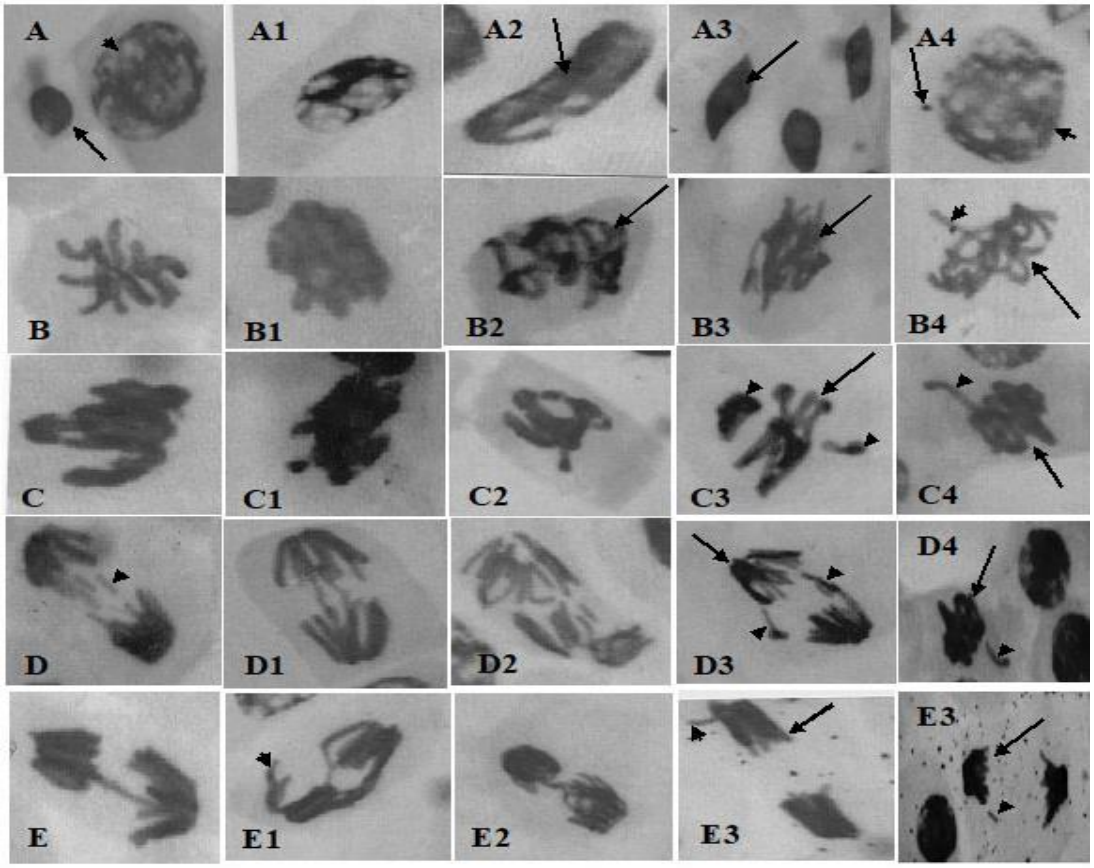

Figure 1. *A: Arrow (micro nuclei) arrow head (normal interphase); A1: Vacuolated nuclei; A2: Stickiness prophase; A3: hard stickiness interphase; A4: Arrow (very small micro nuclei) arrow head (normal interphase); B: Sticky chromosomes in late prophase; B1: Hard stickiness prophase; B2: Stickiness prophase; B3 & B4 Partially stickiness prophase with delay; C & C1 Hard stickiness metaphase; C2: Partially stickiness metaphase; C3: Sticky chromosomes in metaphase arrow head (unoriented chromosomes with pro-micro nuclei); C4: Metaphase with delay; D: Anaphase with chromosome breaks; D1: Multi Bridge anaphase; D2: Disturbed anaphase with pro-micro nuclei; D3: Arrow (anaphase) arrow head (lagging chromosomes); D4: Arrow (stickiness metaphase) arrow head (pro-micro nuclei); E: Late anaphase with chromosome bridge; E1: Early telophase with chromosome bridge; arrow head (pro-micro nuclei); E2: Telophase with chromosome bridge; E3: Telophase with chromosome delay; E4: Arrow (Telophase) arrow head (chromosome breaks)*

Most of the induced chromosomal abnormalities were chromosomal losses, chromosomal breaks, lagging and unoriented chromosomes (*Fig. 1*). Completed division lead to micronuclei, which appeared as DNA damage; the percentage of micronuclei recorded in F1 cells are presented in *Table 1*. The lowest percentage of micronuclei were (0.05 and 0.06) were found at 10% and 25% SS concentrations, respectively, after 0 months. The highest percentage of micronuclei (3.43) was recorded for 100%SS treatment after 6 months.

Microbial diversity in SS

To determine the microbial toxicity in SS, microbial biodiversity was evaluated for 100% SS after each natural attenuation period by inoculating different types of media.

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The raw SS (0 months) had a high diversity of microorganisms with six different colonies found. The diversity of microorganisms decreased with increased natural attenuation periods; after 6 months of natural attenuation, there were 5 different colonies, including two new colony types, but after 12 months, the analysis showed only three colonies including one new colony type. *Figure 2* shows the microbial succession observed during the different periods of natural attenuation compared with microorganisms germinated using the reference soil.

Table 1. *Variations in Vicia faba cells treated by different concentrations of raw sewage sludge after different periods of natural attenuation*

Parameters	Month	Treatments						
		Soil	NC	PC	SS_10%	SS_25%	SS_50%	SS_100%
Mitotic index	0	31.33 ± 0.33	35.82 ± 0.54	37.00 ± 0.58	37.89 ± 0.56	36.00 ± 0.58	35.82 ± 0.13	0.0 ± 0.0
	6		45.00 ± 0.58	43.23 ± 0.87	44.00 ± 0.58	46.33 ± 0.89	45.00 ± 0.58	34.31 ± 4.11
	12		44.37 ± 0.87	47.67 ± 0.88	47.41 ± 0.89	49.33 ± 0.88	50.17 ± 1.11	46.00 ± 1.15
	F_{Month} = 152.4*, *df* = 2; F_{Sample} = 375.8***, *df* = 6; $F_{Month \times Sample}$ = 126.6***, *df* = 12**							
Mutagenic alterations	0	0.85 ± 0.15	0.49 ± 0.00	16.42 ± 0.30	0.75 ± 0.01	1.05 ± 0.05	1.39 ± 0.04	0.0 ± 0.0
	6		0.93 ± 0.07	8.39 ± 0.87	0.89 ± 0.11	2.42 ± 0.30	0.49 ± 0.29	3.00 ± 0.00
	12		0.07 ± 0.07	11.63 ± 0.32	0.85 ± 0.16	0.08 ± 0.08	0.12 ± 0.12	1.00 ± 0.00
	F_{Month} = 33.3*, *df* = 2; F_{Sample} = 961.0***, *df* = 6; $F_{Month \times Sample}$ = 54.6***, *df* = 12**							
Genotoxic alterations	0	3.93 ± 0.58	2.70 ± 0.29	9.39 ± 0.31	4.98 ± 0.26	8.50 ± 0.29	8.03 ± 0.22	0.0 ± 0.0
	6		5.46 ± 0.29	14.56 ± 0.29	5.49 ± 0.29	9.95 ± 0.06	8.42 ± 0.30	11.00 ± 0.00
	12		3.00 ± 0.58	11.42 ± 0.30	2.93 ± 0.07	2.00 ± 0.58	2.06 ± 0.06	5.13 ± 0.13
	F_{Month} = 331.8*, *df* = 2; F_{Sample} = 360.4***, *df* = 6; $F_{Month \times Sample}$ = 90.7***, *df* = 12**							
Micronuclei in F1 cells	0	0.15 ± 0.15	0.37 ± 0.01	8.00 ± 0.00	0.05 ± 0.03	0.06 ± 0.06	3.28 ± 0.11	0.0 ± 0.0
	6		0.86 ± 0.15	7.52 ± 0.29	1.48 ± 0.29	1.53 ± 0.29	1.14 ± 0.14	3.43 ± 0.30
	12		0.12 ± 0.12	10.36 ± 0.32	1.04 ± 0.04	0.91 ± 0.09	0.85 ± 0.15	0.95 ± 0.05
	F_{Month} = 21.8*, *df* = 2; F_{Sample} = 976.9***, *df* = 6; $F_{Month \times Sample}$ = 48.0***, *df* = 12**							

NC: negative control, PC: positive control, *F*-values represent the two-way ANOVA, *df* = degrees of freedom, ***: $P < 0.001$

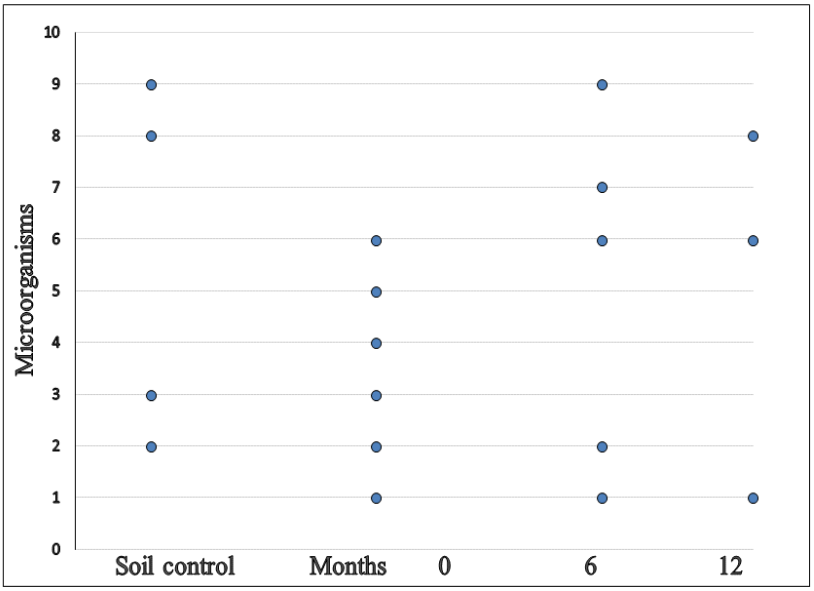

Figure 2. *Microorganisms progression observed during different periods of natural attenuation of 100% SS Where 1 to 9 refers to different types of microorganisms colonies which scored using scoring colony- forming units*

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Detection of furans and dioxins

GC-MS analysis for 100% SS was used to assess the toxicity level of furans and dioxins, which are some of the toxins found in SS (*Table 2*). Different types of furan were detected at different levels; there was a high level of 2-Pentyl furan in raw SS at 0 months but after 6- and 12-months natural attenuation period levels were low. 2-{[1-(Furan-2-yl) ethyl] amino} ethan-1-ol was detected in raw SS and after 6 months but was not detected after 12 months of natural attenuation. N-Benzoyl-9-(2,3,5-tri-O-benzyl pento furanosyl)-9H-purin-6-amine was only detected at a very low level in the initial sample (raw SS). Furanon dihydro-5-tetradecyl was also detected at a high level in raw SS but decreased to a moderate level at 6 months sample and was not detected at 12 months sample. Generally, the types of furans detected decreased throughout the natural attenuation periods. No dioxins were recorded in all examined samples.

Table 2. *Toxin progression observed during different periods of natural attenuation of 100% SS using GC-MS analysis. (There are three levels of detected toxins high level (+++), moderate level (++), low level (+))*

Dioxins and furans	Samples		
	Initial sample (raw SS)	**6-month sample**	**12-month sample**
Dioxins	Not detected	Not detected	Not detected
Furans			
2-pentyl **furan**	+++	+	+
2-{[1-(**furan**-2-yl) ethyl]amino}ethan-1-ol	++	++	Not detected
N-benzoyl-9-(2,3,5-tri-O-benzyl pento **furanosyl**)-9H-purin-6- amine	+	Not detected	Not detected
Furanon dihydro-5-tetradecyl	+++	+	Not detected

Discussion

Assessment of the effects of SS on Vicia faba

There is no germination for *Vicia faba* seeds treated with 100%SS aqueous extracts at 0 months, indicating that the treatment with raw SS without any natural attenuation period was considered highly toxic to the test organism, thus excluding the valuation of the cytotoxic, mutagenic and genotoxic potential of this samples. Walter et al. (2006) recorded similar results by regarding seeds germination of *Lepidium sativum* L. treated with SS. In our study, no any other inhibition recorded in seed germination for the other treated samples.

Assessment of the cytotoxicity in seeds germinated directly in SS after 6 months period at 100% SS mitotic cell division of *Vicia faba* was inhibited comparing with the negative control by regarding the mitotic index values which it was 35.82% decreased to 34.31% which recorded as lowest mitotic index this is may be due to the toxicity still exist even after 6 months of natural attenuation which altered the cell division reflected as high percentage of stickiness phases across all phases of mitotic cell division (*Fig. 1*). Similar effect observed by the treatment with 50%SS after 0-month period where the mitotic index was 35% compared with the other treatments after the same period. *Table 1*, showed that the cytotoxicity was decreased through natural attenuation periods of the SS concentrations which reflected as increasing in mitotic index, this confirms the efficiency of this process for inhibiting the toxicity effect of raw SS. Similar results

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obtained by Mazzeo et al. (2015) where they evaluate the cytotoxicity effect on *A. cepa* seeds germinated in different concentration of SS at different periods.

Concerning genotoxic potential, a substantial frequency in cellular alterations was recorded in the association of 10, 25 and 50% concentrations after a period of 0 months of natural attenuation and for concentrations of 25, 50 and 100%; after 6 months of natural attenuation *Table 1*. Where the 100% SS extract after 6 months treated seeds, severe genotoxic effect induced on the cells as also after 12 months, the 100% SS (raw SS) still induced highly significant genotoxic effects, confirming that the contaminants responsible for genotoxic potential in SS are still active even after 12 months of natural attenuation. Our results regarding the genotoxic potential of SS indicated that; 100% SS cannot be considered an applicant material for use as a soil reconditioner due to its persistent toxicity recorded in our study. Therefore, based on our results, SS/soil mixtures are more convenient for use in agriculture. These results agree with Walter et al. (2006); they confirmed the harmful effect of raw SS in agriculture by regarding (*Lepidium sativum* L.) seeds germination.

Significant mutagenic effects were recorded on the mitotic division for the tested organism (*Vicia faba*) of 25, 50% after a period of 0 months of natural attenuation indicating that raw SS cannot be used in agriculture without natural attenuation. 100% SS after 6 and 12 months of natural attenuation also cannot be used as a soil fertilizer because SS without any soil mixture induced significant and sever mutagenic effect for the tested plant (*Table 1*). 50% SS without any natural attenuation period induced the formation of Micronuclei. Also, after 6 months of natural attenuation period 25, 100% SS also induced the formation of micronuclei (*Fig. 1*). The recorded significant results for F1 cells showed that the damage recorded in *Vicia faba* cells was transferred to and stable in cells of the F1 region. Thus, even at lower concentrations, the SS samples were inducing the destruction of the genetic material of the meristematic cells of the test organism. These data support that 100% SS cannot be detoxified compared with the mixture of soil/SS.

From the obtained above results, natural attenuation period for 12 months appeared as the excellent period for degradation of the most mutagenic and toxic substances existing in the studied SS (see *Fig. 1*; *Table 1*). Genotoxicity can be induced also in *Allium cepa* due to exposure to SS which reported by many authors, for example, Srivastava et al. (2005); they recorded mitotic index reduce and significant chromosomal aberrations frequency induced by SS aqueous extract treatment in India. Caritá (2007) also study genetic damage induced in *A. cepa* due to the treatment by aqueous extract of SS which collected from five WWTPs of Sao Paulo (Brazil). Furthermore, highly genotoxic effects in seeds of *A. cepa* exposed to biosolids as raw materials were recorded by Christofoletti et al. (2012).

The mutagenic and genotoxic effects recorded in our study may be due to the existence of toxic substances as cresol, also due to the synergistic effects of the main components of SS. According to ATSDR (2008) who studied in vitro tests in human and animals which confirmed that cresols can effect on DNA inducing genotoxicity. Our results can confirm that the reduction of the toxicity effect of SS through natural attenuation periods with soil mixture.

Microbiological biodiversity of SS

In our study on microorganisms biodiversity, we used three different types of media to help many different types of microorganisms to germinate. About nine different microorganisms colonies were recorded in the examined sample (100% SS and soil

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control) regarding the reference soil the highest rates of microorganisms biodiversity was six different shapes of microorganisms colonies with no any natural attenuation period (0 months) so raw SS considered highly contaminated, the lowest microorganisms biodiversity rates were 3 colonies which recorded after 12 months of natural attenuation. So our study on the count of microorganisms colonies supports the results obtained from the treatment of *Vicia faba* by SS extract that raw SS cannot be used as soil fertilizer because it has high percentage of toxicity which reduced through natural attenuation period sand reached to lowest percentage after 12 months compared by the reference soil which recorded only 3 colonies. Similar results reported by Petersen et al. (2003) they recorded microbial succession during decomposition of sewage sludge. Also, Mazzeo et al. (2015) recorded 17 different species of bacteria in addition to some fungi when they study the microorganisms succession in SS which decreased to 6 species after 12 months of natural attenuation. Many authors suggested that there are several types of bacteria in SS may have the ability to degrade contaminants that were initially present in these samples (Sharma et al., 2002).

Detection of toxins

Suzuki et al. (2011) recorded the existence of furans and dioxins in the environment; the occurrence of these compounds generally resulting from industrial activities such as incineration of halogenic substances, the ignition of diesel, the insecticides manufacture wood ignition and the microorganisms action. Klimm et al. (1998) also reported furans and dioxins existed in SS.

Using GC-MS analysis some toxins were identified in SS as furans in different levels which decreased with natural attenuation period increased which reflect the importance of natural attenuation for detoxification processes of raw SS to be available as a soil fertilizer. In our study, furans only were detected in about four forms and dioxins not detected at all (*Table 2*). Furan is an organic compound involving a five-membered aromatic ring with one oxygen and four carbon atoms. Furan derivatives are chemical compounds containing furan rings. Exposure to high levels of furans may rise the infection risk of rats and mice by hepatocellular tumors and tumors of the bile duct in rats. So furans recorded as a possible human carcinogen (Bakhiya and Appel, 2010). The reduction of toxins level across natural attenuation periods may be due to the microbial degradation as some study revealed that several microorganisms have been defined as degraders for dioxin and furan (Parsons et al., 1998; Suzuki et al., 2011).

Conclusion

Genotoxicity, cytotoxicity, and micronuclei in F1 cells of *V. faba* were induced owing to SS treatment for different natural attenuation periods, including different types of chromosomal abnormalities, which increased as the concentration of raw SS increased. However, this effect declined through the natural attenuation period. Regarding microorganisms progression of SS, six colonies were scored in row SS, but only three colonies remained after 12 months of natural attenuation. GC-MS analysis identified some toxins in SS as furans in different levels which decreased with natural attenuation period increased. Our study revealed that *V. faba* test can be used to evaluate the toxicity of SS contaminants. Also, raw SS is considered highly toxic and highly contaminated and cannot be used as soil fertilizer without natural attenuation for at least 6 months and must be mixed with soil to reduce its contamination to the

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environment and humans and so the application of SS in agriculture must be with caution. We recommended that, row SS needs more chemical and biological studies to identify all the contaminants in it and their impact on living organisms and the environment when it used as soil fertilizer.

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Conflict of interests. The author declares that they have no conflict of interests.

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ALGAE GROWTH INHIBITION BY AQUEOUS EXTRACTS FROM *ALTERNANTHERA PHILOXEROIDES* AND UNDERLYING MECHANISMS

HUANG, Y. J.* – HUANG, Y. – YAO, Y. T. – ZHOU, Z. Z. – ZHANG, J. – YANG, S. Y. – ZHOU, J. H. – ZHOU, S. B.

School of Ecology and Environment, Anhui Normal University, Wuhu 241000, China

Collaborative Innovation Center of Recovery and Reconstruction of Degraded Ecosystem in Wanjiang Basin Co-founded by Anhui Province and Ministry of Education, Anhui Normal University, Wuhu 241000, China

Anhui Provincial Engineering Laboratory of Water and Soil Pollution Control and Remediation, Wuhu 241000, China

**Corresponding author*
e-mail: yongjiehuang0108@163.com

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Abstract. Using *Microcystis aeruginosa* as a research receptor, the inhibitory effects of 0.3-1.1 g/L root, stem and leaf extracts from *Alternanthera philoxeroides* on algae growth and their underlying mechanisms were studied. The biomass, oxygen radical (O_2^-), malondialdehyde (MDA), nucleic acid, microcystin and polysaccharide contents were determined and cell morphological and structural variations were observed. Results showed that the extracts from *A. philoxeroides* significantly inhibited the growth of *M. aeruginosa* in a concentration-dependent manner. In particular, root extract had the strongest inhibition effect, followed by stem and leaf extract. The growth of more than 80% of all *M. aeruginosa* was inhibited after 96 h exposure to 1.1 g/L root extract. Besides, the damage degree of cells treated with root extract after 72 h was shown by the scanning electron microscope. With the accumulation of O_2^-, the contents of all physiological indicators increased with higher extract concentrations, except for MDA content, which decreased first after 24 h culture at 0.3 g/L treatment. In conclusion, *A. philoxeroides* had a certain ecological control capacity on *M. aeruginosa* growth by aggravating oxidative stress, destroying membrane permeability, changing cell structure and finally inducing plasmatorrhexis, but the specific action of allelochemicals it contained remained to be further explored.
Keywords: *harmful algal blooms, allelopathy, growth inhibition, peroxidation reaction, microcystin, polysaccharide*

Introduction

In recent years, intense agricultural production and living activities had led to a dramatic increase of N and P levels in water, which has caused severe eutrophication and frequent harmful algal blooms (HABs) (Lapointe et al., 2015). Among these, the most common is the occurrence of cyanobacteria cells over propagation whose dominant species include *Microcysis*, *Oscillatoria*, *Anabaenopsis*, *Cylindrospermopsis* etc. (Kaur et al., 2019). The end result not only seriously affects the biodiversity of aquatic ecosystem and the development of aquaculture, but also endangers human health (Carmichael and Boyer, 2015).

At present, the use of various techniques to restrain algal growth has been widely developed and applied. Physical and chemical processes mainly include filtration, ultrasonic treatment, ball clay flocculation and algaecide (copper sulfate and hydrogen peroxide), etc. (Greenfield et al., 2014; Han et al., 2019). Bioflocculation, algicidal

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bacteria, viruses, plant-derived compounds, fish and zooplankton are considered as cost-effective, safe, ecologically healthy emerging biotechnologies for the control of algal blooms (Mecina et al., 2017; Pal et al., 2020), especially the effects of allelochemicals identified from plants on algae have attached much attention (Herrera et al., 2019).

Plants can release chemicals into the biological community to promote or inhibit the growth and development of themselves and other surrounding organisms directly or indirectly (Uddin et al., 2017). Allelopathic chemicals have been sought out in an array of plants, like *Phragmitescommunis*, *Eichhornia crassipes* and *Pistia stratiotes* Linn. Besides, allelochemicals are secondary metabolites of biosynthesis and are easily decomposed. According to their different properties and synthetic pathways, they can be divided into phenolic acids, terpenoids, alkaloids, etc. (Zhao et al., 2019). Results have shown that phenolic acids (Zhang et al., 2010), linoleic acids (Ni et al., 2015), flavonoids and tannins (Tazart et al., 2019) can strongly restrain the growth of *M. aeruginosa*, and may become promising alternatives to algae control.

M. aeruginosa is a typical cyanobacterium with a global distribution range. The large area coverage of *M. aeruginosa* in eutrophication freshwater ecosystems tends to cause lack of oxygen in water and inhibit photosynthesis of aquatic plants (Rzymski et al., 2020). What is more severe is that it can produce and release microcystins (monocyclic seven-peptide compounds synthesized from multifunctional protein complex) into the environment, and then gradually accumulate along with the food chain, which mainly induce various liver disease as a threat to animal and human life by inhibiting the function of protein phosphatases 1 and 2A (Mecina et al., 2019). So it is essential and representative to explore superior strategies for managing the outbreak of *M. aeruginosa*.

A. philoxeroides is a pernicious invasive weed in China, first appeared in South America, and as an amphibious plant, the extraction and release of allelochemicals may provide more opportunities for algae treatments, which has aroused our interests (Prabakaran et al., 2019). Whenever it invades a strange environment, *A. philoxeroides* occupies the ecological niche quickly and becomes the dominant species in the community, leading to the decline of biodiversity and the destruction of ecological balance (Portela et al., 2020). It has been reported that *A. philoxeroides* has certain edible and medicinal value in Southeast Asia, but it is generally harmful in most areas with a poor utilization (Masoodi et al., 2013). The reasonable application of *A. philoxeroides* to algae elimination may weaken algae breed potential and improve its own usage rate at the same time to achieve mutual benefit. Many studies have confirmed the allelopathy of *A. philoxeroides* on other plants (*Zoysia matrella*, *Medicago sativa*, *Cichoriumintybus*, and *Avena sativa*), ethyl propionate has been found to be one of the main allelochemicals in root extract of *A. philoxeroides* due to its higher content, in addition, the common effect substances may also include diethyl phthalate, dibutyl phthalate etc. (Huang et al., 2017), and some experiments have begun to take notice of its inhibition effect on *M. aeruginosa*. The discussion was mainly focused on the effects on photosynthesis and antioxidant activities, and there was no relatively systematic research in the aspect of inhibition and stress response of algae. Moreover, the employ of plant-derived compounds has both positive and negative effects, the extraction of some plant-derived compounds involves organic solvents and poses environmental risks, and overuse may affect the biological survival of the original habitat. So it is needed to further evaluate the amount of input in practical application (Suzuki et al., 2020).

Hence, in our experiment, *M. aeruginosa* was selected as the research object, and the allelopathy effects on *M. aeruginosa* were investigated with different concentrations of

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A. philoxeroides extracts, from the following aspects: (a) the influence of *A. philoxeroides* extracts on the biomass of *M. aeruginosa*; (b) impacts of *A. philoxeroides* on physiological and biochemical characteristics of *M. aeruginosa*, oxygen radical, MDA, nucleic acid, microcystin and polysaccharide content were detected; (c) morphology and structure changes of *M. aeruginosa* under *A. philoxeroides* disposition (microscopic observation). We intend to develop a novel environment-friendly alga-growth inhibitor. We also want to supply scientific and theoretical basis for turning this invasive plant into a resource.

Materials and methods

Test materials

M. aeruginosa was purchased from the freshwater algae seed bank of the institute of aquatic biology, Chinese Academy of Science (PCC7806). The alga was cultivated in a climate chamber until they were in their logarithmic growth phase. *A. philoxeroides* used in the experiment was collected at the Zheshan campus lawn of Anhui Normal University, Wuhu, in eastern China (31°34′N, 118°38′E).

Methods

Algae cultivation

100 mL of *M. aeruginosa* stock was cultivated with the BG-11 culture medium at 25 ± 1 ℃, 4,000 lx, and 12 h: 12 h day light photoperiod for ten days. Fresh culture medium was supplemented and the culture container was shaken 3-4 times per day until *M. aeruginosa* was in its logarithmic growth phase.

The composition of BG-11 medium included $NaNO_3$ 1.5 g/L, K_2HPO_4 40 mg/L, $MgSO_4$ 75 mg/L, $CaCl_2 \cdot 2H_2O$ 36 mg/L, Ammonium ferric citrate 6 mg/L, EDTA-2Na 1 mg/L, Na_2CO_3 20 mg/L, A5 + Co (mother liquor) 1 mg/L. Among them, mother liquor formula was $MnCl_2 \cdot 4H_2O$ 1.81 g/L, $ZnSO_4 \cdot 7H_2O$ 0.22 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g/L, $CuSO_4 \cdot 5H_2O$ 0.079 g/L, $Co(NO_3)_2 \cdot 6H_2O$ 0.049 g/L.

Extracts preparation

Fresh *A. philoxeroides* plant was collected and was first rinsed with tap water, then washed three times with distilled water. Roots, stems and leaves of *A. philoxeroides* were sampled separately and dried (15-30 min at 105 ℃, and then dried to constant weight at 60-70 ℃). Each plant part was grounded with a grinder until sufficient amount was available for the experiment. The powder of 40 g was soaked in 200 mL distilled water at a ratio of 1:5 (W/V) for 48 h, and then qualitative filter paper and quantitative filter paper were used for double filtration. Final concentration of the filtered solution was considered as 200 g/L. The concentrations of aqueous extracts were adjusted to 0.3, 0.5, 0.7, 0.9, 1.1 g/L by dilution with distilled water and stored at 4 ℃ prior to experiment.

Algae growth inhibition test

100 mL of *M. aeruginosa* was transferred to 250 mL sterilized conical flask. Initial concentration of these algae was 1.43×10^6 cells/mL. Different concentrations of the root, stem and leaf extracts (0, 0.3, 0.5, 0.7, 0.9, 1.1 g/L) were then added to conical flasks containing *M. aeruginosa*. Since only a small amount of the extract was added to

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each conical flask, there was little effect on algal density, so extract volume was not considered. The test was replicated three times. The number of algal cells was counted with a blood cell counter once every 24 h and recorded to 96 h.

The growth inhibition rate of *M. aeruginosa* by the extracts of *A. philoxeroides* was calculated as the following formula:

$$IR\ (\%)=(1-{}^{N1}/_{N0})\times 100\% \quad \text{(Eq.1)}$$

where *N1* represents the algae density in the treatment group at day *N*, while *N0* represents the algae density in the control group at day *N*. Units of both *N1* and *N0* are cell counts/mL.

Determination of physiological parameters of M. aeruginosa

Nucleic acid, MDA and O_2^- were determined at 24, 72 and 120 h after cultivation. 12 mL algae solution was collected and centrifuged at 4,000 r/min for 10 min. The supernatant was used to measure the content of nuclear acid using UV-Vis spectrophotometer (UV-3802, Unico, USA) at 260 nm (Sun et al., 2004). The sediment was dissolved in 1 mL phosphate buffer saline (PBS) (pH 7.0), for three times of repeated freeze-thaw at -80 ℃. After centrifugation at 12,000 r/min for 15 min, the supernatant was the enzyme solution of MDA. 2 mL enzyme solution (control plus 2 mL distilled water) was added to 2 mL 10% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA). The mixture was placed in a boiling water bath at 100 ℃ for 15 min, cooled rapidly and then placed in a centrifuge at 4,000 r/min for 10 min. The supernatant was taken and the absorbance was measured at 450, 532 and 600 nm respectively. MDA (umol/g) = 6.45 × (A532 - A600) - 0.56 × A450. In the determination of O_2^- content, 2 mL supernatant of algal liquid was taken with the same centrifugation at 4,000 r/min for 10 min, and 0.4 mL 10 mmol/L hydroxylamine hydrochloride was added. After mixing, the supernatant was placed in a water bath at 25 ℃ for 20 min. Then 2 mL α-naphthylamine (7 mmol/L) and 2 mL p-aminobenzene sulfonic acid (17 mmol/L) were added, mixed and the reaction was carried out at 25 ℃ for 25 min. The same volume of butyl alcohol was used, shaken thoroughly and the reaction mixture was set aside for layering. The upper butyl alcohol was taken to measure the light absorption value at 530 nm.

At 72 h, 20 mL algae solution was collected and centrifuged at 4,000 r/min for 15 min, the supernatant was transferred to Eppendorf tube and saved at -20 ℃ for the determination of extracellular polysaccharides and microcystins in *M. aeruginosa*. The sediment was transferred with 1 mL PBS (pH 7.2-7.4), repeated freeze-thaw was carried out at -20 ℃ for 3 times, then the supernatant was taken and stored at -20 ℃ for determination of intracellular polysaccharides and microcystins in *M. aeruginosa*. Both microcystins and polysaccharides were analyzed by enzyme-linked immunosorbent assay (ELISA) kit (96T/48T, Shanghai Xinyu Biotechnology Co., Ltd), and the specific operation method was referred to the kit instructions.

Observation of cell superficial structure of M. aeruginosa

The algae cells treated with root extracts of different concentrations for 72 h were collected, fixed with 2.5% glutaraldehyde for 24 h and 1% osmium acid for 1 h, and then washed with PBS (pH 7.4) for 3 times. The ethanol gradients of 15%, 30%, 50%, 75%, 95% and 100% were used to elute for 15 min respectively. After the supernatant

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was discarded, 10 mL 1:1 mixture of isoamyl acetate and ethanol was added and soaked for 10-20 min. The sediment left by centrifugation was further soaked and shaken in pure isoamyl acetate for 20 min, and then observed and photographed under a scanning electron microscope (JSM-6390LV, JEOL) after coating.

Data analysis

The obtained data were expressed in terms of mean value ± SE (standard error), and SPSS 19.0 analysis software was used to analyze the differences between the control group and treatment group for one-way ANOVA followed by a T-test. $P < 0.05$ represents statistical significance, and $P < 0.01$ represents a greater significant difference.

Results and analyses

Effects of A. philoxeroides extracts on M. aeruginosa growth

When *M. aeruginosa* was exposed to the extracts of root, stem and leaf from *A. philoxeroides* for 24 h, 48 h, 72 h and 96 h, the growth inhibition rate of algal cells increased with increasing concentration of extracts (*Table 1*). In particular, when *M. aeruginosa* was treated for 24 h by ≥ 0.5 g/L root extract, ≥ 0.7 g/L stem extract and ≥ 0.5 g/L leaf extract from *A. philoxeroides*, significant differences generated compared with lower concentration treatments ($P < 0.05$). Also, during the entire experimental process, the growth inhibition rate of each treatment group showed an obvious trend of increasing with culture time. After 96 h exposure, the optimal concentration of root extract with the highest growth inhibition was 0.9 g/L, the IR was close to 80.48%, while the optimal concentrations of stem and leaf extracts were both 1.1 g/L, and the IR was 55.79% and 45.64%, respectively.

Table 1. *The effect of A. philoxeroides extracts on M. aeruginosa growth*

Treatment	Growth inhibition rate (%)				
	Concentration (g/L)	24 h	48 h	72 h	96 h
Root extract	0.3	25.22 ± 1.21a	43.61 ± 1.78a	55.72 ± 2.09a	64.42 ± 1.13a
	0.5	28.11 ± 1.84b	56.45 ± 0.65b	57.46 ± 1.04b	70.05 ± 2.36a
	0.7	34.62 ± 1.47c	63.37 ± 0.55c	66.53 ± 1.35c	74.62 ± 1.76b
	0.9	51.57 ± 1.58d	73.07 ± 1.19d	74.75 ± 0.33d	80.48 ± 1.13c
	1.1	51.57 ± 0.87d	72.95 ± 0.63d	74.70 ± 0.32d	80.34 ± 1.18c
Stem extract	0.3	29.96 ± 3.32a	23.67 ± 0.45a	32.33 ± 1.01a	43.61 ± 0.97a
	0.5	30.60 ± 0.72a	23.67 ± 1.06a	35.19 ± 0.20b	47.53 ± 0.73b
	0.7	42.97 ± 4.87b	38.98 ± 0.45b	45.57 ± 1.21c	52.15 ± 0.52c
	0.9	42.81 ± 0.91b	45.66 ± 1.82c	49.07 ± 1.06d	54.91 ± 0.56d
	1.1	51.08 ± 1.05c	48.31 ± 0.96d	51.66 ± 0.62e	55.79 ± 1.67d
Leaf extract	0.3	13.33 ± 6.06a	10.03 ± 2.16a	20.58 ± 1.05a	31.61 ± 0.83a
	0.5	25.65 ± 2.64b	30.90 ± 0.45b	32.27 ± 0.31b	35.44 ± 0.92b
	0.7	38.88 ± 1.24c	34.22 ± 0.48c	36.46 ± 0.45c	38.81 ± 1.18c
	0.9	42.01 ± 0.37c	40.66 ± 1.88d	41.53 ± 0.77d	42.96 ± 1.18d
	1.1	42.25 ± 1.24c	44.04 ± 0.28e	44.78 ± 0.22e	45.64 ± 0.62e

Data in the table are mean value (n = 3) ± SE. Different letters in the same column indicate significant difference among treatments of different concentrations of extracts from the same plant part at $P < 0.05$, while the same letters indicate no significant difference within the treatment (plant organs)

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Effects of A. philoxeroides extracts on nucleic acid contents in M. aeruginosa

As shown in *Table 2*, a series of diluted extracts from various organs of *A. philoxeroides* had different effects on the nucleic acid release of *M. aeruginosa*. Among them, after 24 h, 72 h and 120 h culture, the nucleic acid content of the treated group was significantly higher than that of the control group when the concentration of root extract was ≥ 0.3 g/L, stem extract was ≥ 0.5 g/L, ≥ 0.3 g/L, ≥ 0.5 g/L, and leaf extract was ≥ 0.7 g/L, ≥ 0.5 g/L, ≥ 0.5 g/L ($P < 0.05$). And nucleic acid content was the highest after 120 h treatment when the concentration of root, stem and leaf extracts was 1.1 g/L, which was increased by 96.94%, 62.14% and 51.82% respectively compared to that in the control group. *M. aeruginosa* cells exhibited the most nucleic acid release in the root extract treatment group, followed by the stem and leaf extract treatment groups with the same concentration.

Table 2. *Effects of A. philoxeroides extracts on nucleic acid content of M. aeruginosa*

Treatment	Nucleic acid content (OD_{260})			
	Concentration (g/L)	24 h	72 h	120 h
Control	0	0.0773 ± 0.0031a	0.0823 ± 0.0025a	0.0872 ± 0.0031a
Root extract	0.3	0.0850 ± 0.0026b	0.1177 ± 0.0015b	0.1307 ± 0.0015b
	0.5	0.0857 ± 0.0040b	0.1233 ± 0.0021c	0.1363 ± 0.0025c
	0.7	0.0913 ± 0.0012c	0.1143 ± 0.0040d	0.1580 ± 0.0026d
	0.9	0.1323 ± 0.0021d	0.1600 ± 0.0020e	0.1680 ± 0.0020e
	1.1	0.1303 ± 0.0025d	0.1613 ± 0.0021e	0.1717 ± 0.0021e
Stem extract	0.3	0.0770 ± 0.0017a	0.0870 ± 0.0021b	0.1037 ± 0.0021a
	0.5	0.0883 ± 0.0021b	0.1023 ± 0.0025c	0.1157 ± 0.0012b
	0.7	0.0913 ± 0.0031b	0.1117 ± 0.0031d	0.1270 ± 0.0030c
	0.9	0.0917 ± 0.0035b	0.1210 ± 0.0026e	0.1370 ± 0.0020d
	1.1	0.1000 ± 0.0010c	0.1277 ± 0.0021f	0.1413 ± 0.0015e
Leaf extract	0.3	0.0753 ± 0.0014a	0.0847 ± 0.0015a	0.1010 ± 0.0030ab
	0.5	0.0797 ± 0.0012ab	0.0927 ± 0.0025b	0.1083 ± 0.0025b
	0.7	0.0833 ± 0.0012b	0.1037 ± 0.0025c	0.1173 ± 0.0021c
	0.9	0.0893 ± 0.0015c	0.1163 ± 0.0021d	0.1297 ± 0.0071d
	1.1	0.0940 ± 0.0014d	0.1183 ± 0.0012d	0.1323 ± 0.0035d

Data in the table are mean value (n = 3) ± SE. Different letters in the same column indicate significant difference among treatments of different concentrations of extracts from the same plant part at $P < 0.05$, while the same letters indicate no significant difference within the treatment (plant organs)

Effect of A. philoxeroides extracts on O_2^- contents in M. aeruginosa

After the treatment of *M. Aeruginosa* with root, stem and leaf extracts of different concentrations for 24 h, 72 h and 120 h, the O_2^- content in the treatment group increased with the rise of extract concentrations, and with the extension of culture time, the O_2^- content in algal cells of the treatment group reached a significant higher level than the control group at a lower extract concentration (*Fig. 1*). Of which, under 120 h exposure, significant differences appeared at the concentration ranges of root extract ≥ 0.3 g/L, stem extract ≥ 0.3 g/L and leaf extract ≥ 0.5 g/L ($P < 0.05$). O_2^- content attained the highest value when the extracts of root, stem and leaf was 1.1 g/L, which was 2.67, 1.96

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and 1.83 times higher than the control group, respectively. Besides, according to our results, root extract had the most powerful effect on the induction of O_2^- in *M. aeruginosa*, followed by stem and leaf extract, respectively.

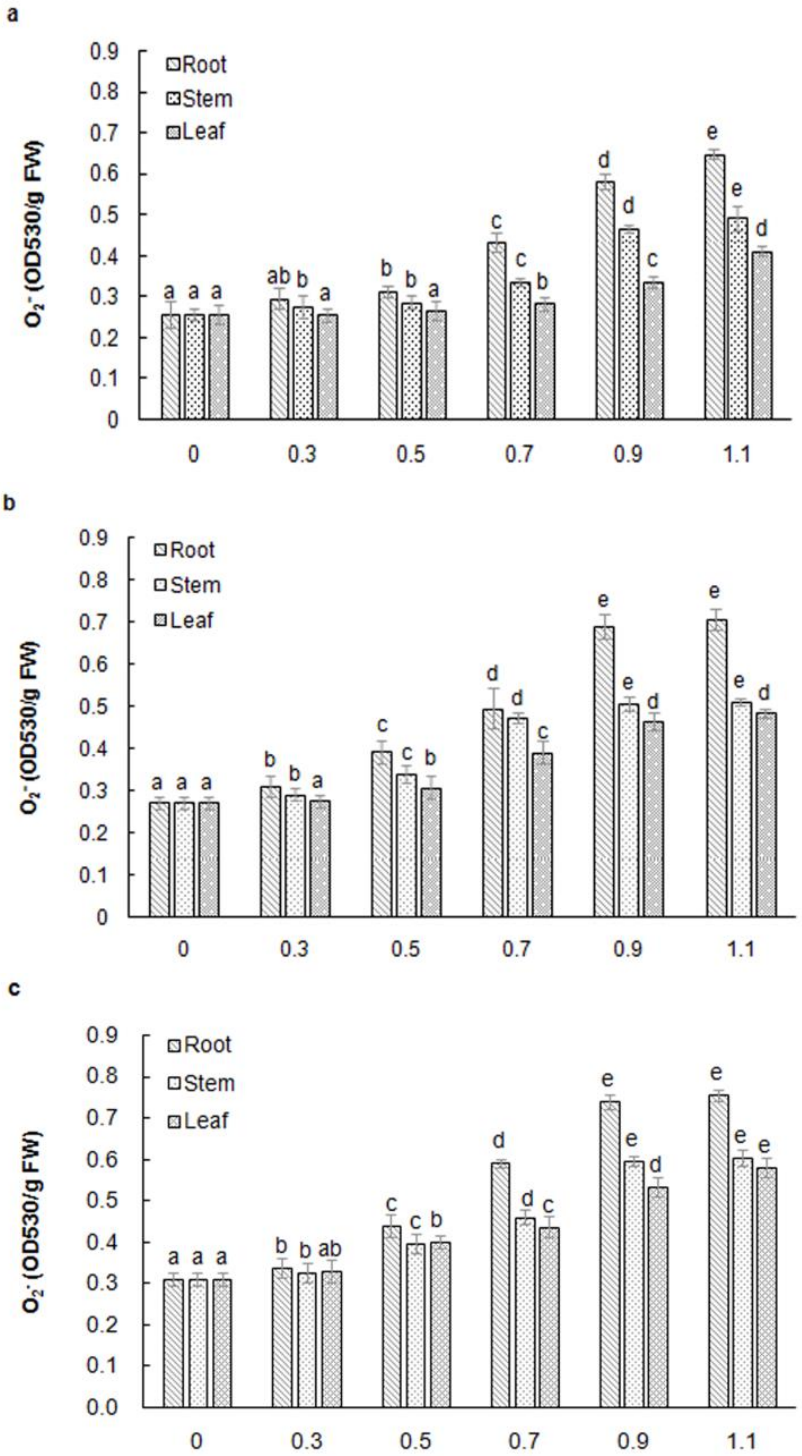

Figure 1. *O_2^- contents in M. aeruginosa exposed to different concentrations of A. philoxeroides extracts for 24 h (a), 72 h (b) and 120 h (c). Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at $P < 0.05$; otherwise, the same letters represent no significant difference*

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Effect of A. philoxeroides extracts on MDA contents in M. aeruginosa

The MDA content of *M. aeruginosa* treated with extracts from *A. philoxeroides* for 24 h, 72 h and 120 h overall increased with increasing concentration of extracts. In particular, after 24 h exposure, MDA content decreased initially (from 0 to 0.3 g/L) in all treatments and was significantly increased when *M. aeruginosa* cells were treated by ≥ 0.5 g/L root, stem extracts and ≥ 0.7 g/L leaf extract ($P < 0.05$), finally reached the peak at 1.1 g/L (*Fig. 2a*). However, after 120 h exposure, MDA content in the treatment group was significantly higher than that in the control group from the beginning with 0.3 g/L extracts ($P < 0.05$) and elevated continually (*Fig. 2c*). In addition, beneath high concentration for a long time, the allelopathy of three extracts from *A. philoxeroides* showed the following sequence: root > stem > leaf, the effect of root extract on MDA contents in *M. aeruginosa* was the most obvious.

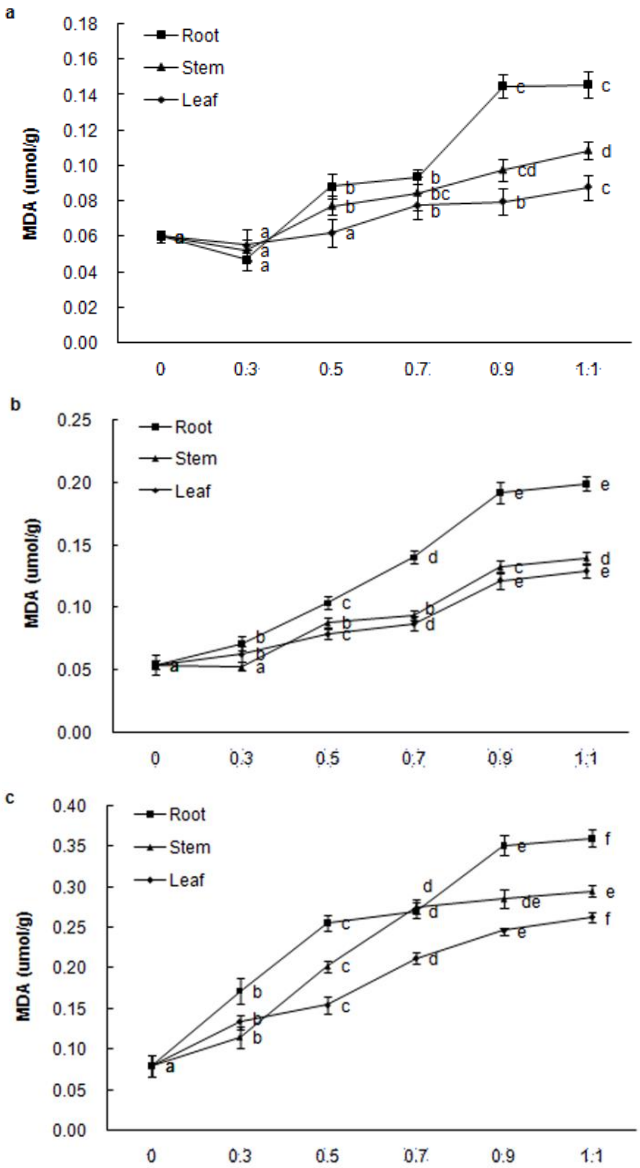

Figure 2. *MDA content in M. aeruginosa cells exposed to different concentrations of A. philoxeroides extracts for 24 h (a), 72 h (b), and 120 h (c). Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

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Effects of A. philoxeroides on intracellular and extracellular microcystin contents in M. aeruginosa

The release of microcystins in *M. aeruginosa* at different concentrations of extracts from *A. philoxeroides* showed a similar growth situation as above (*Fig. 3*). In general, the intracellular and extracellular content of microcystins in the treatment group was significantly higher than that in the control group when *M. aeruginosa* cells were treated by ≥ 0.3 g/L root extract, ≥ 0.5 g/L stem extract and ≥ 0.5 g/L leaf extract, or ≥ 0.3 g/L of all kinds of extracts respectively ($P < 0.05$). 1.1 g/L of root, stem and leaf extracts had the most adverse stimulation, that is intracellular (by 57.70%, 23.00% and 13.05%) and extracellular (by 62.82%, 47.50% and 38.65%) microcystins had the largest increase compared to the control. Under different treatments, the extracellular content of microcystins released by *M. aeruginosa* cells was always lower than that in the intracellular. And according to the consequences, it could be intuitively reflected that the restrain to algal cells from strong to weak was root, stem and leaf extracts.

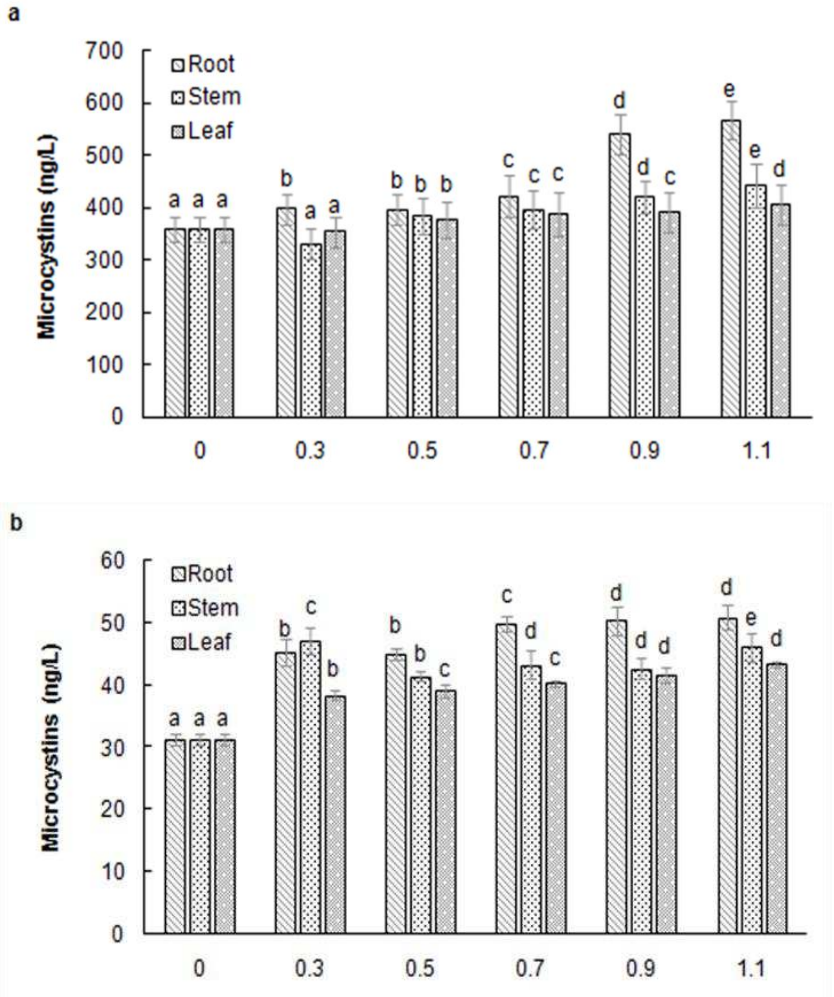

Figure 3. *Effects of extracts from A. philoxeroides on intracellular (a) and extracellular (b) microcystin contents in M. aeruginosa. Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

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Effects of A. philoxeroides on intra- and extra-cellular polysaccharide contents in M. aeruginosa

The results showed that both intra- and extra-cellular contents of polysaccharides in *M. aeruginosa* increased with increasing concentration of extracts, and the content of extracellular polysaccharides was relatively high (*Fig. 4*). After 72 h exposure of *M. aeruginosa* to ≥ 0.3 g/L of root, stem and leaf extracts, the intracellular content of polysaccharides in the treatment group was significantly higher than that in the control group ($P < 0.05$). While extracellular content of polysaccharides achieved significant differences when *M. aeruginosa* cells were exposed to ≥ 0.3 g/L root extract, ≥ 0.3 g/L stem extract and ≥ 0.7 g/L leaf extract ($P < 0.05$). The action strength of the extracts from *A. philoxeroides* was root > stem > leaf, and 1.1 g/L had the greatest influence on the production of polysaccharides in *M. philoxeroides*.

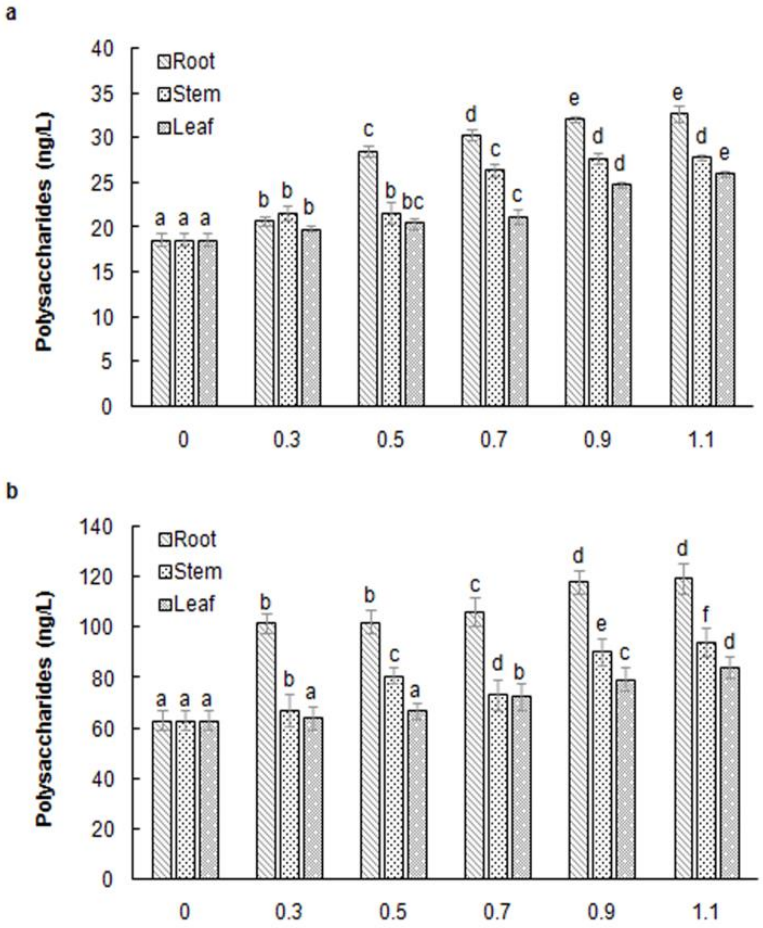

Figure 4. *Effects of extracts from A. philoxeroides on intracellular (a) and extracellular (b) polysaccharide contents in M. aeruginosa. Mean values (n = 3) followed by different letters represent significant difference in treatments of different extract concentrations from the same plant part at P < 0.05; otherwise, the same letters represent no significant difference*

Effect of A. philoxeroides on cell superficial structure of M. aeruginosa

It was observed by scanning electron microscope that the *M. aeruginosa* cells in the control group remained their integrity with a round and smooth appearance, while the

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surface structure of *M. aeruginosa* cells in the treatment group was severely damaged after being exposed to root extract from *A. philoxeroides* for 72 h (*Fig. 5*). With the increasing concentrations of root extract, the morphology of *M. aeruginosa* cells changed gradually, cell shrank, cell wall ruptured and intracellular material flowed out until the cell disintegrated. The 1.1 g/L of root extract was the most destructive on the structure of *M. aeruginosa* cells.

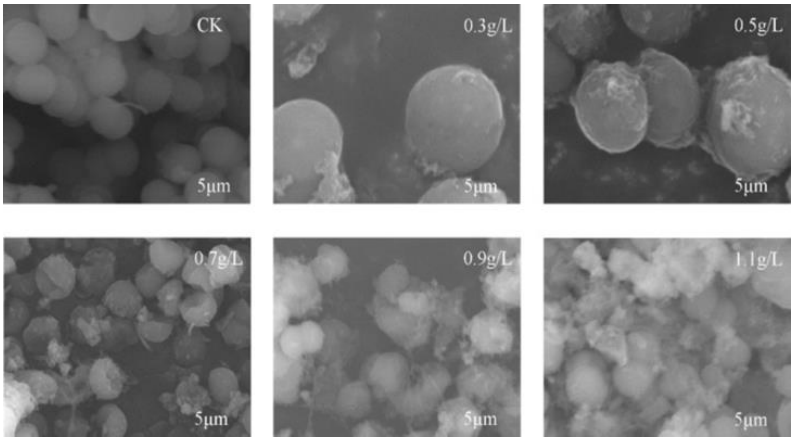

Figure 5. *Scanning electron microscope images of cell morphology and structure of M. aeruginosa damaged by root extract (0-1.1 g/L) from A. philoxeroides (× 5000)*

Discussion

Recent research has found that the growth of *M. aeruginosa* was inhibited by the root, stem and leaf extracts from *A. philoxeroides*. In general, the inhibition intensity was weak at low concentrations and increased at high concentrations, that is the growth of algae was greatly restricted. The same inhibitory trend was also found in the study of Li et al. (2016) about *Sagittaria trifolia* tubers extract influence on *M. aeruginosa*. After being treated with 0.9 g/L root extract for 96 h, the growth inhibition rate of *M. aeruginosa* cells was 80.48% (*Table 1*), which showed that *M. aeruginosa* cells could hardly survive in this environment. Scanning electron microscopy (SEM) displayed that the algae cells were damaged in varying degrees with the increase of the concentration of root extract for 72 h (*Fig. 5*), which means the extracts of *A. philoxeroides* could inhibit cell growth by destroying the morphological structure of *M. aeruginosa* cells, promoting cell lysis and finally leading to cell death. As one type of small molecules, nucleic acid is normally enclosed in the cell. Sometimes, on account of adverse stimulus, the cell membrane cannot maintain the relative stability of the internal structure and function of cells, and control the transport of substances inside and outside the cells due to the loss of selective permeability, thus nucleic acid is released (Shi et al., 2018). With the increase of extracts concentration and culture time, the increase of nucleic acid content further verified the damage degree of algal cells (*Table 2*).

In order to survive in an unfavorable living environment, organisms have evolved a set of strategies to outwit their adversaries. One of them is the antioxidant enzyme system (García et al., 2016). Under normal circumstances, the process of cell metabolism to complete all kinds of life activities can produce reactive oxygen species (ROS). Due to the antioxidant enzymes, cells can effectively fight against the negative impacts of reactive oxygen species and maintain the intracellular ROS level in

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equilibrium (Pereira et al., 2018). Nevertheless, when the cells are subjected to adverse external stimulation (ultraviolet-B, heavy metal and allelochemicals), the original balanced state is broken, and excessive reactive oxygen exists, leading to peroxidation damage of the cells (Zhang and Benoit., 2019).

We found in this study, O_2^- transformation in *M. aeruginosa* could be affected by *A. philoxeroides* extracts, especially root extract (at 1.1 g/L) with a strong competence. The O_2^- content in the treatment group was significantly higher than that of the control group after exceeding a certain concentration range and constantly elevated with the extension of culture time (*Fig. 1*). Huang et al. (2013) also showed the accumulation of intracellular reactive oxygen species in the study of allelopathy of *Solidago canadensis* L. against *M. aeruginosa*, and pointed out that the response mainly depended on the allelopathic substance released by the plants rather than the plant species. Furthermore, induced accumulation of reactive oxygen species in cells may cause damage to photosynthetic pigment, protein, DNA and lipid (Apel and Hirt, 2004). Algal cell membranes are composed of unsaturated phospholipids and are susceptible to ROS, MDA as one of the products of lipid decomposition, it is usually used to mark the process of membrane lipid peroxidation, and the changes in its concentration can reflect the damage of cell membrane and the ability of cells to resist harmful external interference (Davey et al., 2005). The results indicated that MDA content in *M. aeruginosa* increased gradually, which coincided with the increase of O_2^- content, but after 24 h treatment with the root, stem and leaf extracts of 0.3 g/L, MDA content decreased slightly compared with the control group (*Fig. 2*). Under the stimulation of short time and low concentration of the extracts, stress response occurred in algal cells thus membrane permeability and enzyme activities heightened, which could promote cells to absorb the nutrients contained in the extracts and temporarily maintain cells growth (Yuan et al., 2020). In addition, a small amount of ROS was removed by antioxidant enzymes, and the content of MDA was also decreased. It was needed noting that severe membrane lipid peroxidation would lead to a continuous increase of MDA content as the effect of extracts exceeded the tolerance threshold of algal cells (Chen et al., 2019).

The formation of microcystins (MCs) and polysaccharides (PSs) is thought to be a way for algal cells to maintain their growth and a defense mechanism to fight against adverse external factors. MCs are secondary metabolites produced by *M. aeruginosa*, which are usually trapped in living cells, ROS can transmit signals to cause oxidative stress in algal cells, thus promote the combination of MCs with some specific proteins to regulate self-synthesis and release process (Tsai, 2015). Wu et al. (2013) found that allelochemical extracts of *Pistia stratiotes* had influences on *M aeruginosa*, during the whole culture period, high concentration extract had no significant role in the release of extracellular MC contents while the intracellular MC contents increased in a concentration-dependent manner and finally remained stable. Hou et al. (2019) pointed out that the allelopathic inhibition of juglone on *M. aeruginosa* resulted in the increase of both intracellular and extracellular MC contents with the rise of juglone concentration. Our experiment showed the same results with Hou et al. (2019) that intracellular and extracellular MC contents increased in a dose-dependent manner after 72 h treatment (*Fig. 3*). The main reason was that membrane lipid peroxidation promoted the production of MCs, and the extracellular MC contents were released gradually with the metabolic activities and life process of the cells. The content changes display a positively interactive effect between intracellular and extracellular MC

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contents (Chen et al., 2015). It was worth saying that the production and release of MCs could be used as an important indicator to evaluate the treatment of algal blooms by allelochemicals. In our study, although the contents of MCs increased gradually in the test, the extracellular MC contents was far less than the intracellular. Especially at high concentration of root extract (0.9 g/L 96 h), the inhibition rate of algal cells was more than 80%, that is the cell survival rate was low, but most MCs still existed in the cells. In the follow-up research, it is necessary to further isolate and identify allelochemicals that play a major inhibitory role in *A. philoxeroides* and estimate the usage amount of *A. philoxeroides* in order to better evaluate the ecological safety of its application as an algicide.

Polysaccharides (PSs) are a kind of macromolecular substances with high activity. It has been reported that various biological factors (predation, competition) and non-biological factors (nutrition, temperature, light) can affect the production and release of PSs from *M. aeruginosa* (Zhu et al., 2014). The presence of PSs can not only promote algal cells to form large coenobium so as to protect cells from invasion, but also had the ability to remove excess ROS in cells to relieve oxidative stress reaction (EI-Sheekh et al., 2012). It was found in our study that both intracellular polysaccharides (IPSs) and extracellular polysaccharides (EPSs) contents increased with the increasing concentration of extracts from different plant parts of *A. philoxeroides* after 72 h (*Fig. 4*). And the change of the PSs was consistent with that of MCs contents, indicating that algal cells could produce MCs and PSs, and improve the yield with the increase of stress intensity for self-protection in the face of awful environment. Both MCs and PSs may be regulated by cell peroxidation caused by oxygen radicals, and a certain amount of MCs could activate PSs synthesis genes (Mohamed, 2008). For the reason why the content of EPSs was much higher than that of IPSs, some studies have mentioned that EPSs were produced by IPSs and then secreted to the outside of the cells, and there was a significant positive correlation between them, particularly, under terrible environmental conditions, cells would try to release more PSs outside (Liu et al., 2020).

Conclusions

Our current study revealed that the root, stem and leaf extracts of *A. philoxeroides* could inhibit the growth of *M. aeruginosa*. It was also found that root extract had the strongest inhibitory effect, followed by stem and leaf, which may be related to the specific allelochemicals contained in different plant parts of *A. philoxeroides*. With the extension of culture time and the increase of extracts concentration, the superfluous generation of O_2^- led to cell damage and the contents of MDA and nucleic acid increased. At the same time, *M. aeruginosa* cells could resist harmful irritation by releasing microcystins and polysaccharides in a dependent manner with the concentration of the extracts.

Nonetheless, the contents discussed in this study are still insufficient. The important allelochemicals in the extracts of *A. philoxeroides* and more internal mechanisms limiting the growth of *M. aeruginosa* will be the focus of future research. Meanwhile, measuring the pros and cons of ecological application of plant aqueous extracts remains the key, and a large number of studies under natural conditions are requisite.

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ASSESSMENT OF WASTEWATER CONTAMINANT CONCENTRATION THROUGH THE VADOSE ZONE IN A SOIL AQUIFER TREATMENT SYSTEM

AMIN, H. M.[1*] – GAD, A. A. M.[2] – EL-RAWY, M.[3,4] – ABDELGHANY, U. A.[1] – SADEEK, R. A.[3]

[1]*Civil Engineering Department, Faculty of Eng., Al-Azhar University, Qena, Egypt*
(e-mail: HaithamAmin.e20@azhar.edu.eg)

[2]*Civil Engineering Department, Faulty of Eng., Assiut University, Egypt*
(e-mail: aligad1964@gmail.com)

[3]*Civil Engineering Department, Faculty of Eng., Minia University, Minia 61111, Egypt*
(e-mail: mustafa.elrawy@mu.edu.eg)

[4]*Civil Engineering Department, College of Engineering, Shaqra University, Dawadmi 11911, Ar Riyadh, Saudi Arabia*
(e-mail: mustafa.elrawy@su.edu.sa)

[*]*Corresponding author*
e-mail: HaithamAmin.e20@azhar.edu.eg; phone:+20-111-343-0149

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Abstract. Soil aquifer treatment (SAT) is one of the most important methods for reusing wastewater. This study evaluated the influence of 300 cm vadose zone thickness on the removal of wastewater contaminants. Physicochemical parameters and heavy metal were assessed at 0, 30, 60, 100, 150, 200, and 300 cm soil depth. Results showed that the upper 300 cm soil layer effectively removed some contaminants from wastewater. An increase in soil pH with depth from 7.38 to 8.25. The removal efficiency of COD and BOD was 83.27% and 83.24%, respectively. TSS decreased from 146.7 to 76.5 mg/l, with a removal efficiency of 47.9%. A high removal efficiency of 91.4% was observed for TDS. Removal of TC and FC concentrations were high, with a maximum reduction of 99.5% and 99.4%. On the other hand, B concentration increased from 0.15 to 0.5 mg/l. Removal efficiencies for cations were 84.5%, 43.5%, and 15%, for Mg^{+2}, Na^{+}, and Ca^{+2}, respectively. Heavy metal concentrations within the soil layer were restricted to several centimetres. The removal efficiency was as follow; Cd^{2+} (100%)> Zn^{2+} (59.8%)> pb^{2+} (52.9%)> Mn^{2+} (10.8%)> Cu^{6+} (8.9%)> Al^{3+} (5.53%). Moreover, no significant removal values were obtained for chromium (Cr^{6+}) concentration.
Keywords: *soil pollution, wastewater treatment, biochemical oxygen demand, chemical oxygen demand, heavy metals*

Introduction

Wastewater is considered a source of harmful diseases. It contains a variety of contaminants, including heavy metals and pathogens, which can potentially harm the human, environment, and animal health (Singh et al., 2004; Chen et al., 2005; Hamilton et al., 2007; Qadir et al., 2007). The use of soil aquifer treatment (SAT) system for wastewater treatment is a widespread practice worldwide (Idelovich, 1981; Bouwer, 2002; Sheng, 2005; Dillon et al., 2006; USEPA, 2006; Goren et al., 2014). SAT is an artificial water recharge of partially treated effluents by water spreading in infiltration basins and percolation through the vadose zone followed by additional purification processes occurring in the saturated zone of the underlying aquifer (Bouwer, 2002; Sharma and Kennedy, 2017).

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The most important advantages of SAT are improved water quality (Barba et al., 2019; El-Rawy et al., 2019; Al-Maktoumi et al., 2020), groundwater level restoration in depleted aquifers (El-Rawy et al., 2016; Salameh et al., 2019; Siebe et al., 2019), and the probability of partially treated wastewater storage in the aquifer (Elkayam et al., 2015; Page et al., 2018; El-Rawy et al., 2019; Al-Maktoumi et al., 2020) for future use. In addition, it can also be exploited as part of a salt water interference barrier system along costal zones (Sharma and Kennedy, 2017; El-Rawy et al., 2019; Al-Maktoumi et al., 2020).

The soil in the SAT system provide a medium for natural purification processes (Fichtner et al., 2019) and allows partially treated wastewater to infiltrate through several hundred meters of the unsaturated zone and the aquifer, through which the recharged wastewater quality is much improved (Amy and Drewes, 2007; Nadav et al., 2012; Donn et al., 2020; He et al., 2020). During recharge process, the heavy metals, pathogens, organics, and some hazardous ions are efficiently removed and consumed (Bancolé et al., 2003; Reemtsma et al., 2010; Barba et al., 2019).

The unsaturated zone acts as the medium in which biological and physicochemical reactions occur (Cha et al., 2006) to significantly reduce wastewater parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), pathogens, nutrients, and heavy metals (Kopchynski et al., 1996; Drewes and Fox, 2000; Leenheer et al., 2001; Drewes et al., 2006) before reaching the aquifer. A minimum soil depth of 3 m to groundwater is usually recommended (Tchobanoglous et al., 1999) to safeguard the underlying aquifer's quality.

Some researchers showed the vital role of the thin upper layer of the soil in the removal process in SAT (Essandoh et al., 2013; Friedman et al., 2018; Mienis and Arye, 2018; Siebe et al., 2019; Takabe et al., 2019). Most microbiological activity occurs in the topsoil layer in SAT basins, where trade-off conditions for water, nutrients, and oxygen supply are more comfortable to be met. From soil column studies and field studies, it was concluded that biological degradation is the major process responsible for the decrease in organic carbon content (Wilson et al., 1995; Quanrud et al., 1996) coupled with mechanical filtration, which remove suspended organic matter in the top few centimeters of the soil layer where a surface-clogging layer is formed (Houston et al., 1999).

During SAT for target contaminants, the primary removal mechanisms include adsorption to soil grains or soil organic matter and biodegradation under oxic and anoxic redox conditions. Adhesion of charged particles to clay or organic media can also obstruct movement (Bales et al., 1991). Biodegradation is an essential process in the SAT system that causes the breakdown of organic chemicals in soil by the action of microorganisms naturally present in the soil (Charbeneau, 2000). In every 1 g of soil, there are about 10^7 to 10^8 bacteria, with the most enormous numbers being found in the surface layer (Ishizawa and Toyoda, 1964).

The soil and vadose (unsaturated) physicochemical properties allow for the natural dilution of chemical and biological contaminants (Bitton and Harvey, 1992; Wilson et al., 1995; Schijven and Hassanizadeh, 2000; Morrison et al., 2020). In many arid regions, soils are calcareous or alkaline and may possess harmful chemical, physical, and microbial properties (DeNovio et al., 2004).

The mixture of natural soil contains several types of particles: sand, gravel, silt, clay, etc. The soil grain size and components physically and chemically influence the infiltration system functions. For example, clay soil shows a good chemical collecting

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of contaminants, but it has more unsatisfactory physical performances such as low permeability and porosity. While sand soil has high permeability and air exchange accompany with a weak capacity in contaminants adsorption (Hillel, 1988; Wang, 2015). Using different soil layers for wastewater purification is very useful and economical when adding synthetic layers such as metal and coal into the system (Ho and Wang, 2015).

In the west of the Sohag infiltration site, the horizontal hydraulic conductivity is estimated to be 20.46 m/day, and the specific storage is estimated to be 4.37×10^{-2} (Abdel Moneim, 1999). El-Haddad and El-Shater (1988) and Youssef et al. (2011) indicated that the subsurface sediments have a high vertical hydraulic conductivity that supports the potential of aquifer pollution. On the other hand, it is observed that the majority of the sediment samples are classified as silty sand, muddy sand, or sand. The hydraulic conductivity ranges from 1.13 to 21.4 cm/h. The porosity (%) ranges from 30.9 to 46.8 (Youssef et al., 2011).

Researchers have made significant observations concerning the effects of wastewater effluent application to the soil. Generally, irrigation with partially treated wastewater promotes the total and available heavy metal concentrations in soils. Liu et al. (2005) found that the application of wastewater irrigation for 40 years in Beijing, China, resulted in increased Cd^{2+}, Cr^{6+}, Cu^{2+}, Zn^{2+}, and pb^{2+} concentration accumulation. Soil contaminants by heavy metals are of serious concern due to their toxicity and persistence in the environment (Facchinelli et al., 2001; Mico et al., 2006).

Previous studies of SAT systems primarily focused on removing contaminants from the water, mainly neglecting the soil concentration profiles' changes. A minimum of 300 cm unsaturated zone was available at the field site to study wastewater contaminants concentration behavior.

The objective of this study was to conduct a field study to measure and assess partially treated wastewater contaminants concentration through the upper surface layer of soil on the change of hydrogen ion (pH), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), total dissolved solids (TDS), total coliform (TC), fecal coliform (FC), boron (B), major cations (Na^{+}, Ca^{2+}, Mg^{2+}), and heavy metals (Al^{3+}, Cd^{2+}, Cr^{6+}, Cu^{2+}, Mn^{2+}, pb^{2+}, and Zn^{2+}) concentrations during its movement through the vadose zone.

Materials and methods

Study area

Sohag Governorate belongs to the arid area of Egypt. It is located in Upper Egypt midway between Cairo and Aswan and is approximately 125 km long with an average width varying from 16 to 20 km. The Nile River divides it into two parts; one to the west of the river and the other to the east of the Nile. In the west of Sohag, there is a wastewater treatment plant (*Fig. 1a*) that was constructed in 1995 (the oldest wastewater treatment facility in Sohag Governorate). Wastewater is treated by primary treatment followed by an aerobic activated-sludge process and clarifiers. The treatment capacity of the plant is more than 40,000 m^3 /day. The secondary effluent then allowed to irrigating of wooden forests at the El-Dair region (*Fig. 1b*), which infiltrate the soil through flooding basins into the groundwater aquifer. There is an infiltration site (SAT system) in the forest constructed and operated in 2006 (15 years recharging). It is consisting of eight infiltration basins, as shown in *Fig. 1c*. This site lies in the wadi

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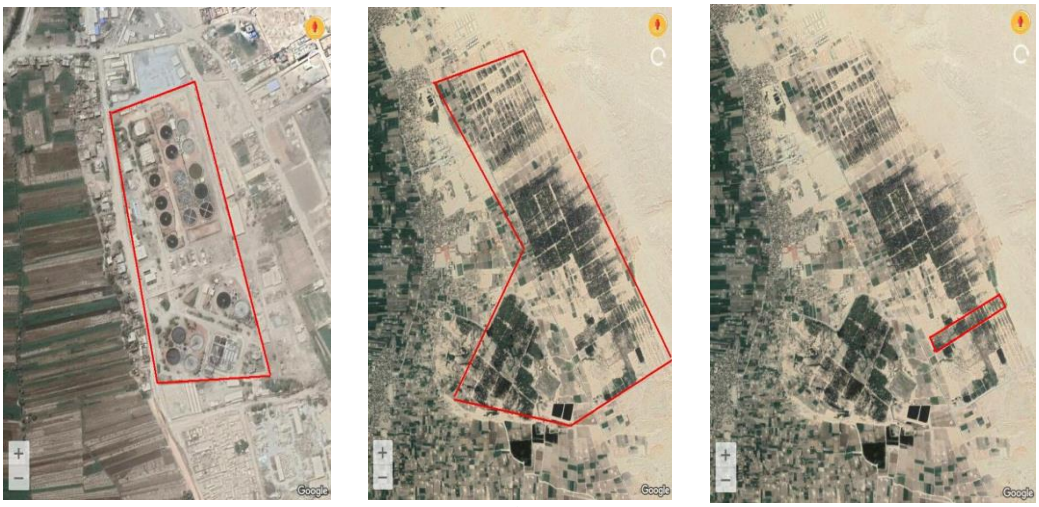
deposits (sandy gravel) ranging in thickness from 1 m to more than 10 m, which is bordered by the Eocene limestone plateau from the west and the cultivated floodplain from the east (Omer, 1996; Ali, 2005).



(a) (b) (c)

Figure 1. *a) Wastewater treatment plant; b) Wooden forests c); Infiltration basins*

Sampling and field methods

To assess wastewater effluent contaminants movement and retention through the soil, sampling was taken from the field (west of Sohag SAT). Samples were collected from the upper 300 cm of the unsaturated zone at different depths 0, 30, 60, 100, 150, 200 and 300 cm. Excavation was carried out in the infiltration basin after the flooding period ended.

A conventional backhoe (the type of excavation equipment or digger consisting of a digging bucket on the end of a two-part articulated arm) was used in the excavation as shown in *Fig. 2a*, for removing soil to a depth of 300 cm in a series of vertical soil sections at distances of 0, 30, 60, 100, 150, 200, and 300 cm from the infiltration basin surface (*Fig. 2b)*. Prior to collecting the soil samples, the soil face was cleaned using trowels to remove potential smearing. Soil sample characteristics obtained from the infiltration site are shown in *Table 1*.

The sand particles founded the highest portion than clay and silt and rendered the texture class to be sandy loam as determined using the soil texture triangle. Texture class (sandy loam) could be a helping factor in the leaching of recharged wastewater.

Samples were stored immediately in an icebox as shown in *Fig. 2c,* and transported to the laboratory within 24 h, and analyzed for hydrogen ion (pH), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), total dissolved solids (TDS), total coliform (TC), fecal coliform (FC), boron (B), major cations (Na^{+}, Ca^{2+}, Mg^{2+}), and heavy metals (Al^{3+}, Cd^{2+}, Cr^{6+}, Cu^{2+}, Mn^{2+}, pb^{2+}, and Zn^{2+}) in South Valley University, Faculty of Science, Central Laboratory, and El-Minia University, Faculty of Agriculture. All laboratory measurements were performed according to standard method for the examination of water and wastewater 23rd edition (Rice et al., 2017).

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(a) (b) (c)

Figure 2. *a) A conventional backhoe; b) Excavated borehole c); Soil samples*

Table 1. *Soil samples characteristics collected from the infiltration site*

Soil depth (cm)	Sand (%)	Silt (%)	Clay (%)
0-30	63.8	21.2	15.0
30-60	75.9	13.9	10.2
60-100	69.5	14.3	16.2
100-150	70	14.1	15.9
150-200	69.8	14.8	15.4
200-300	68.4	15.9	15.7

Results and discussions

The recharged wastewater quality characteristics for the SAT system is shown in *Table 2*.

Table 2. *Recharged wastewater quality*

Parameter	Concentration	Unit
pH) (Hydrogen power	7.38	-
Biochemical oxygen demand (BOD)	120	mg/l
Chemical oxygen demand (COD)	294	mg/l
Total suspended solids (TSS)	97	mg/l
Total dissolved solids (TDS)	680	mg/l
Total coliform (TC)	90500	CFU/100 ml
Fecal coliform (FC)	40000	CFU/100 ml
Boron concentration (B)	0.1	mg/l

Hydrogen ion concentration (pH)

From *Fig. 3*, it is obvious that the initial pH value of the recharged wastewater was generally lower than the soil pH, and it is increased with depth. The average pH value at the 0, 30, 60 100, 150, 200, and 300 cm depth was 7.38, 7.8, 7.94, 8.06, 8.09, 8.15, and 8.25 mg/l, respectively.

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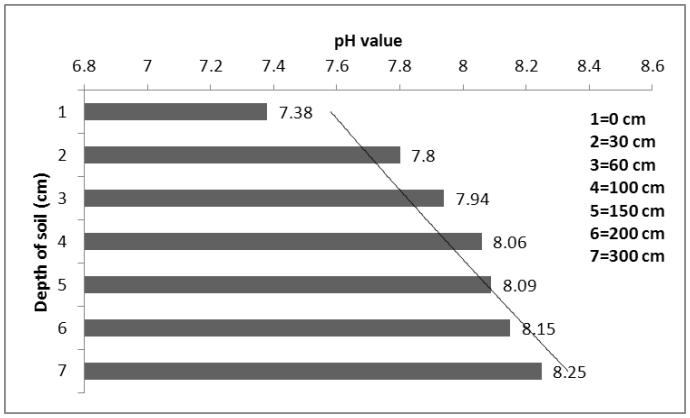

Figure 3. *Hydrogen ion (pH) values with soil depth up to 300 cm*

It is observed that when the partially treated wastewater comes into contact with the soil, the pH value was increased with depth. This situation can occur possibly due to sufficient soil buffering capacity to consume the H ions from nitrification reactions. Also, pH changes through the vadose zone depended on microbiological activities and chemical reactions. On the other hand, the increase in these values with depth can be attributed to alkali minerals' presence in the soil media. The results agree with (Schipper et al., 1996; Becerra-Castro et al., 2015; Shahrivar et al., 2020), which reported that soil pH increases following long-term wastewater application.

Biochemical oxygen demand (BOD)

Changes of average biochemical oxygen demand (BOD) concentrations with soil depth are depicted in *Fig. 4*. Results show that most BOD removal occurs during the upper cm of the soil and reaches a maximum removal of 78% at the 100 cm depth.

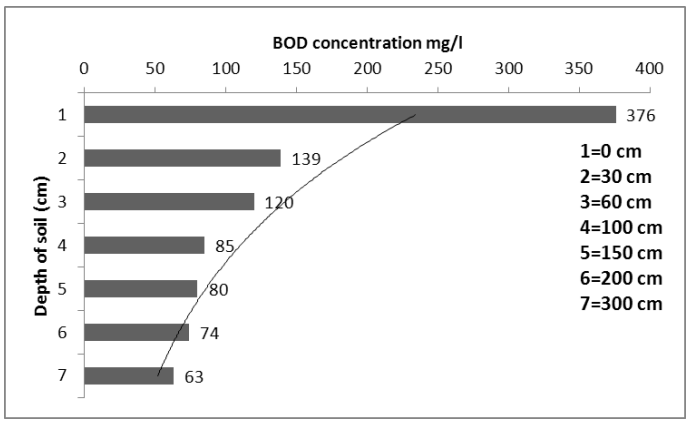

Figure 4. *Biochemical oxygen demand (BOD) concentration with soil depth up to 300 cm*

It is noticed a more significant proportion of the removal that occurs in SAT system occurs within the upper few cm of the soil. *Fig. 4* shows that the removal of BOD was highest within the upper 100 cm of the soil. The profile shows an overall decrease in concentration with depth. A steep decrease in BOD concentration in the upper 100 cm, and then a steady concentration was observed down to 300 cm of the layer. This

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phenomenon is mainly due to aerobic biological activity. Mechanisms of BOD removal from the recharged wastewater by SAT is a combination of biodegradation, filtration, and sorption processes.

Chemical oxygen demand (COD)

Chemical oxygen demand COD concentrations through the unsaturated zone were measured to be always lower than influent concentration. Variations of COD concentrations with soil depth are shown in *Fig. 5*. It is noticed that the higher removal efficiencies were achieved at upper 100 cm of the soil depth, and no significant differences were detected in depth greater than 100 cm up to 300 cm. COD concentrations through the soil depth operated with partially treated wastewater was decreased from about 538 to 90 mg/l with a removal efficiency of about 83%.

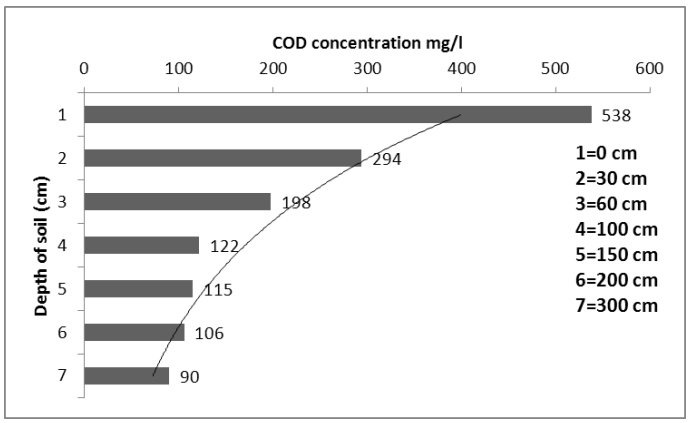

Figure 5. *Chemical oxygen demand (COD) concentration with soil depth up to 300 cm*

The high COD reduction in the unsaturated zone was attributed to aerobic microorganisms' ability to oxidize all organic material into carbon dioxide and water and oxidizable inorganic matter. As shown in *Fig. 5*, removal of COD is rapid near the soil/water interface in the SAT system, and the removal efficiency was nearly 78% achieved through the top 100 cm. High removal efficiency is due to dissolved oxygen and organic matter found at their highest concentrations in the topsoil surface, resulting in high biological activity (due to the large percentage of microorganisms that increase near the infiltration surface). Besides, there were no limitations on dissolved oxygen availability in the soil.

Total suspended solids (TSS)

SAT effectively removes suspended solids (SS) from the recharged water within the upper soil layer. The main consequence of SS removal during soil passage is the clogging of the infiltration basin.

During SAT, the partially treated wastewater is spread in the infiltration basins to allow percolation through the soil down to the aquifer. The unsaturated zone acts as the medium in which physicochemical and biological reaction occurs to reduce wastewater parameters such as total suspended solids (TSS). From *Fig. 6*, it is observed that TSS concentration was high at the soil surface. This is due to the accumulation of TSS with time because of repeated recharge. The concentration decreased at the upper 30 cm of

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the soil layer from 146.7 to 125.4 mg/l. The concentration decreased gradually with depth from 125.4 to 76.5 mg/l with a maximum removal efficiency of 47.90%. This is due to the soil layer's effect, which caused a significant particle detachment, but some small particles penetrated it. When saturation is achieved, preferential drainage pathways through natural deliver the particles to deep distance.

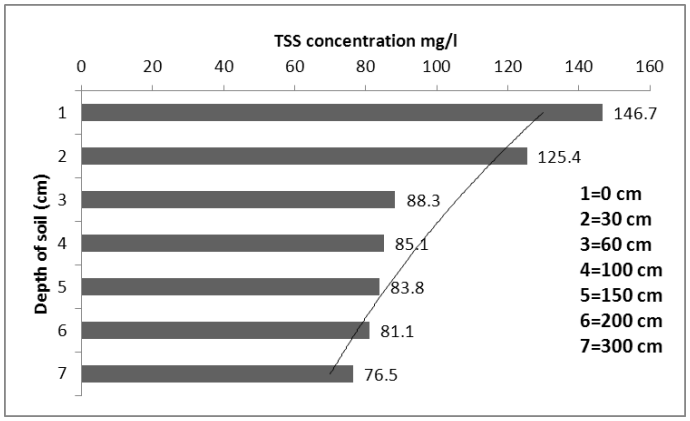

Figure 6. *Total suspended solid (TSS) concentration with soil depth up to 300 cm*

Total dissolved solids (TDS)

Figure 7 shows changes in TDS concentrations with soil depth in the field study. Generally, TDS decreased during the percolation through the soil layer in the field study. TDS concentration was declined from about average 680 to 58.5 mg/l with a removal efficiency of 91.4%. This decrease through the soil is due to adsorption and filtration through the soil matrix of dissolved wastewater materials. It is clearly noticed that the concentration of TDS at the top of the soil surface is more compared to the lower soil layer. This increase is due to TDS accumulation at the topsoil layer, and it is evidence for the desorption of adsorbed materials to the soil media.

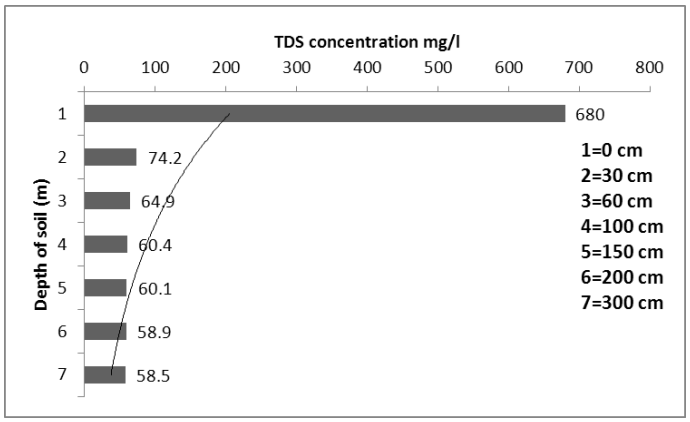

Figure 7. *Total dissolved solid (TDS) concentration with soil depth up to 300 cm*

It is seen from *Fig. 7* that the decreases in TDS values continued with depth. The removal of dissolved solid is due to the mechanical filtration in the top few centimeters of the soil, where a surface-clogging layer is formed. Adhesion of charged particles to

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clay or organic matrices can also obstruct movement. On the other hand, if the recharged water studies continued for a more extended depth, TDS might have decreased through the vadose zone.

Total coliforms (TC)

Total Coliform concentrations (TC) at the topsoil surface was measured as 3480 CFU/100 ml. Results show that passage through the soil decreased TC counts when compared to the applied wastewater. *Fig. 8* shows that the highest number of microorganisms was observed in the upper 60 cm of the soil depth because the top of the soil contained more oxygen and organic carbon.

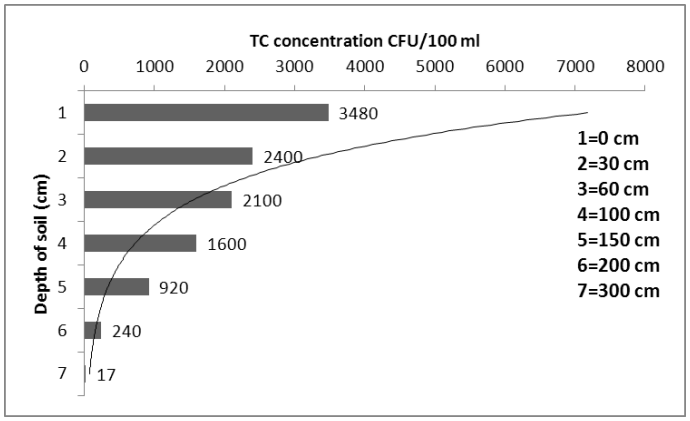

Figure 8. *Total coliforms (TC) concentration with soil depth up to 300 cm*

Due to the recharge, the concentration increased in the topsoil and then decreased up to 300 cm depth. During the SAT system, bacteria and viruses are removed by various processes such as filtration, predation, and adsorption. Physical (straining in soil) and chemical processes (adsorption by negatively charged soil particles) help keep microorganisms in the upper soil layer, where they are subjected to microbial competition, and decrease the potential for them to be transported to groundwater.

It is noticed that TC was almost completed removed from the applied wastewater at depth 300 cm with a removal efficiency of 99.5%. According to Harun (2007), removal efficiencies are affected by the retention time, grain size distribution, microbe's size, and microbes' ability to persist in soil. On the other hand, the most removals of the total coliforms from the partially treated wastewater effluents occur during depth greater than 300 cm. Variations in soil structure, texture can explain the high performances, and other conditions can also impact adhesion.

Fecal coliforms (FC)

Initially, coliforms did not easily remain viable following passage through a soil, which is consistent with others' results, who also reported significant decreases in coliform counts after exposure to the soil (Spackman et al., 2003; Durso et al., 2016). As shown in *Fig. 9,* passage through the soil decreased FC counts compared to the applied wastewater. Physical (straining in soil) and chemical processes (adsorption by negatively charged soil particles) help keep microorganisms in the upper soil layer,

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where they are subjected to microbial competition, and decrease the potential for them to be transported to groundwater.

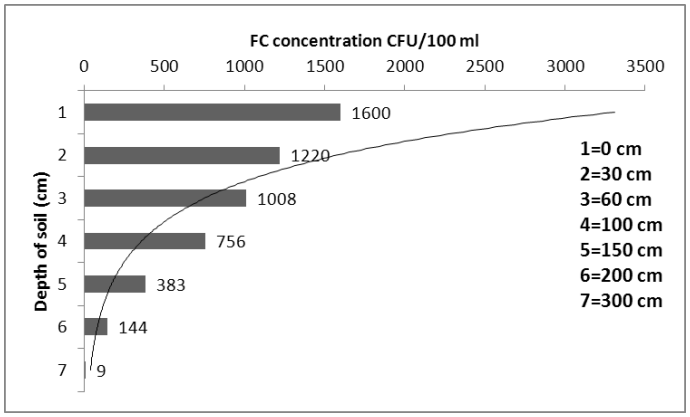

Figure 9. *Fecal coliforms (FC) concentration with soil depth up to 300 cm*

On the other hand, microorganisms removal efficiency is better in the unsaturated zone with effluent traveling through the smaller pores because slow average pore water velocities increase contact opportunities with soil surfaces. However, the greater depth of bacterial penetration may result from increased recharge loading and subsurface transport time. As suggested by Vasseur et al. (1996), soil exposed to coliforms for a long time may demonstrate that coliforms can adapt to the soil environment after long-term exposure.

Results indicated that the highest FC number was observed in the upper 60 cm of the soil depth because the top of the soil contained more oxygen and organic carbon. There was a significant reduction in FC concentration with depth because of decreasing organic carbon concentrations. The concentration reached 9 CFU/100 ml, at 300 cm depth with a removal efficiency of 99.4%.

Boron concentration (B)

Boron transport in the soil is illustrated in *Fig. 10*. Results indicated extensive movement to lower depth.

The B concentration in the soil increased with depth from 0.15 to 0.50 mg/l. This is due to adsorption reactions. Furthermore, the higher B concentration in leachate was due to B that was desorbed from the soil to the soil solution. The amount of B adsorbed by soils varies with soil constituents' contents, the most essential being clay minerals and organic matter (Keren et al., 1985). On the other hand, boron adsorption on soil constituents increases with increasing pH (Keren and Communar, 2009).

Major cations concentration

Samples collected from the infiltration site showed a decrease in the major cations (Ca^{2+}, Na^{+}, Mg^{2+}) concentration with increasing depth. The results showed that the application of wastewater caused an increase of Ca^{2+}, Na^{+}, Mg^{2+} of the soil at the top 30 cm. Increasing the cations at the top 30 cm of soil recharged with wastewater compared to the lower soil layer is attributed to minerals in the wastewater.

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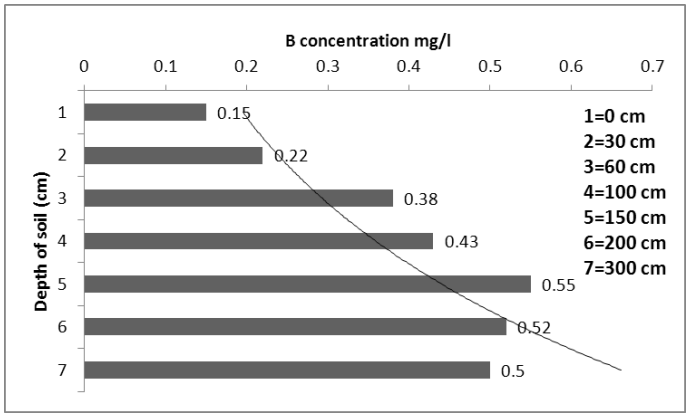

Figure 10. *Boron (B) concentration with soil depth up to 300 cm*

Calcium (Ca^{2+}) concentration was 8 mg/l at the upper 30 cm, then decreased with varying concentration during remain 300 cm with removal efficiency 15%. The concentration of sodium (Na^{+}) was 6.7 mg/l at the upper 30 cm. The concentration decreased with steady concentration from 6.7 to 4.4 mg/l at 60 cm up to 200 cm percolation, then decreased to 3.9 mg/l at 300 cm depth with removal efficiency 43.5%. This is in line with Najafi and Nasr (2009) findings and Mojiri (2011).

Magnesium (Mg^{2+}) has a constant concentration of 3.16 mg/l at the upper 30 cm, and then decreased with depth. The concentration decreased with irregular value and reached 0.49 mg/l at 300 cm depth. The removal efficiency was 84.5%. *Table 3* observed that salts accumulation increased in the topsoil as a result of high evaporation and capillary rise. Generally, the exchangeable cations maintain a descending complex as follows: Ca^{2+}> Na^{+}> Mg^{2+}.

Table 3. *Concentration of major cations at different depths of soil*

Parameter (mg/l)	**Recharged wastewater quality**	**Samples at different depths of soil (cm)**						
		0	**30**	**60**	**100**	**150**	**200**	**300**
Sodium (Na^{+})	6.9	6.9	6.7	4.4	4.4	4.4	4.4	3.9
Calcium (Ca^{2+})	8.1	8.0	8.2	6.0	5.4	8.0	7.4	6.8
Magnesium (Mg^{2+})	3.16	3.16	3.16	1.46	2.19	0	0	0.49

Heavy metals concentration (HM)

Heavy metals (HM) are non-biodegradable contaminants found in secondary wastewater effluent and thus may accumulate in the soil. It can negatively affect plant growth and groundwater quality. The concentration of heavy metals in the soil layer due to recharged with wastewater for the SAT system is shown in *Table 4*. The removal of HM concentration can vary within the soil depth. From *Table 4*, the following results can be concluded.

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Table 4. *Concentration of heavy metals at different depths of soil*

Parameter (mg/l)	Recharged wastewater quality	Samples at different depths of soil (cm)						
		0	**30**	**60**	**100**	**150**	**200**	**300**
Aluminium (Al^{3+})	0.15	84.3	93.46	119.05	123.31	85.98	85.90	79.64
Cadmium (Cd^{2+})	0.01	0.001	0.0024	0	0	0	0	0
Chromium (Cr^{6+})	0.01	0.52	0.54	1.12	1.28	0.99	2.12	1.59
Copper (Cu^{2+})	0.01	0.45	0.41	0.32	0.38	0.44	0.69	0.41
Manganese (Mn^{2+})	0.3	1.67	1.70	2.29	1.95	1.80	1.49	1.49
Lead (pb^{2+})	0.01	0.07	0.074	0.041	0.054	0.029	0.029	0.033
Zinc (Zn^{2+})	0.05	0.97	1.18	0.55	0.63	0.61	0.54	0.39

Aluminium concentrations (Al^{3+})

The concentration of Al^{3+} increased to a depth of 100 cm from the soil surface. As shown in *Table 4*, the concentration was 84.3 mg/l at the soil surface and reached 123.31 mg/l at depth 100 cm. The relatively high Al^{3+} concentration of several separate topsoil samples is due to the accumulation of Al^{3+} in the topsoil layer with time. On the other hand, the concentration of Al^{3+} decreased after 100 cm, and with increasing soil depth, the concentration decreased. After a depth of 100 cm, the concentration was 85.98 mg/l and reached 79.64 mg/l at a depth of 300 cm. This decrease is due to sorption and surface precipitation reactions on the soil.

Cadmium concentrations (Cd^{2+})

The application of partially treated wastewater had no significant effect on soil cadmium accumulation (Cd^{2+}). *Table 4* shows that Cd^{2+} concentration was efficiently removed within the top 30 cm soil depth. Compared to the recharged water, the concentration of Cd^{2+} is reduced from 0.001 mg/l to 0.0024 mg/l in the upper 30 cm. This reduction may be due to the plants' uptake in the infiltration basin and accumulation via surface adsorption and precipitation. On the other hand, efficient Cd^{2+} removal 100 percent was observed at a depth of more than 30 cm. This is due to adsorption by the soil during the penetration through the unsaturated layer.

Chromium concentrations (Cr^{6+})

According to *Table 4*, the chromium concentration (Cr^{6+}) in the soil samples increased, which is higher than either the recharged water. Cr^{6+} concentration increases with increasing soil depth with irregular distribution and reached 1.59 mg/l at 300 cm. This is due to the accumulation of it with continuous recharge or its presence in the soil, with a concentration higher than the recharged water. Accumulation of heavy metals from partially treated wastewater application could be caused directly by the wastewater composition (Dotaniya et al., 2018) or indirectly through increasing solubility of the

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indigenous insoluble soil heavy metals due to the chelation or acidification action of the applied wastewater (Rusan et al., 2007; Xue et al., 2013). On the other hand, the accumulation is due to precipitation and sorption or cation exchange.

Copper concentrations (Cu^{2+})

Due to the following recharge with time, heavy metal of Cu^{2+} increased in the topsoil, especially within the surface soil layer much higher than that of the deeper soil layers. This is due to surface adsorption and bioaccumulation, and precipitation of the heavy metal on the humic acid and fulvic acid-related organics (An et al., 2015). *Table 4* shows that the average copper (Cu^{2+}) concentration in the soil is 0.45 and 0.41 mg/l for the surface and bottom of the excavated soil, respectively. Cu^{2+} concentration decreases with increasing soil depth with irregular distribution and reached 0.41 mg/l at 300 cm. This is due to adsorption by the soil layer and precipitation. The result agrees with (Bouwer and Chaney, 1974; Dotaniya et al., 2018), which reported that Cu^{2+} accumulation in the topsoil increased due to the long-term wastewater application.

Manganese concentrations (Mn^{2+})

The concentration of Manganese (Mn^{2+}) increased, especially within the surface soil layer at the upper 60 cm. The increase in concentration is due to the accumulation of Mn^{2+} with time. Then the concentration decreased with increasing soil depth. As shown in *Table 4*, Mn^{2+} concentration was 1.67 mg/l at the soil surface then increased to 2.29 mg/l up to 60 cm depth (concentration increased 37.1%). On the other hand, the concentration decreased with a depth of more than 60 cm and reached 1.49 mg/l at 300 cm. This is due to accumulation via surface adsorption and precipitation. The majority of heavy metals in recharged wastewater were accumulated in the soil via physical adsorptions, chemical interactions, and bonding reactions (Lin et al., 2004; Zhang et al., 2014).

Lead concentrations (Pb^{2+})

The application of partially treated wastewater had a significant effect on the accumulation of lead Pb^{2+} with the upper 100 cm (0.054 mg/l) compared to the recharged concentration (0.01 mg/l). As shown in *Table 4*, lead concentration in the upper 100 cm of the soil layer was much higher than that of the deeper soil layers. This is due to surface adsorption, bioaccumulation, and local vertical transport. Its concentration decreased sharply after 100 cm depth from the soil surface and with increasing soil depth. The Pb^{2+} concentration decreased from 0.054 to 0.029 mg/l after depth of 100 cm while in the next 200 cm remained nearly at 0.03 mg/l.

Zinc concentrations (Zn^{2+})

Heavy metals of Zn^{2+} accumulated in the topsoil, especially within the surface soil layer much higher than that of the deeper soil layers. As shown in *Table 4*, the zinc concentration (Zn^{2+}) increased from 0.97 to 1.18 mg/l at the upper 30 cm of the soil. This is due to the accumulation of Zn^{2+} in the soil layer resulting from partially treated wastewater with time (Dotaniya et al., 2018). The 0-30 cm layer accumulation was high and then decreased to 0.55 mg/l at depth 60 cm. Zn^{2+} concentration decreases with increasing soil depth with irregular distribution and reached 0.39 mg/l at 300 cm. This

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decrease in concentration is due to the absorption by the soil layer during the recharge and precipitation. Bouwer and Chaney (1974) found a similar accumulation of Zn^{2+} in the top 45 cm horizon of sandy soil basins after 70 years of application of wastewater, and Banin et al. (2002) also observed that the accumulation of heavy metals occurs in the top 0-60 cm horizon of the recharge basins during 20 years recharging of partially treated wastewater.

Conclusions

From analyses of a 300 cm soil layer, it could be concluded that the upper soil layer was effective in the removal of chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), total dissolved solid (TDS), total coliform (TC), fecal coliform (FC), significant cations (Na^{+}, Ca^{2+}, Mg^{2+}), and heavy metals (Al^{3+}, Cd^{2+}, Cr^{6+}, Cu^{2+}, Mn^{2+}, pb^{2+}, and Zn^{2+}) concentrations. The pH value of soil increased with depth from 7.38 to 8.25. Chemical oxygen demand (COD) and Biochemical oxygen demand (BOD) was removed with an average of 83.27% and 83.24%. TSS concentration decreased from 146.7 to 76.5 mg/l, with a removal efficiency of 47.9% was achieved in the top 300 cm. Results showed high removal efficiency of TDS over the soil depth. The concentration decreased from 680 to 58.5 mg/l, with a removal efficiency of 91.4%. Removal of total coliform and fecal coliform concentrations from the recharged water were high with a maximum average reduction of 99.5% and 99.4%, respectively, at the 300 cm depth. Significant increases in boron (B) concentration (from 0.15 to 0.5 mg/l) with depth were observed at 0 - 300 cm. The exchangeable cations maintain a descending complex as follows: Mg^{2+} >Na^{+}> Ca^{2+}. The significant difference in Mg^{+2}, Na^{+}, and Ca^{+2} concentrations at 0 - 30 cm is high compared to the next lower depth. Removal efficiency was 84.5%, 43.5%, and 15%, for Mg^{+2}, Na^{+}, and Ca^{+2}, respectively. Heavy metals Al^{3+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and pb^{2+} distribution within the soil layer was fairly restricted to several centimeters. Infiltration of Cd^{2+} was restricted to about 30 cm depth. On the other hand, no significant removal values were obtained in chromium (Cr^{6+}) concentration, with increased penetration compared to the recharged water. The order of these metals in soil was as follow; Cd^{2+} (100%)> Zn^{2+} (59.8%)> pb^{2+} (52.9%)> Mn^{2+} (10.8%)> Cu^{6+} (8.9%)> Al^{3+} (5.53%). Finally, the SAT system based on the infiltration of partially treated wastewater into the soil is considered one of the most important land treatment techniques to polish partially treated wastewaters. Aerobic microbial activity within the soil layer's top portions was the main removal mechanism in the SAT system. High removal efficiencies were achieved within the topsoil layer, where oxygen levels were the highest. Filtration, adsorption, precipitation, and ion exchange were other effective mechanisms for polishing the SAT system's wastewater.

Recommendations for future studies

1. Study the transport of wastewater contaminants at depth greater than 300 cm.
2. Study the travel time of wastewater contaminants and filtration rate through the vadose zone.
3. Study the effect of temperature on wastewater contaminants removal during the vadose zone.
4. Study the effect of different soil types on wastewater contaminants removal.

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HOW ENVIRONMENTAL VARIABLES CAN DETERMINE THE CHIR PINE (*PINUS ROXBURGHII* SARG.) DISTRIBUTION IN SWAT HINDUKUSH RANGE OF PAKISTAN: CURRENT AND FUTURE PROSPECTIVE OF THE SPECIES

KHAN, N.[1*] – ALI, K.[2] – SAEED, S.[3]

[1]*Department of Botany, University of Malakand, Chakdara Dir Lower 18800, Khyber Pakhtunkhwa, Pakistan*

[2]*School of General Education, College of the North Atlantic Qatar, P.O. Box 24449, Doha, Qatar*

[3]*Department of Botany, University of Balochistan, Quetta, Pakistan*

**Corresponding author*
e-mail: nasrullah.uom@gmail.com

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Abstract. Distribution patterns of plant species and their relationships with physiographic, soil and climatic variables were investigated in the subtropical pine forests across the natural limits in the Swat, Hindukush range of Pakistan. A vegetation survey identified 9 tree species belonging to 8 families and 8 genera, which were classified into 3 distinct vegetation communities with an average density ranging from 512-1231 individuals ha^{-1} and basal areas of 31.6-36 m^2 ha^{-1}. Total density and basal area values for tree species located at higher elevations were lower than those in the middle and low elevation communities for all the species. We found that *P. roxburghii* had unimodal size distributions, suggesting that these populations are not at equilibrium and are changing over time. CCA-Ordination with the associated Monte Carlo permutation test was employed to explore the patterns of variation in vegetation distribution and identified elevation and soil organic matter as the most influential variables responsible for the changes in species composition. The MaxEnt predictive modeling results clearly indicate a significant difference in the present predicated inter-site floristic composition and distribution of the species, suggesting an overall restriction to the south, whereas, the future distribution model suggest a shift towards the neighbouring districts in the west. This study identified few significantly important environmental variables linked with *P. roxburghii* associations and their distribution across the study area. We recommend additional research that includes multiple sampling from varied locations and other abiotic and biotic variables throughout Pakistan and neighbouring countries for better understanding of the species niche modeling.

Keywords: *species distribution modeling, environmental correlates, Hindukush range, climate change, conservation and management*

Introduction

Disentangling of the principal mechanisms responsible for structuring plant communities has been a central research dilemma in community ecology (McCune and Grace, 2002; Condit et al., 2011). Several factors such as biogeochemical variations and edaphic, topographic, soil, and climatic variables allow for a suite of potential limiting factors and have varied effects on the vegetation structure and function at both local and regional scales (Song et al., 2004; Miehe et al., 2009). However, the effects of such factors on vegetation distribution were not examined until the advent of multivariate statistical techniques and bioclimatic models for the forest tree species in the northern mountainous ranges of Pakistan (Khan et al., 2011). The prominent role of environmental

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and climatic factors in determining distribution by the Pine, coniferous and broadleaved woodlands in northern Pakistan is still not properly documented except for the scanty work done by Siddiqui et al. (2010), Ahmed et al. (2011), Khan et al. (2013, 2014).

Mountain ecosystems are often regarded as being particularly sensitive to environmental factors (Shaheen et al., 2011) and climate change (Trevidi et al., 2008). However, this has rarely been investigated at the scale of individual mountain ranges or parts of ranges using vegetation environmental and climate relationships in northern Pakistan. The adverse effects of climate change on vegetation has been reported by several workers in different countries including Pakistan (e.g. Miehe et al., 2009) as this phenomenon has been supported by the climate data monitors and reported 1.5-4.5 °C increase in annual temperature using climatic models (Song et al., 2004). The IPCC (2013) has reported that increase in anthropogenic activities and industries will warm the climate and could lead to an increase in global average surface temperature of 1.1-6.4 °C by 2100 century. Hence, it is predicted that climate change will have profound biological effects, such as shifts in range of species distribution that are expected to be northwards (Barry et al., 1995).

Under the climate change scenario, Pakistan is recognized as one of the most vulnerable countries in South-Asia due to which several tree species are expected to vanish, particularly from the northern mountain ranges (Ali et al., 2014). In these mountains, the vegetation in Swat Hindukush range is comparatively undisturbed, which provides an ideal habitat for research on tree species distribution and its response to environmental variables and climate change. However, the drastic change in the forest composition and structure driven by climatic, environmental and human disturbances has recently been shown to be complex in nature (Ali et al., 2014). In addition, large scale fragmentation in these forest patches has resulted in increased number of threatened species and hence they are at a great risk of losing plant diversity (Ahmed et al., 2011). Numerous shifts in the distribution patterns and abundance of species have been recorded (Thomas et al., 2004) due to climatic variables such as temperature and precipitation that have significant effect on the distribution and population density of species. It is suggested that if the shift in the distribution of species does not occur towards suitable environmental regions, such species will face a serious risk of extinction (Thomas, 2011).

Some environmental regions are very prone to climate changes, i.e. the Mediterranean and Asian regions where droughts and unpredicted precipitation patterns will bring many changes. Species of many mountainous ecosystems will show different plastic and evolutionary strategies to cope climate change and most of them should move upward in altitude (Song et al., 2004). Despite the presence of such climatic and anthropogenic disturbances, the remaining primary forests of Swat Hindukush range of Pakistan lack studies that explain vegetation-environment and climatic relationships in a quantitative manner. Some studies (e.g. Ahmed et al., 2006, 2011; Siddiqui et al., 2009) used semi-formal non-numerical approaches with insufficient environmental factors that did not expose the underlying group structure or the overriding factors responsible for the distribution of these forests. Such scanty information is not productive in implementing conservation strategies and the provision of baseline for planning and assessment of the success of restoration activities (Sarker et al., 2014). Therefore, a more thorough system is required to predict the present-day species distribution representing processes that are assumed to control species range limits (Song et al., 2004). The present study was conducted in the large-scale *P. roxburghii* natural forests

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in Swat Hindukush range of Pakistan. These forests are distributed on both sides of river Swat and in the inner valleys, classified under the subtropical dry temperate forests of the country (Champion et al., 1965) and typically occurring in the Sino-Japanese phyto-geographical region of the world (Sher et al., 2014). Although, *P. roxburghii* is commercially and ecologically important and has high conservation value, but so far remains little explored by foresters and biologists (Khan et al., 2014).

In the present study, an attempt was made to explore *P. roxburghii* associations and their future distribution with environmental and climatic variables using advanced multivariate statistical methods and species distribution models (SDMs). Among the SDMs, MaxEnt model reliably predicts suitable habitat using presence records and pseudo-absence points (Phillips et al.; 2006; Elith et al., 2006) and have been widely used for the identification of suitable habitats, potential distribution range, and plant's future distribution changes (e.g., Ashraf et al., 2016; Abdelaal et al., 2019; Li et al., 2019; Kamyo and Asanok, 2020; Khan et al., 2020). Using both these techniques our specific research objectives were to examine (i) whether the occurrence of *P. roxburghii* is restricted to certain compositional and environmental circumstances (ii) whether *P. roxburghii* is regenerating in various community types linked with certain topographic, edaphic and climatic variables by analyzing its stand structures (iii) that density and recruitment of *P. roxburghii* in the forests can vary the structural characteristics of the stand, which may reflect an environmental gradient from various sites strongly dominated by *P. roxburghii* to those rich in broadleaved species and (iv) to simulate and compare the possible present and potential future distribution of *P. roxburghii* associations in Swat Hindukush range of Pakistan to assist its conservation and management.

Materials and methods

Study area

The Swat valley is part of the subtropical dry temperate areas in Hindukush range of northern Pakistan, located at 34° 34′ to 35° 55′ N and 72° 08′ to 72° 50′ E (Shinwari et al., 2003). The total area of the district is 5337 km^2 with long stretches of pines, and it is generally considered as a hub to biodiversity due to its unique hills and climate (Ali et al., 2014). Geographically, the area shares borders with Chitral, Indus Kohistan, Shangla, Bunir and District Dir (*Fig. 1*). The area has a Mediterranean climate comprising two phytoclimatic subtypes: dry and moist (Champion et al., 1965). The Swat meteorological station data revealed that the average annual temperature is 19 °C and annual precipitation averages 897 mm. Mean relative humidity remains high from January to March (75%) and dips below 40% from May to June. The most abundant substrate consists of marble sandstones, which form soils of the sandy type only, and are accompanied by clays, marls and limestone in Vertisols (Ali et al., 2014). *P. roxburghii* stands constitute the most extensive plant formation in the area between altitudinal ranges from 700 m to 1800 m above sea level. *P. roxburghii* occurs either in pure stands or mixed with other species (Appendix 1) in the shady zones and the valley bottoms, as well as interspersed with *Olea ferruginea* and *Quercus baloot* in drier transition zones.

Field methods

After general reconnaissance of the Swat District, 25 sites were selected for sampling that approximately covered the entire range of *P. roxburghii* distribution in its natural

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zones of occurrence. The field sampling was conducted during July 2017 to March 2018. Prior to field data collection, stand locations were selected systematically using Arc GIS v. 9. Coordinate pairs and other geophysical characteristics were downloaded as waypoints into GPS receivers so that the forest stands could be located in the field. In addition, collection of geo-referenced data about the stands was ensured robust with RedHen DX-GPS system and Nikon D300 camera to gather and save the background information with pictures as metadata. Over 2,000 photographs of forest communities were obtained and metadata was extracted with the help of BR's EXIF extractor, a freeware available online (http://www.br-softwaare.com/extracter.hotml).

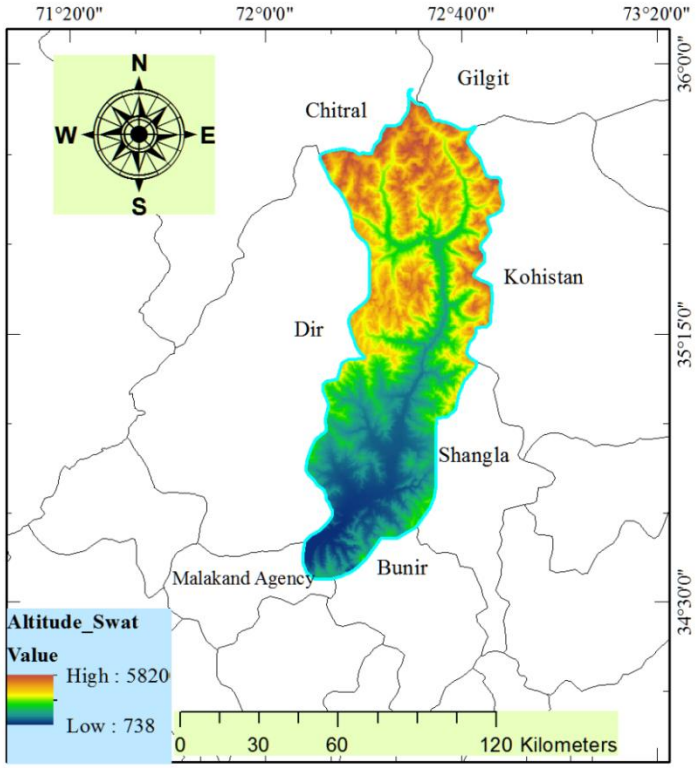

Figure 1. *Map showing altitudinal topographical variations in Swat District Hindukush mountain range of Pakistan*

At each forest stand, 30 quadrates of 10 m × 10 m in size along a 200 meter straight transect in a suitable direction in both disturbed and undisturbed forests were phytosociologically analyzed (Uprety et al., 2014). All constituent trees of ≥ 10 cm diameter at breast height (DBH) were counted and DBH (above 1.37 m height) was measured with forestry tape to quantify species composition and structural characteristics. In addition, dead trees were identified and their density was obtained to quantify structural feature and disturbance history of the forest. For advance growth, the regeneration layer, i.e. sapling (≤10 cm) and seedling (≤5 cm) of *P. roxburghii* and associated tree species, were systematically sampled by laying down 5 m × 5 m quadrates in the entire stands. We extracted two cores radii from living trees of *P. roxburghii* at breast height parallel to the slope contour in the opposite direction using Swedish increment borers (*Appendix 1*) to document age and radial growth. At least 2 cores from 30 randomly chosen individual trees were obtained with an attempt to

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achieve the pith of tree. Samples were placed with 2 end sealed plastic straws for safety with relevant information, i.e. DBH, site name and tree number. Six saplings were harvested from three different forests at low, middle, and high altitudinal zones at ground level to help determine the mean age at coring height following the method of Rigg et al. (1998). For each forest stand, soil samples from two pits (1 kg/pit at two different plots) were extracted at a depth of 30 cm using a bucket auger, and a pooled sample of 500 g was analysed in the Swat Agriculture Research Centre (SARC).

Quantitative and laboratory methods

Soil samples were air-dried, sieved through a 2-mm sieve, and analysed for texture (hydrometer method), pH (1:5 mixed soil-water solution using a digital pH meter model AS218), and total organic matter following the Springers-Klee method (Springer and Klee, 1954). The Kjeldahl method was used to determine total nitrogen (Bremner and Mulvaney 1982), available phosphorus was estimated following Olsen (1954), and exchangeable potassium by ammonium ions exchange using a galvanometer. Phytosociological attributes i.e. relative frequency; relative density, relative basal area, and importance value index (IVI) were calculated (Curtis and Mclntosh, 1950) for the overstory and understory species. Absolute values of density/ha and basal area m^2/ha were obtained for tree and understory strata. The IVI values for tree species and environmental variables were subjected to PC-ORD v. 5.10 for objective classification and ordination of the forests. From various classification methods, hierarchical polythetic agglomerative cluster analysis was used and stands were merged (clustered) into groups with the results being displayed as a dendrogram (McCune and Grace, 2002). The quantitative Sorensen (Bray-Curtis) an effective distance measure for ecological community analysis (McCune and Grace, 2002) and flexible beta as a linkage method ($\beta = -0.25$) was applied (Lance and Williams, 1967) which is compatible with Sorensen distance and is space-conserving (Legendre and Legendre, 1998). We used the Kruskal-Wallis test an alternative of one-way ANOVA to compare the environmental variables among different communities.

Detrended correspondence analysis (DCA), an indirect gradient analysis, was applied to identify the major gradient that influence species distribution. Preliminary analyses were made by applying the default option of DCA (Hill and Gauch, 1980) to check the magnitude of change in species composition along the first axis (i.e. gradient length in standard deviation (SD) units). In the present study, DCA estimated the compositional gradient in the vegetation data to be larger than 4.0 SD units for the first axis; thus, canonical correspondence analysis (CCA) was the appropriate ordination method to perform direct gradient analysis (terBraak, 1986). CCA was performed using 12 environmental variables after the exclusion of calcium, magnesium and electric conductivity as these variables were highly correlated and thus showed no significant differences among community types. All the default settings were used for CCA, and a Monte Carlo permutation test (499 permutations) was used to test for significance of the eigen-values of the first conical axis. Intra-set correlations from the CCAs were used to assess the importance of environmental variables.

All the tree species were grouped into different diameter classes of 10-20, and 21-30, and so on in each community type and Weibull function was fitted following Ryniker et al. (2006). Cores obtained were mounted, sanded, and polished with sandpapers of progressively finer grit until a fine surface was obtained as detailed in Stroke and Smiley (1996). These samples were measured to a precision of 0.001 mm under a

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stereo-microscope attached with a Velmex Measuring System (V. 10.6). The age of the trees was determined by counting the number of rings from the outermost ring to the pith. When the pith was not obtained in the core samples, we estimated the age of the ring closest to pith according to its shape of curvature (Xing et al., 2012). It is worth noting that the age of these trees thus obtained is the age of the stem at breast height, because the age does not include the time that the tree grow from the ground to the sampling height. Therefore, following Ogden and Ahmed (1989), rings obtained from sapling were added to the trees age in order to obtain total age of the trees. Linear regression was used to calibrate the relationship between age and size of the trees. For climate change modeling, the metadata obtained were transformed into CSV comma-delimited text file format that can be used with the Maximum Entropy (MaxEnt) software (Phillips et al., 2006). The HADCM3 A2 a climate change scenario (Collins et al., 2001) was used, which represents a grid point model that has a horizontal resolution of 3.75 × 2.5 degrees in longitude × latitude. This corresponds to a spacing between points of approximately 300 km. Bioclimatic layers (see *Table 1*) in GIS compatible format were downloaded from the Worldclim website and used in the analysis.

Table 1. *Different bioclimatic variables used in the simulation of P. roxburghii in Swat Hindukush range of Pakistan. (Source: WorldClim, 2011)*

S. No	Bio-climatic variables	Description
1	bio-1	Annual mean temperature
2	bio-2	Mean diurnal range (mean of monthly (max temp-min temp)
3	bio-3	Isothermality (100*mean diurnal range/annual temperature range) or (bio_2/bio_7*100)
4	bio-4	Temperature seasonality (standard deviation *100)
5	bio-5	Max temperature of warmest month
6	bio-6	Min temperature of coldest month
7	bio-7	Temperature annual range (bio_5 - bio_6)
8	bio-8	Mean temperature of wettest quarter
9	bio-9	Mean temperature of driest quarter
10	bio-10	Mean temperature of warmest quarter
11	bio-11	Mean temperature of coldest quarter
12	bio-12	Annual precipitation
13	bio-13	Precipitation of wettest month
14	bio-14	Precipitation of driest month
15	bio-15	Precipitation seasonality (coefficient of variation)
16	bio-16	Precipitation of wettest quarter
17	bio-17	Precipitation of driest quarter
18	bio-18	Precipitation of warmest quarter
19	bio-19	Precipitation of coldest quarter

Results

Composition and structural patterns

Among the 500 plots measured in 25 forest stands, 9 woody plants belonging to 8 families of 8 genera were identified and classified into 3 communities by cluster analysis (*Fig. 2*). These communities were clearly isolated in the nonmetric multidimensional scaling (NMS) ordination (results not shown). The summary of associated physiographic, and soil physical and chemical characteristics of these vegetation types were shown in *Table 2*. Group I was located at high elevations and slopes and low pH, nitrogen (%), K^+, enriched with herbaceous species and grasses i.e.

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Viola biflora, *Hetropogon cinata* and *Hetropogon* species. Group II occurred at medium elevations and slopes, pH levels, and phosphorus contents with frequent *Dodonea viscosa*, *Gymnosporia royleana*, *Plectranthus rugosus*, *Periploca aphylla*, *Teucrium stockcianum* and *Ajuga bracteosa*. The Group III vegetation type mainly distributed at low elevations and comparatively high slopes with low pH, Phosphorus and high organic matter, nitrogen (%), and clay (%); *Dodonea viscosa*, *Indigofera gerardiana*, *Ajuga* and *Salvia* were the common native species apart from the tree seedlings and saplings in the understory stratum.

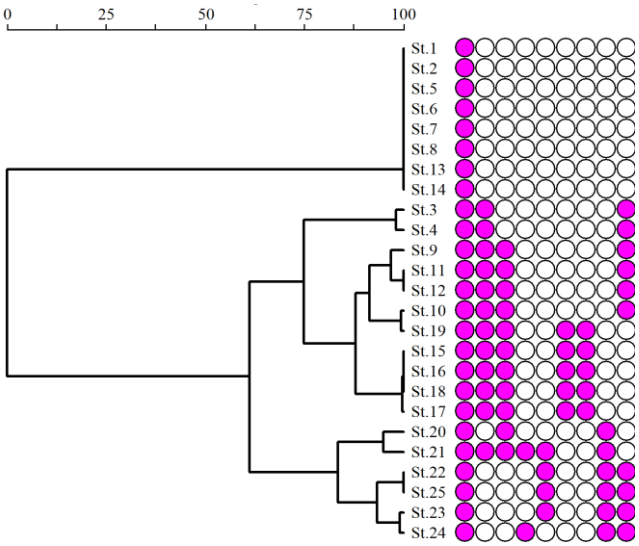

Figure 2. *Ward's Agglomerative cluster analysis of 25 forest stands and 9 tree species grouped into three major vegetation types using quantitative Sorensen (Bray-Curtis) distance measure with a flexible beta (β = –0.25) linkage extracted at 65% information*

Table 2. *Summary statistics of the Kruskal-Wallis test performed on environmental variables of the comparing tree communities*

Groups	**I**	**II**	**III**	**H-statistics**	**P-value**
Dominant trees	***P. roxburghii***	***P. roxburghii Q. incana***	***P. roxburghii A. modesta***		
Elevation (m)	1475 ± 46.17	1234 ± 46.17	1140 ± 35.38	6.51	**0.03**
Slope (°)	34 ± 1.37	27 ± 1.97	29 ± 2.11	3.83	**0.14**
Aspect	4.87 ± 0.91	5.4 ± 0.59	7 ± 0.68	3.46	**0.17**
Clay (%)	12.8 ± 1.10	12.5 ± 0.69	13.9 ± 1.03	1.25	0.53
Silt (%)	13.4 ± 1.23	12.9 ± 1.32	16.8 ± 2.08	3.02	**0.22**
Sand (%)	73 ± 1.59	72 ± 1.59	69 ± 2.30	3.08	**0.21**
pH (1:5)	6.81 ± 0.21	6.6 ± 0.15	6.9 ± 0.20	1.12	0.56
Org. matter	1.5 ± 0.45	1.2 ± 0.26	2.3 ± 0.71	2.21	0.33
Lime (%)	4.4 ± 1.07	6.4 ± 1.42	4.0 ± 0.93	1.16	0.55
N (%)	0.08 ± 0.02	0.10 ± 0.03	0.13 ± 0.03	1.20	0.54
P (mg/kg)	4.98 ± 0.17	5.5 ± 0.22	4.75 ± 0.04	7.32	**0.02**
K (mg/kg)	82.7 ± 12.2	81.5 ± 8.9	109 ± 23.5	0.79	0.67

org. matter: organic matter, N: nitrogen, P: phosphorous, K: potassium; P-values (bold) are significant α = 0.05

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Significant differences between the species composition ($A = 0.4037$, $P < 0.001$), structural parameters (i.e. density; $A = 0.3520$, $P < 0.001$; basal area; $A = 0.3260$, $P < 0.001$) and environmental matrix ($A = 0.1865$; $P < 0.001$) were obtained using MRPP. The result of cluster analysis was further clarified by a pair-wise comparison of the communities with high A-values recorded for Group I and II ($A = 0.4508$, $P \geq 0.001$) and Group I and III ($A = 0.4106$, $P \geq 0.001$), whereas substantial similarity was found between Group II and III ($A = 0.1727$, $P < 0.001$).

The average Importance values, density and basal areas for the tree species are presented in *Tables 3* and *4* according to the Phytosociological groups. The plots from 8 forest sites contained a single species declared as mono-specific community (Group-I) of *P. roxburghii* with an average density of 634 individuals ha^{-1} and 47 ± 7.66 basal area m^2/ha. The proportion of saplings was higher (25%) as compared to seedlings (15%) and dead logs (18%) which significantly contributed to the overall density in this community (*Table 4*).

Table 3. *Importance values (Mean ± SE) of tree species in three groups obtained from hierarchical cluster analysis*

Species	Group - I	Group - II	Group - III
Pinus roxburghii	100 ± 00	60.6 ± 1.86	58.33 ± 2.1
Quercus incana	-*	16.8 ± 3.18	1.66 ± 1.66
Quercus baloot	-*	9.0 ± 2.0	5 ± 3.41
Acacia modesta	-*	-*	15 ± 2.23
Persia dutii	-*	-*	2.5 ± 1.70
Monotheca buxifolia	-*	-*	6.66 ± 2.1
Punica granatum	-*	5.27 ± 1.92	10.83 ± 4.1
Olea ferruginea	-*	4.0 ± 1.47	-*
Ficus palmate	-*	4.0 ± 1.47	-*

-*/ - = absence

Group-II had eleven sites and 6 species dominated by *P. roxburghii* (IV = 60.6 ± 1.86) and *Q. incana* (IV = 16.8 ± 3.18), with a total density of 1231 individual's ha^{-1} and 35.31 basal area m^2/ha. The density of *P. roxburghii* and juveniles, i.e. seedlings and saplings was significantly lower as compared to Group I and III, respectively. However, dead trees shared 17% of the total tree density, which is comparatively higher than that of Group III, formed by six sites with seven species led by *P. roxburghii* (IV = 58.33%) and *Acacia modesta* (IV = 15%) in the arboreal forest (*Table 2*). The overall, density in this group is higher than the prior group (Group II) with an average density of 502 individuals/ha and 25.6 basal area m^2/ha of the dominant species. The main companions in these communities are *Q. baloot* with an average IVI that ranged from 5 – 9.0% followed by *Punica granatum* and *Monotheca buxifolia*. All these species contributed < 2% density and basal area in these communities. However, *Olea ferruginea*, *Ficus palmata*, *P. dutii* and *P. granatum* were minor associates with < 5% of IVI and 1% density ha^{-1} and basal area m^2/ha (*Tables 3* and *4*).

Vegetation-environment relationship

Twelve environmental factors measured were used in CCA to explore the patterns of species distribution. The correlation among the environmental variables showed

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virtually total independence, which generally, simplifies the interpretation of the present results. The iteration report showed that a stable solution was quickly found with a tolerance level of 0.100000E-12 (=10^{-13}) after 22, 55 and 18 iterations for the first three canonical axes, respectively. The unrestricted Monte Carlo test permutation *F*. ratios showed strong relationship between the matrices, i.e. eigen-values ($P = 0.0190$) and species-environment ($P = 0.0480$) correlation, indicating that observed patterns did not arise by chance. The results of the first three axes explained 49.3% of the variability in species data, of which 26.6% was accounted for the first axis. The results indicated a significant correlation between species and environmental variables in the first CCA axis ($R = 0.913$, $P = 0.02$). The results of canonical coefficients revealed that physiographic (elevation, aspect), soil physical (sand, silt, and clay) and chemical (lime and P) factors were the major variables in the first axis, whereas, potassium (K^+) dominate the third axis. The bi-plot species data shows the species that have greater loading on the axes, are *Q. baloot*, *O. ferruginea*, *F. palmata* and *Q. incana* which occupied the negative end of Axis 1, whereas, *A. modesta*, *P. dutii* and *M. buxifolia* occupied the lower and *P. roxburghii* the upper positive ends respectively (*Fig. 3*). This means that the positive axis species are extending their population while the negative axis species showed a similar underlying gradient but in an opposite direction to *P. roxburghii* along different environmental regimes in the study area.

Table 4. *Average density/ha and basal area m^2/ha of living and dead trees of P. roxburghii in the three groups. Only the density of seedling, sapling and dead tree of P. roxburghii are shown*

Species	Group - I		Group - II		Group - III	
	Dha^{-1}	**BA m^2 ha^{-1}**	**Dha^{-1}**	**BA m^2 ha^{-1}**	**Dha^{-1}**	**BA m^2 ha^{-1}**
Pinus roxburghii	634 ± 00	47 ± 7.66	433 ± 34.0	19.6 ± 6.4	512 ± 28.1	25.6 ± 8.9
Quercus incana	-*	-	55 ± 10.1	8.4 ± 3.6	10 ± 5.60	1.27 ± 0.22
Quercus baloot	-	-	17 ± 7.0	4.9 ± 2.0	20 ± 6.40	5.33 ± 3.1
Acacia modesta	-	-	-	-	22 ± 8.21	3.81 ± 1.8
Persia dutii	-	-	-	-	5 ± 2.72	0.01 ± 0.2
Monotheca buxifolia	-	-	-	-	13 ± 6.3	2.81 ± 1.9
Punica granatum	-	-	12 ± 4.94	1.45 ± 2.4	16 ± 7.5	2.55 ± 1.7
Olea ferruginea	-	-	10 ± 3.6	0.89 ± 0.44	-	-
Ficus palmate	-	-	7.0 ± 2.40	0.07 ± 0.04	-	-
Seedlings	234 ± 45	Nc	187 ± 66	Nc	209 ± 88	Nc
Saplings	377 ± 57	Nc	299 ± 105	Nc	266 ± 67	Nc
Dead trees	276 ± 79	Nc	211 ± 59	Nc	188 ± 78	Nc
Total	1522	47	1231	35.31	1261	41.38

-*/- = absence, Dha^{-1} = density per hectare, BA m^2 ha^{-1} = basal area meter square per hectare, Nc = not count

Diameter patterns

The diameters of *P. roxburghii* distributed in different stands were pooled based on cluster analysis and presented in the form of histograms that show a unimodal pattern (*Fig. 4a-c*). The mean diameter of the species was 8.1, 7.8 and 8.7 cm in Group I, II and III, respectively (*Table 5*). The standard deviation around the means was approximately

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2.3 cm for all the groups. The values for scale parameter (α and β) roughly followed the distribution diameter means, fitting the Weibull function as expected. The shape statistics for *P. roxburghii* in Group I was α = 4.1, for Group II α = 3.6, and Group III individuals was α = 3.8 respectively show that diameter distribution for *P. roxburghii* populations in all the groups are generally skewed. Results of the K-S test for function fitting are provided in *Table 5* indicating that the 2 parameters of Weibull function suggest the lowest mean values for the diameter distributions of *P. roxburghii* in all groups.

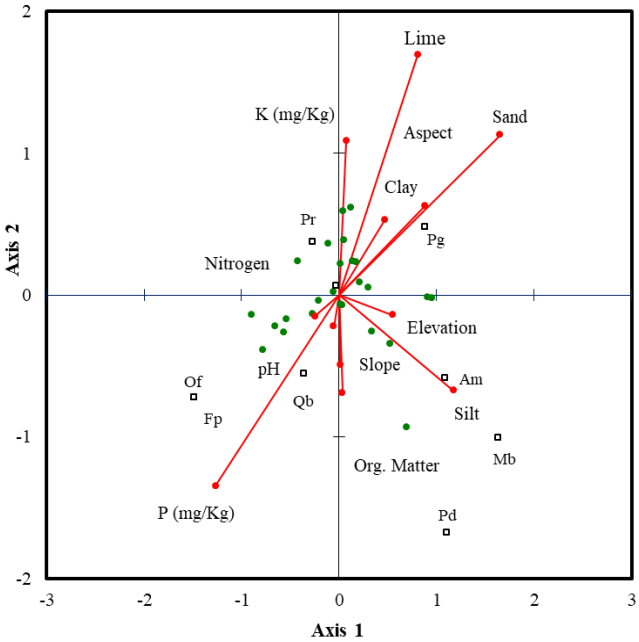

Figure 3. *Sites-environment on the bi-plot of CCA ordination of 25 forest stands. The eigenvalue for axis 1 was 0.22 and for axis 2 was 0.10*

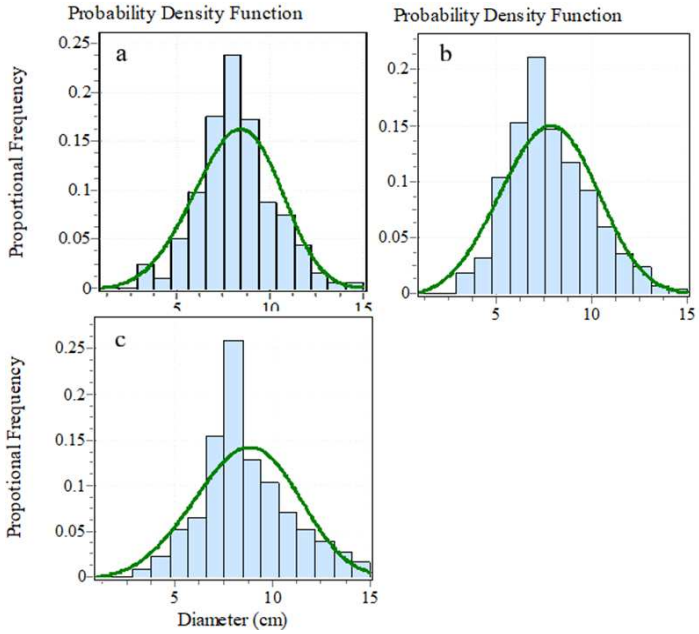

Figure 4. a-c *Diameter class histograms and corresponding fitted Weibull distribution for three groups of P. roxburghii*

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Table 5. *Representing, samples size (N), mean diameter (±SD), Weibull shape (α) and scale parameters (β) with K-S goodness of fit summary*

Groups	No. of samples (N)	Mean diameter (X)	Shape parameter (α)	Scale parameter (β)	Kolmogorov-Smirnov (K-S)
I	801	8.2 ± 2.1	4.1	9.0	0.14
II	854	7.8 ± 2.3	3.6	8.6	0.14
III	774	8.7 ± 2.3	3.8	9.6	0.16

Age and growth rate patterns

Age structure and growth rate pattern of *P. roxburghii* in different vegetation types (Group I-III) indicated a pattern similar to that of diameter. Bell-shaped age distribution was found in Group I, where the juvenile stages, i.e. 1-10 cm and 10-20 cm classes, accounted for 3 to 4% and 6 to 8% of the total individuals. Substantial numbers of individuals were young (58%) while old trees (>100 years) were rarer (13%) in this group. The mean annual increment was 5.0 (SD = ± 2.92 cm) which is higher than that of the other forest groups located in the area. Comparing Group II with prior (Group I) and proceeding group (Group III), the age structure was generally different. In Group II the youngest individuals were comparable (14%) while young were predominant, becoming 64% of the total. Only 10% of the individuals were between the ages ranged from 100-140 years (*Fig. 5*), and old trees above this range were entirely absent. Mean annual increment was 3.8 ± 1.92 cm which is higher than the individuals in the prior group.

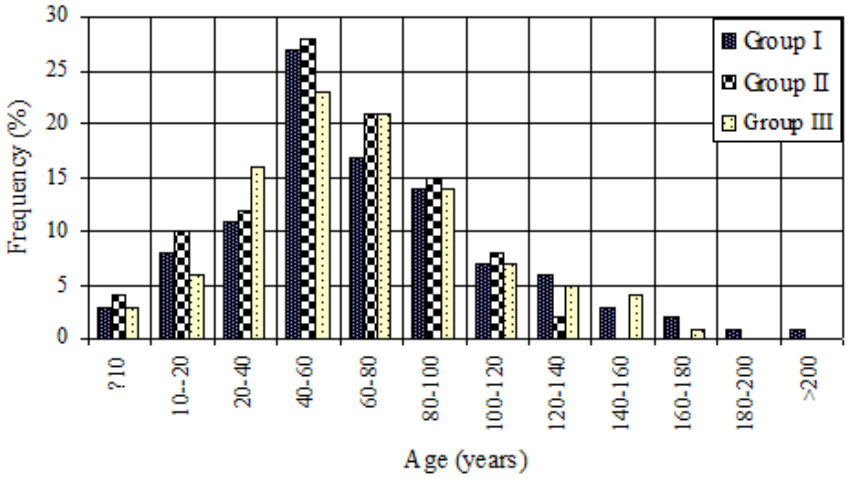

Figure 5. *The age structure of P. roxburghii populations in different groups*

Group III, shared a similar proportion of individuals in the youngest category (9%), while the majority (48%) of them were between 40 and 100 years old. Old trees (>100) were rare and no individuals were found > 180 years old, followed an almost bell-shaped pattern. Mean annual increment of *P. roxburghii* was 2.7 ± 0.98 which is highest among all the groups. Statistically, significant relationships were obtained between age and diameter ($y = 0.053x - 58.001$; $R = 0.852$ $P < 0.001$) and age and height ($y = 0.0453x - 50.002$; $R = 0.810$; $P < 0.001$) of *P. roxburghii* in the entire groups using regression equations.

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Predictive modeling

MaxEnt predicted the existing distribution of *P. roxburghii* as restricted to the south of the valley due to its significantly higher population density in these areas (*Fig. 6a*) whereas, the future distribution model shows even worse situation for the species, i.e., the entire shift of population is to the neighbouring district located in the west. Only a few patches at the western border may still survive because of the favourable conditions available at the end of the century (*Fig. 6b*). Ground-truth surveying (qualitative) was carried out to check the validity of the present predictive model. The Jackknife analysis (JA) of the present probability distribution of the area under cover (AUC) for the species indicates that all environmental variables are contributing to the AUC (over 0.80) except bio-7 (Temperature annual range), which has a very small share in the gain of AUC. The highest contribution was recorded for climatic variable bio-19 which is the precipitation of the coldest quarter (*Fig. 7*).

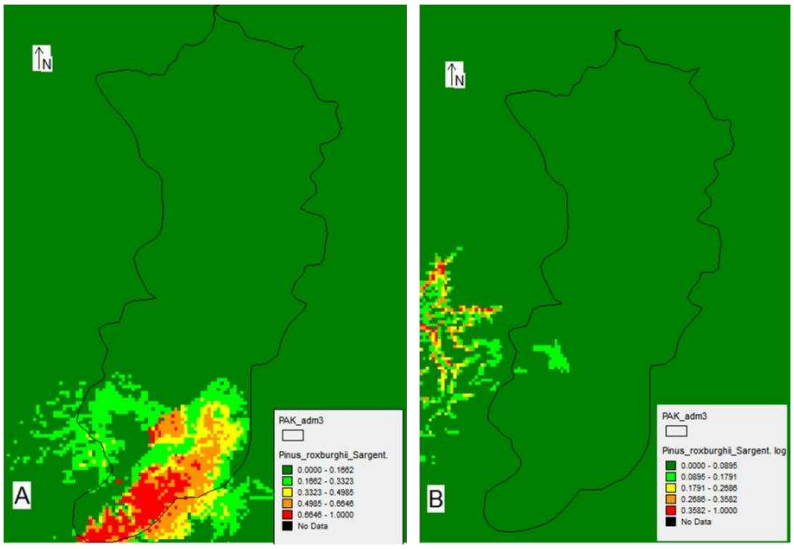

Figure 6. a *Present predicted distribution of P. roxburghii.* ***b*** *Future projected distribution of P. roxburghii*

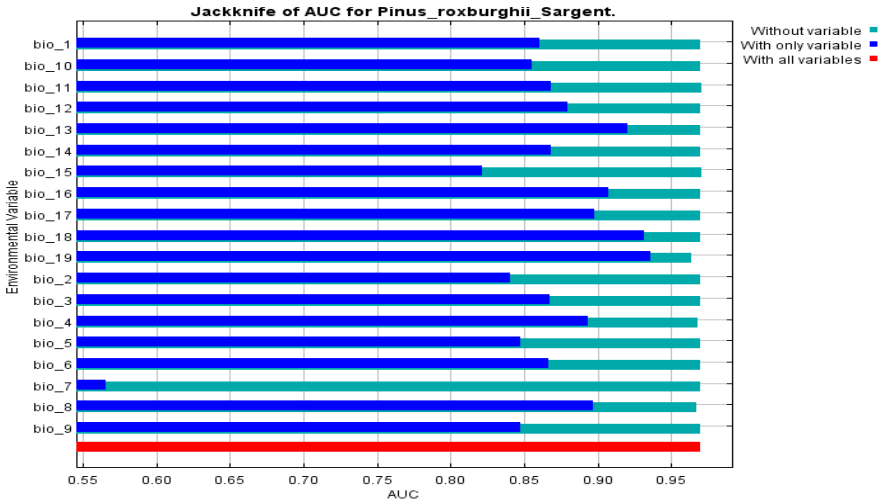

Figure 7. *Jackknife of AUC for P. roxburghii, present distribution model*

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The sensitivity and 1- specificity graph shows the best fit of the model with gain of 0.977 and 0.970 gain for AUC of training and test data, respectively (*Fig. 8*). The figure also clearly indicates the best fit of the model; both training and test omission that are very close with each other and close to the predicted omission (threshold of 0.5). In the future prediction model of *P. roxburghii*, the trend remains the same; the most important variable was found to be bio-19 and the least contributor was bio-7 (*Figs. 9* and *10*).

Figure 8. *Sensitivity and 1- specificity for P. roxburghii for present distribution model*

Discussion

Variability of environmental conditions could lead to spatial segregation of the flora and structural alterations in plant communities (Dufour et al., 2006). In the present subtropical pine forest, we have identified three distinct plant communities that vary considerably in species composition and occupied different ecological niches in this region. Contrary to monospecific community of *P. roxburghii* (Group I), *P. roxburghii* and *Q. incana* association (Group II) appeared to prefer medium elevation, degree of slopes, sand (%), pH and potassium, while *P. roxburghii* and *A. modesta* preferred the sites with low elevations, pH, lime (%) and high physical and chemical properties (*Table 2*). *P. roxburghii*, typically as a pure community, was mainly restricted to relatively high elevation slope sites, with low amount of clay, silt, nitrogen (%) lime (%) and potassium content. It has been reported by Siddiqui et al. (2009) that *P. roxburghii* mostly occurs as pure populations in most of the forest patches in northern Pakistan. However, on some drier hill sites it is associated with broadleaved and deciduous tree species like *O. ferruginea*, *M. buxifolia*, *P. granatum* and *F. palmata* in low and middle elevation zones (Khan et al., 2014). Several, other studies of vegetation classification were also conducted in Hindukush and Himalayan mountain ranges in Pakistan, showing that *P. roxburghii* occurs as a pure community and also a dominant associate with broadleaved and deciduous trees almost in the same elevation ranges (Malik et al., 2007; Nafeesa et al., 2007). These results correspond to our findings owing to similar eco-geographical regions. However, in neighboring countries like India, Nepal, and Bhutan

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this species is restricted to the Monsoon belt with summer rain forming associations with *P. ponderosa*, *Q. leucotricophora* and *Q. semecarpifolia* etc., which may be due to the different climate regimes and eco-geographical regions. These differences indicate that vegetation structure, community pattern and differential species distribution are affected by a broad array of biotic, environmental and climatic interactions that overlap and govern community structure in a complex manner (Sarker et al., 2014).

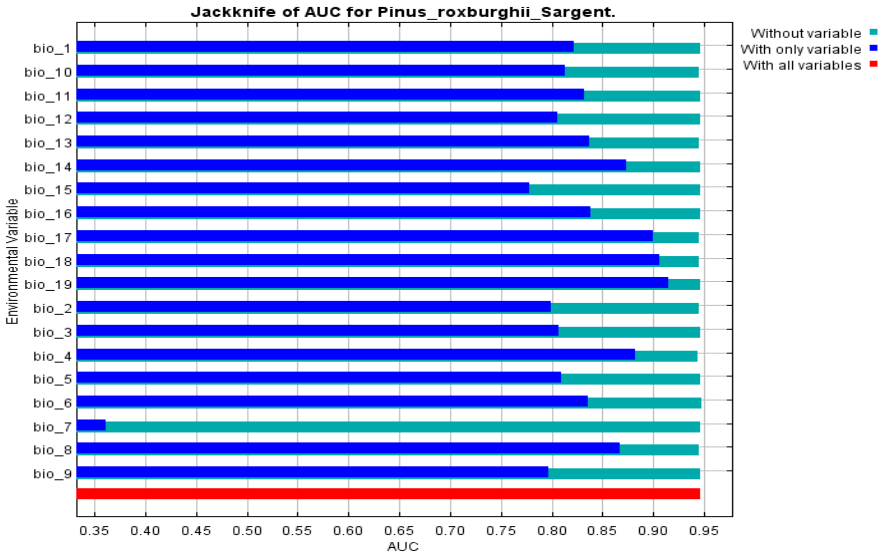

Figure 9. *Jackknife of AUC for P. roxburghii, future prediction model*

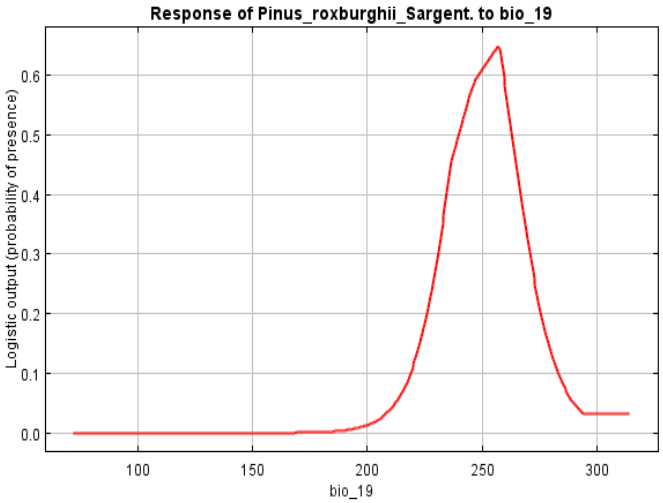

Figure 10. *P. roxburghii showing response to bioclimatic variable-19 (precipitation of coldest quarter) for future prediction*

In subtropical forests, natural vegetation often responds to several gradients simultaneously and different combinations of gradients produce divergent responses to the set of gradients (Khan et al., 2013). Our results of ordination support such a continuum and indicate that spatial distribution patterns of *P. roxburghii* associations do

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not follow a single environmental gradient, rather, an assortment of gradients account for its compositional variation. This variation could be attributed to physiographic (e.g. elevation, aspect), soil physical (e.g. clay, silt and sand particles) and chemical properties (lime and P) as found previously (Siddiqui et al., 2009; Ahmed et al., 2011; Khan et al., 2014) or climatic variables (Ali et al., 2014). These results show that species composition was affected not only by physiographic but also by soil properties, probably due to high variability of the sub-alpine and alpine environments (Champion et al., 1965; Marini et al., 2007). The ordination bi-plot species data shows that species with greater loading on the axes are *Q. baloot*, *O. ferruginea*, *F. palmata* and *Q. incana* which occupied the negative end of Axis 1, whereas, *A. modesta*, *P. dutii*, and *M. buxifolia* the upper positive end along with *P. roxburghii*. Such a distribution pattern shows that positive axis species are extending their populations while the negative axis species show a similar underlying gradient but in the opposite direction to *P. roxburghii* along different environmental regimes. These compositional changes in the current forests seem to be governed by altitudinal gradient acting as an elevation driver among the physiographic factors responsible for compositional variation in the high mountains range (Hong et al., 2015). Several factors like temperature, humidity, snowfall, solar radiation, etc. are associated with elevation, as demonstrated by several authors (e.g., Rana et al., 2011).

Few forest stands with trees in excess of > 110 cm DBH were observed in subtropical *P. roxburghii* forests and the majority are represented by trees < 50 cm DBH. Ahmed et al. (2006) suggest that the majority of mature subtropical pine forests have basal area values that fall within the range of 25-32 m^2/ha (based only on stems ≥ 10 cm DBH). The maximum observed basal area in *P. roxburghii* for any one stand, was 41.0 m^2/ha; this exceeds most observed values in other mature pine and coniferous forests in northern Pakistan and even in the neighboring countries (Siddiqui et al., 2009; Khan et al., 2011; Ahmed et al., 2011). All but one vegetation type (oak-olea) fall within or above the range of basal area suggested by Khan (2012) as being indicative of structural maturity in subtropical forests of northern Pakistan. The analysis of forest disturbance history from nondestructive sampling techniques is generally difficult, but DBH distributions may be useful for differentiating among broad-scale differences in stand structure (McCarthy et al., 1987).

The basal area results were confirmed by diameter size analysis, which indicted that all of the communities examined had a unimodal (Bell shaped) distribution, most likely due to high mortality or growth suppression of individuals in the smaller and greater size classes. It has been observed that *P. roxburghii* grow rapidly in height and diameter at early stage of life, with growth rate decreasing with age (Personal observations). In the current study the mean annual increment in the diameter ≥ 10 and ≤ 110 was 5.0 ± 2.92 in the highlands, 3.8 (±1.92), at middle elevations and 2.7 (±0.98) years/cm in the lowlands. These values indicated that *P. roxburghii* growth is also sensitive to climate as climatic parameters vary considerably on high altitude (Wang et al., 2004; Huang and Zhang, 2007). Strong relationships were obtained between diameter and age and growth rates, analogous to previous studies (e.g., Khan et al., 2014). Generally, the predication of diameter distribution of stands is of great interest to forest managers for the evaluation of forest resources and predicting future silvicultural treatments (Nano and Montero, 2002). Hence, the use of appropriate statistical models play an important role (Sheykholeslami et al., 2011) by indicating whether the density of smaller trees in a stand is sufficient to replace the current population of larger trees and to help evaluate

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potential forest sustainability (Rubin and Manion, 2006). We used the three-parameter Weibull function in the present study, which proved effective for fitting the diameter distributions of pine forests. This work will provide baseline information and will substantially increase our knowledge of diameter distributions of pine forests in northern Pakistan. The proposed model diameter distribution will be exceedingly useful for further inventories and the management of these forests as the present study was the first attempt in the area. Other species found in the present study including *Q. incana*, *O. ferruginea*, *A. modesta*, *P. granatum* and *M. buxifolia*, were all present as overstory and understory except in Group I, but at low densities. *Q. incana* and *P. granatum* have been shown to establish themselves in open canopy on northern aspects. However, their ability to replace *P. roxburghii* cannot be predicted from the present study, although it seems likely. Due to low sample size Weibull function for these species was not interpreted.

It is now a well-established fact that the climate change is real and the average global temperature in on the rise affecting organisms in one way or another (IPCC, 2013; Root et al., 2003). Generally, organisms show a well pronounced response to the change though the fact that some responses are less pronounced or remain unknown requires dedicated scientific investigations. The current study concludes that most of the tree species in the District will respond to the changing climate in the area and will either shift their habitats, reduce/increase their distribution or in some cases go extinct in the area. In corroboration with the findings of Song et al. (2004), altitude had significant effect on the distribution of species. They have also reported the effect of climate change on the northward movements of the tree species, including *Abies spp*. and *Picea spp*. The variable Bio-19 (precipitation of the coldest quarter) was found to be the most important variable in the present predictive distribution model of *P. roxburghii*. This environmental variable remains equally important for the future predictive model of *P. roxburghii* distribution. Another personal observation related to *P. roxburghii* is that it is the most preferred plant by the Forest Department of Pakistan for the purpose of reforestation, but the future distribution model tells a different story about the future of the species i.e., extreme reduction in distribution. It is evident from the results of the study that the associated species with *P. roxburghii* will have considerable impact on its distribution and density in the future but this warrants further studies to evaluate the impact of reduction and loss of this important plant on the other plant species and communities.

Conclusions

The use of numerical methods and species distribution model (SDM) were exceedingly useful in the exposition of the current classification, compositional variation with relation of environmental factors and in simulating the future distribution trend of *P. roxburghii* communities in Swat Hindukush range of Pakistan. In this paper, we described the first comprehensive investigation into environmental factors that significantly affect the distribution *P. roxburghii* communities in a priority conservation area of northern Pakistan renowned for eco-tourism. Despite the low variance in the data explained, the studied variables provide useful insight on plant distribution. Thus, the research approach demonstrated here can help in conserving the remaining natural patches of the forest by providing a basis for vegetation monitoring, mapping and assessing site qualities a priori.

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APPENDIX

Appendix 1. *Examples of ecosystems of a Pinus roxburghii landscape, Swat Hindukush Range Mountains of Pakistan (a) contains trees with obvious fire scars and with dry surface soils lose by landslide (b) young forest stand with poor understorey cover due to overgrazing, (c) private forest conserved by the local residents, (d) successfully regenerating young forest following harvesting or natural disturbance (e) coring a huge diameter tree using an increment borer at Karakar sampling site*

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A CONSORTIUM OF PLANT GROWTH-PROMOTING RHIZOBACTERIA STRAINS SYNERGISTICALLY ASSISTS JUJUNCAO (*PENNISETUM GIGANTEUM)* TO REMEDIATE CADMIUM CONTAMINATED SOILS

YANKEY, R.[1] – KARANJA, J. K.[2] – OKAL, E. J.[1] – OMOOR, I. N. A.[1] – LIN, H.[1] – BODJREMOU, D. M.[3] – LI, J.[1] – LIN, D. M.[1] – CAO, X. M.[1] – LIN, Z. X.[1*]

[1]*China National Engineering Research Center of Juncao Technology, College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China*

[2]*Center for Plant Water-Use and Nutrition Regulation, Joint International Research Laboratory of Water and Nutrient in Crops, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China*

[3]*Institute of Horticultural Biotechnology, College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China*

[*]*Corresponding author*
e-mail: lzxjuncao@163.com

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Abstract. Plant growth-promoting bacteria (PGPB) have received much attention in recent years due to their ability to interact with plants and remediate contaminated soil. This research aimed to assess the potential synergistic effect of three rhizobacterium strains; *Enterobacter cloacae* RCB980 (A3), *Klebsiella pneumonia* kpa (A4), and *Klebsiella sp* XT-2 (A7) in the remediation of cadmium (Cd) contaminated soils using *Pennisetum giganteum* plant. *P. giganteum* seedlings were transplanted into pots with seven different concentrations of Cd (0, 25, 50, 75, 100, 150, and 200 mg/kg), and the rhizosphere treated with combinations of bacteria A3, A4, and A7, for 60 days. Plant height, shoot and root biomass, chlorophyll content, bioaccumulation (BAF) and translocation factors (TF) were then determined. Root and shoot BAF for plants inoculated with bacteria strains at soil Cd concentrations of 25 and 50 mg/kg were all above 1.0 whereas TF values were greater than 1.0 only at 25 mg/kg Cd concentration. The study revealed that the application of double and triple strain consortium of bacteria significantly enhanced plant growth parameters and phytoremediation as compared to single strain. These results suggested that the strains had the synergistic potential to be utilized in enhancing *P. giganteum* growth and phytoremediation of Cd stressed soils.
Keywords: *phytoremediation, heavy metals, chlorophyll, bacteria, pollution*

Introduction

In recent years, contamination of the environment by heavy metals has increased sharply as a result of increased industrialization and excessive population growth. This continuous release of heavy metal pollutants into the environment has become so alarming that, the past decade recorded yearly global figures of 22,000, 783,000, 939,000, and 1,350,000 metric tons for cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn) respectively (Singh et al., 2003). This poses major environmental and human health problems worldwide. It has already been established that high concentrations of heavy metals in the soil affect the growth of plants and reduce agricultural productivity (Edelstein and Ben-Hur, 2018). The response of plants to heavy metals in soils differs. When most plants accumulate heavy metals into their tissues, cellular activities are

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negatively affected leading to retarded growth (Hall, 2002; Bücker-Neto et al., 2017). The common heavy metal pollutants are Cd, Pb, Cu, Hg, and Zn (He and Yang, 2007), therefore complete removal of such metals is the only way to effectively treat them since they cannot be easily broken down into harmless products (Wu et al., 2018).

Although several methods, with varying degrees of success and cost, are employed to address heavy metal pollution, the use of green plants in association with plant-growth promoting rhizobacteria (PGPR) has been generally accepted to be highly productive (Saxena et al., 2019). The use of grasses is most encouraged as they grow faster in low nutrient soils and usually have dense root and shoot biomass. Microorganisms play several roles in soil-water-plant-pollution relationships. Their importance in the heavy metal phytoremediation process cannot be over-emphasized, as their ability to endure metal toxicity, and change metal species into less toxic and soluble forms for plant uptake while promoting plant growth leading to enhanced biomass even under stressed environments.

Many studies have shown that bacterial strains of *Enterobacter and Klebsiella* species exhibit physiological and genetic characteristics which significantly enhanced growth of various plants (McKenzie-Reynolds, 2018; Liu et al., 2018; Dhungana and Itoh, 2019). *P. giganteum* also known as Jujuncao in Chinese is a tall herbaceous grass that is hardy and grows quickly under poor soil conditions while producing high biomass. The grass is widely cultivated and used for ecological remediation, animal feeding, and for production of edible and medicinal mushrooms. Although heavy metal accumulation capacities of certain plants such as *Brassica juncea*, *Helianthus annuus*, and *Zea mays* have been extensively studied, their large scale use for phytoremediation is limited due to their low biomass (Cui et al., 2004; Turgut et al., 2004; Szabó and Fodor, 2006). Despite high biomass trees such as *Salix sp* and *Populus sp* being demonstrated by Liphadzi et al. (2003) and Vervaeke et al. (2003) to potentially be ideal for phytoextraction, such trees generally take a longer period of time to grow and are therefore not good candidates for phytoremediation. Grasses, however, have generally become a good fit for this purpose and Jujuncao in particular, has been identified as potential good phytoremediators because of its wide growth adaptability, fast growth, and extensive roots and shoot biomass (Hayat et al., 2020). It would thus be interesting to explore the synergistic plant growth promotion association between these PGPR and Jujuncao for phytoremediation.

The success of bacteria assisted phytoremediation differs depending on the levels of tolerance of the plant and bacteria to the heavy metal in question. For these concerns, the selection of plant and bacteria species for phytoremediation of heavy metals depends mainly on the tolerance capacity of the bacteria and plant to the heavy metal and the plants' biomass production capabilities (Rezania et al., 2016). Moreover, it has been established that multiple contaminations of the same soil by different heavy metals are common and because different bacteria employ different mechanisms for remediation activities, some authors stress that bioremediation of heavy metals would be more successful if a cocktail of bacterial strains is utilized rather than using a single strain culture (Kang et al., 2016; Varjani et al., 2020). Previous studies have reported the ability of *Klebsiella sp* and *Enterobacter sp* to tolerate high Cd concentrations and to promote plant growth in heavy metal contaminated soils (Pramanik et al., 2018; Chuanboon et al., 2019). Therefore, this research aims to study the synergistic effect of these strains in the remediation of Cd contaminated soils using *P. giganteum* plants.

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Materials and Methods

Isolation of rhizobacteria strains

Following a random harvesting of 20 *P. giganteum* plants from the Lianjiang abandoned copper mines, Lianjiang, Fujian province, China and their subsequent movement to the laboratory in zip lock bags, bacteria was isolated from the composite pool of rhizospheric soil attached to the roots of the plants using Davis Minigioli (DM) agar medium according to the procedure of Sarkar et al. (2018). High Cd tolerance was taken as preliminary screening criteria during the isolation of the PGPR using the plate technique screening method according to the procedure of Rajesh et al. (2014). Subsequently, three PGPR strains of the Klebsiella and Enterobacter species were selected, identified as *Enterobacter cloacae* RCB980, *Klebsiella pneumonia* kpa, and *Klebsiella sp* XT-2 and were subsequently tagged as A3, A4 and A7, respectively, for the study of their synergistic growth and phytoremediation potentials on *P. giganteum.* The BLAST sequences were submitted to NCBI and assigned Genbank accession numbers MT103318, MT103319, and MT103320, respectively.

Soil preparation and pot experiment

The experimental soil was collected from Minhou, Fuzhou city, Fujian province, China. The basic physicochemical properties of the soil were pH 6.5, total organic carbon 16.3 g/kg, total nitrogen 1.2 g/kg, cation exchange capacity (CEC) 10.5 cmol/kg, and total Cd 0.95 mg/kg. The methods for determining the basic physicochemical properties of soils followed Tang et al. (1999). Soil total Cd was determined by ICP-MS (Agilent 7500a, USA) after digestion with HNO_3-$HClO_4$-HF. Cuttings of *P. giganteum* were prepared and nursed at the Juncao experimental field and were grown in mini pots for one month before being transferred to the experimental pots (40 (diameter) x 40 (height) cm) containing 9 kg of non-sterile field soil. The experiment was carried out in a greenhouse with a temperature range of 25 to 32°C and 50 to 80% relative humidity, while soil water content was maintained at 60% of water holding capacity.

The experiment employed a completely randomized design that had seven treatments of the three bacteria strains and their combinations; (A3, A4, A7, A3&A4, A3&A7, A4&A7, A3&A4&A7); applied on *P. giganteum* in pots under seven concentrations of Cd (0, 25, 50, 75, 100, 150 and 200 mg/kg) with three replicates each. Different concentrations of the Cd were prepared in distilled water, added to the soil in the pots, and mixed thoroughly. The control without Cd was mock-treated with the same amount of distilled water. The Cd contaminated soils and the controls were subsequently kept for 2 weeks for stabilization purpose before planting. *P. giganteum* plants of average height (75 cm) were then transplanted into the plastic pots with three replicates per treatment, resulting in a total of 168 pots. The three bacteria strains were cultured at 28°C for 48 h on Luria-Bertani (LB) agar medium. A single colony from a freshly streaked plate was selected, inoculated into LB broth, and incubated at 28°C for 48 h on a gyro-rotatory shaker at 200 rpm. For single strain treatment, 15 ml of each bacterium culture containing 10^6 CFU ml^{-1} was inoculated at the rhizosphere of plants in the pots while for the double strains, and triple strains treatments, 7.5 ml and 5.0 ml of each bacterium media were used respectively (Gamez et al., 2019). For bacteria treatment controls (CK), we added 15 ml of sterilized LB solution to the plant rhizosphere. The experimental design had two different controls, whereby the first group of CK consisted

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of plants with no bacterial inoculation in the soil while the second group of CK consisted of plants grown on soil with zero concentration of Cd.

Determination of chlorophyll and Cd contents

Plant heights were measured every 7 days for 60 days. After 60 days of growth, chlorophyll contents were determined by a SPAD-502 Plus chlorophyll content analyzer (Zhejiang Top Cloud-Agri Technology Co. Ltd, China) by measuring three locations along the third flag leaf of each plant. The plants were removed from the pots and the roots washed adequately with deionized water to remove any soil adhering to the root surface. The shoots were cut from the root and fresh weights of the shoot and root were recorded. The root and shoot samples were then dried at 70°C in an oven for six days in order to obtain their dry weights. The samples were then ground, sieved, and 0.5 g of each sample digested with a mixture of HCl/HNO_3 (3:1, v/v). The concentrations of Cd in the digests were then determined using atomic absorption spectroscopy (AAS). To quantify Cd, 0.5 g of each sample was placed in a 100 ml conical flask, 10 ml of a 1:2 mixture of Perchloric and Nitric acid was added to each conical flask, and then left overnight. Glass funnels were placed on each flask ensuring that funnel stem did not touch the liquid in the flask. The flasks were then placed on the digestor and temperature gradually increased until the contents of the flask fully digested. The volume of the digested material was increased to 50 ml with deionized water, then Cd content determined using AAS. Soils from pots of each treatment were thoroughly mixed and Cd content was determined in the soil samples following the digestion procedure by Sabienë et al. (2004).

Quantification of the efficiency of phytoremediation

Bioaccumulation factor (BAF) and Translocation factor (TF) were respectively determined as indicated below (González-Mendoza et al., 2007; Padmavathiamma and Li, 2007).

$$BAF = \frac{The\ metal\ concentration\ in\ plant\ tissue\ (mg/kg)}{The\ metal\ concentration\ in\ soil\ (mg/kg)} \quad \text{(Eq.1)}$$

$$TF = \frac{The\ metal\ concentration\ in\ shoots\ (mg/kg)}{The\ metal\ concentration\ in\ the\ roots\ (mg/kg)} \quad \text{(Eq.2)}$$

Statistical analyses

All the data were expressed as mean with standard errors of three replicates. Two-way ANOVA (Bacteria x Cd) was used to determine the statistical differences across and within different treatments by using SPSS Version 20.0. Least Significant Difference (LSD) and Duncan Multiple Range Test were determined at $P \leq 0.05$. Diagrams were prepared using Excel 2010.

Results

Increasing levels of Cd exposure is detrimental to plant growths

Plants grown on soils with medium to high Cd concentrations exhibited stunted growth with yellow leaves whereas the growth and height were better in the control.

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Plants inoculated with triple (A3A4A7) and double (A4A7) exhibited significantly higher plant heights (approx. 138 cm) than all other bacteria treatments. Again, the influence of the double A3A7 and A3A4 applications were significantly better than single bacteria inoculations. Heights of plants with no bacteria inoculation (CK) were lowest (102.52 cm) and differed significantly from all other treatments (*Table 1*). Furthermore, the results show a strong effect of the Cd stress on plant heights. Plant height significantly differed under the various Cd treatments with 0 mg/kg concentration the highest (194.15 cm) and 200 mg/kg the lowest at 85.54 cm.

Table 1. *Height of P. giganteum grown under different bacteria strains and their cocktails, and in soil amended with different concentration Cd (mg/kg soil)*

Treatments	**Plant height (cm)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	156.8 ± 3.4^{f}	$116.3 \pm 6.3^{j\text{-}m}$	$103.3 \pm 4.8^{o\text{-}u}$	$98.8 \pm 1.8^{s\text{-}y}$	89.0 ± 6.5^{yza}	80.0 ± 3.2^{zab}	$73.3 \pm 2.4^{\text{-}b}$	102.52^{e}
A3	179.0 ± 1.7^{e}	125.3 ± 2.6^{hi}	$109.5 \pm 1.7^{k\text{-}p}$	$103.1 \pm 2.0^{m\text{-}s}$	$96.6 \pm 1.7^{p\text{-}v}$	$88.3 \pm 1.3^{u\text{-}z}$	79.6 ± 0.8^{za}	116.10^{cd}
A4	181.0 ± 2.1^{cd}	132.0 ± 2.3^{fg}	$113.3 \pm 2.9^{j\text{-}l}$	$106.0 \pm 3.2^{n\text{-}s}$	$101.3 \pm 6.3^{q\text{-}w}$	92.6 ± 1.4^{za}	80.0 ± 1.4^{zab}	119.00^{c}
A7	192.3 ± 5.0^{e}	147.0 ± 1.4^{ij}	$118.3 \pm 9.2^{l\text{-}r}$	$106.6 \pm 3.6^{o\text{-}u}$	$102.6 \pm 6.4^{s\text{-}z}$	$93.3 \pm 1.3^{v\text{-}z}$	87.3 ± 2.0^{zab}	112.62^{d}
A3A4	200.3 ± 2.9^{ab}	180.0 ± 1.8^{e}	121.6 ± 4.3^{gh}	$111.6 \pm 2.8^{k\text{-}p}$	$104.6 \pm 2.6^{n\text{-}t}$	$93.6 \pm 2.7^{t\text{-}z}$	$89.3 \pm 5.0^{x\text{-}z}$	133.38^{b}
A3A7	210.3 ± 4.3^{bc}	181.5 ± 1.7^{e}	140.0 ± 4.0^{jk}	$111.8 \pm 3.5^{k\text{-}n}$	$109.1 \pm 2.6^{k\text{-}o}$	$94.6 \pm 2.0^{u\text{-}z}$	90.6 ± 1.2^{yza}	130.38^{b}
A4A7	215.0 ± 1.5^{a}	184.0 ± 3.5^{de}	148.6 ± 2.3^{f}	$114.5 \pm 6.5^{k\text{-}p}$	$111.3 \pm 1.7^{l\text{-}q}$	$94.0 \pm 6.3^{r\text{-}x}$	$92.0 \pm 4.7^{w\text{-}z}$	137.86^{a}
A3A4A7	218.0 ± 3.2^{a}	188.6 ± 2.1^{de}	150.3 ± 3.2^{fg}	$117.6 \pm 2.6^{j\text{-}l}$	$113.1 \pm 5.0^{l\text{-}r}$	$100.6 \pm 5.2^{t\text{-}z}$	$92.0 \pm 3.4^{w\text{-}z}$	138.45^{a}
Mean (Cd)	194.15^{a}	156.85^{b}	125.44^{c}	108.79^{d}	103.50^{e}	92.25^{f}	85.54^{g}	

Values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

The effects of the interactions of bacteria and Cd indicate that heights of plants inoculated with combinations of bacteria cultures were generally significantly higher than those under single strain inoculations. Height of plants with no bacteria inoculation (CK) always significantly differed from those under the influence of some bacteria showing the strong positive effect of PGPB on plant growth. Among all other applied combinations of bacteria and Cd with respect to plant height of Jujuncao, the highest was recorded at the influence of the triple A3A4A7 inoculation (218.0 cm) at 0 mg/kg Cd and the lowest at CK (73.3 cm) at 200 mg/kg Cd (*Table 1*).

PGPB enhance growth of P. giganteum even in the presence Cd

PGPB and their cocktails significantly improve growth (plant weight and biomass) of plants grown in soils with no Cd compared to those with Cd concentrations. The triple (A3A4A7) strain application showed a significantly higher shoot fresh weight (109.25 g) than all other treatments. The influences of the double strain inoculations were generally better and significantly different from the single strain inoculations. The CK recorded the lowest weight (48.05 g) and was significantly different from all other treatments. Each Cd treatment significantly differed from the other in terms of shoot fresh weight with 0 mg/kg Cd concentration recording the heaviest (128.01 g) and 200 mg/kg recording the least weight (60.39 g) (*Table 2*).

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Table 2. *Shoot fresh weights of P. giganteum grown on soil amended with different concentrations of Cd (mg/kg soil) under the application of different bacteria strains and their cocktails*

Treatments	**Shoot fresh weight (g)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	$91.1 \pm 2.1^{p-s}$	73.3 ± 1.7^{x}	51.6 ± 1.4^{-a}	44.5 ± 2.3^{-b}	31.9 ± 1.0^{-c}	24.6 ± 2.3^{-d}	19.1 ± 0.7^{-e}	48.05^{f}
A3	115.3 ± 4.0^{3f}	$96.9 \pm 2.3^{l-n}$	82.6 ± 2.9^{vw}	69.3 ± 0.6^{xy}	62.2 ± 1.2^{z}	50.3 ± 1.2^{-a}	40.1 ± 1.5^{-b}	73.98^{e}
A4	123.5 ± 2.1^{de}	$103.1 \pm 2.2^{h-j}$	$84.0 \pm 1.2^{u-w}$	71.1 ± 1.2^{xy}	63.3 ± 1.3^{yz}	50.5 ± 2.1^{-a}	43.3 ± 1.2^{-b}	77.67^{d}
A7	121.0 ± 1.7^{e}	$99.9 \pm 1.1^{i-l}$	$86.6 \pm 1.4^{r-v}$	68.4 ± 1.6^{xy}	66.3 ± 1.8^{z}	54.4 ± 2.0^{-a}	42.8 ± 1.2^{-b}	75.92^{d}
A3A4	140.1 ± 0.8^{b}	180.0 ± 1.8^{cd}	104.3 ± 1.0^{hi}	$98.5 \pm 1.4^{j-n}$	$94.6 \pm 3.4^{m-p}$	$87.9 \pm 1.5^{r-u}$	$85.1 \pm 3.6^{t-w}$	105.57^{bc}
A3A7	142.3 ± 0.8^{ab}	128.3 ± 1.3^{c}	$102.4 \pm 2.0^{h-k}$	$95.5 \pm 0.4^{l-p}$	$93.7 \pm 0.9^{n-q}$	$88.5 \pm 1.1^{r-u}$	81.5 ± 1.1^{w}	104.76^{c}
A4A7	144.0 ± 0.6^{ab}	129.8 ± 1.1^{c}	106.9 ± 1.3^{gh}	$99.5 \pm 0.9^{i-m}$	$96.4 \pm 1.4^{l-o}$	$89.2 \pm 0.6^{q-t}$	$84.8 \pm 2.3^{t-w}$	107.25^{b}
A3A4A7	146.7 ± 1.2^{a}	127.7 ± 1.6^{cd}	111.8 ± 2.1^{fg}	$103.2 \pm 0.8^{h-j}$	$97.5 \pm 1.2^{k-n}$	$91.4 \pm 1.2^{o-r}$	$86.4 \pm 1.3^{s-w}$	109.25^{a}
Mean (Cd)	128.01^{a}	111.08^{b}	91.30^{c}	81.25^{d}	75.74^{e}	66.87^{f}	60.39^{g}	

Values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

The best influence of the strains on root fresh weights were recorded at the double inoculations A4A7 (12.73 g), A3A4 (12.46 g) and A3A7 (12.44 g) which were significantly better than all other treatments. Generally, the single strain inoculations significantly differed from the cocktail inoculations. The CK recorded the lowest weight and differed significantly from all others. The effects of the Cd treatments on root fresh weights were significantly different from each other (*Table 3*).

Table 3. *Root fresh weights of P. giganteum grown on soil amended with different concentrations of Cd (mg/kg soil) under the application of different bacteria strains and their cocktails*

Treatments	**Root fresh weight (g)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	$13.5 \pm 1.0^{f-i}$	$9.4 \pm 0.5^{q-t}$	$8.4 \pm 0.3^{u-z}$	$7.0 \pm 0.1^{-b-f}$	$6.8 \pm 0.4^{-b-f}$	6.1 ± 0.2^{-fg}	5.6 ± 0.5^{-g}	8.13^{e}
A3	14.4 ± 0.5^{ef}	$13.0 \pm 0.1^{h-j}$	$12.0 \pm 0.3^{k-m}$	$10.0 \pm 0.5^{p-s}$	$8.7 \pm 0.2^{t-y}$	$7.3 \pm 0.4^{-a-d}$	$6.4 \pm 0.1^{-d-g}$	10.26^{d}
A4	14.5 ± 0.4^{e}	$13.8 \pm 0.2^{e-h}$	$12.5 \pm 0.3^{i-k}$	$10.4 \pm 0.3^{o-q}$	$9.6 \pm 0.3^{q-t}$	$7.5 \pm 0.3^{z-c}$	$6.6 \pm 0.2^{-c-f}$	10.71^{c}
A7	$14.2 \pm 0.6^{e-g}$	$13.3 \pm 0.3^{g-i}$	$11.9 \pm 0.1^{k-n}$	$10.1 \pm 0.2^{p-r}$	$8.7 \pm 0.1^{t-x}$	$7.1 \pm 0.1^{-a-e}$	$6.3 \pm 0.1^{-e-g}$	10.22^{d}
A3A4	$17.3 \pm 0.6^{a-c}$	$17.2 \pm 0.4^{a-c}$	$13.1 \pm 0.5^{h-j}$	$11.8 \pm 0.2^{k-m}$	$10.9 \pm 0.4^{n-p}$	$9.1 \pm 0.3^{r-v}$	$7.7 \pm 0.5^{y-b}$	12.46^{ab}
A3A7	$17.0 \pm 0.3^{a-c}$	16.5 ± 0.5^{cd}	$13.1 \pm 0.4^{h-j}$	$12.2 \pm 0.3^{j-l}$	$11.2 \pm 0.3^{m-o}$	$9.0 \pm 0.1^{s-w}$	$8.0 \pm 0.3^{x-a}$	12.44^{ab}
A4A7	17.8 ± 1.1^{a}	$16.6 \pm 0.4^{b-d}$	$13.3 \pm 0.4^{g-i}$	$12.2 \pm 0.2^{j-m}$	$11.5 \pm 0.2^{l-n}$	$9.4 \pm 0.2^{q-u}$	$8.4 \pm 0.3^{v-z}$	12.73^{a}
A3A4A7	17.5 ± 0.7^{ab}	15.9 ± 0.4^{d}	$13.7 \pm 0.3^{e-h}$	$11.6 \pm 0.2^{k-m}$	$10.4 \pm 0.1^{o-q}$	$9.2 \pm 0.3^{r-v}$	$8.0 \pm 0.2^{w-a}$	12.31^{b}
Mean (Cd)	15.74^{a}	14.46^{b}	12.25^{c}	10.67^{d}	9.74^{e}	8.10^{f}	7.16^{g}	

The values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

The effects of the bacteria on shoot dry weights indicated that combinations A3A4A7 and A4A7 showed significantly higher weights (approx. 36 g) than all other bacterial treatments. Here too, the single strain inoculations significantly differed from

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the cocktail inoculations. The CK recorded the least weight (18.70 g) and significantly differed from all other recordings. The effects of the Cd stress were significantly different from each other (*Table 4*). These observations were generally similar for the root dry weights as presented in *Table 5*.

Table 4. *Shoot dry weights of P. giganteum grown on soil amended with different concentrations of Cd (mg/kg soil) under the application of different bacteria strains and their cocktails*

Treatments	**Shoot dry weight (g)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	32.3 ± 0.8^{mn}	21.5 ± 0.4^{-b}	19.0 ± 0.2^{-c}	16.6 ± 0.4^{-d}	6.8 ± 0.4^{-d}	13.5 ± 0.2^{-e}	12.3 ± 0.3^{-e}	18.70^{f}
A3	40.1 ± 1.3^{g}	34.2 ± 0.8^{l}	$30.1 \pm 0.9^{o-q}$	$27.4 \pm 0.7^{s-u}$	$8.7 \pm 0.2^{u-w}$	$24.6 \pm 0.2^{x-z}$	23.7 ± 0.3^{ya}	29.48^{e}
A4	43.8 ± 0.7^{cd}	39.3 ± 0.4^{gh}	34.0 ± 0.6^{l}	$28.8 \pm 0.2^{q-s}$	$9.6 \pm 0.3^{v-x}$	23.2 ± 0.2^{za}	$24.7 \pm 0.3^{w-z}$	31.33^{c}
A7	40.6 ± 0.6^{fg}	39.5 ± 1.2^{gh}	$33.7 \pm 0.6l^{m}$	$27.4 \pm 0.6^{s-u}$	$8.7 \pm 0.3^{v-x}$	$24.9 \pm 0.2^{w-y}$	23.0 ± 0.5^{-ab}	30.69^{d}
A3A4	46.6 ± 0.7^{b}	42.5 ± 0.8^{de}	37.1 ± 0.6^{ij}	33.6 ± 0.3^{lm}	10.9 ± 0.4^{no}	$28.7 \pm 0.4^{q-s}$	$27.8 \pm 0.3^{r-u}$	35.41^{b}
A3A7	46.9 ± 0.7^{ab}	42.2 ± 0.9^{ef}	36.5 ± 0.3^{jk}	33.8 ± 0.9^{lm}	$11.2 \pm 0.3^{n-p}$	$27.9 \pm 0.3^{r-t}$	$26.6 \pm 0.3^{t-v}$	35.00^{b}
A4A7	48.0 ± 0.3^{ab}	44.4 ± 0.5^{c}	37.3 ± 0.6^{ij}	34.5 ± 0.5^{l}	$11.5 \pm 0.2^{n-p}$	29.0 ± 0.2^{qr}	$27.8 \pm 0.3^{r-u}$	36.02^{a}
A3A4A7	48.3 ± 0.6^{a}	44.4 ± 0.6^{c}	38.5 ± 0.5^{hi}	35.1 ± 0.3^{kl}	10.4 ± 0.1^{no}	29.7 ± 0.3^{pq}	$27.9 \pm 0.5^{r-t}$	36.46^{a}
Mean (Cd)	43.34^{a}	38.49^{b}	33.29^{c}	29.66^{d}	27.23^{e}	25.21^{f}	24.23^{g}	

The values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

Table 5. *Root dry weights of P. giganteum grown on soil amended with different concentrations of Cd (mg/kg soil) under the application of different bacteria strains and their cocktails*

Treatments	**Root dry weight (g)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	$6.8 \pm 0.2^{j-n}$	$6.2 \pm 0.1^{q-t}$	5.8 ± 0.1^{tu}	5.7 ± 0.1^{uv}	$5.4 \pm 0.1^{w-z}$	4.8 ± 0.2^{-ab}	4.5 ± 0.2^{-b}	5.63^{e}
A3	$7.3 \pm 0.1^{f-h}$	$7.0 \pm 0.1^{i-m}$	$6.4 \pm 0.1^{p-s}$	5.9 ± 0.1^{tu}	$5.6 \pm 0.1^{u-w}$	$5.1 \pm 0.1^{y-a}$	5.1 ± 0.2^{za}	6.05^{d}
A4	$7.5 \pm 0.1^{d-f}$	$7.0 \pm 0.1^{i-l}$	$6.6 \pm 0.1^{n-p}$	6.1 ± 0.1^{st}	$5.7 \pm 0.1^{u-w}$	$5.5 \pm 0.1^{v-y}$	$5.2 \pm 0.1^{x-a}$	6.22^{c}
A7	$7.3 \pm 0.1^{f-i}$	$6.9 \pm 0.1^{j-n}$	$6.6 \pm 0.1^{n-p}$	$6.2 \pm 0.1^{q-t}$	$5.5 \pm 0.1^{v-x}$	5.1 ± 0.1^{za}	5.0 ± 0.1^{-a}	6.07^{d}
A3A4	8.1 ± 0.1^{bc}	$7.5 \pm 0.1^{d-f}$	$7.1 \pm 0.1^{h-k}$	$6.8 \pm 0.1^{k-o}$	$6.6 \pm 0.1^{m-p}$	$6.5 \pm 0.1^{o-r}$	6.1 ± 0.2^{st}	6.95^{b}
A3A7	8.2 ± 0.3^{ab}	$7.4 \pm 0.1^{e-h}$	$7.0 \pm 0.1^{h-k}$	$6.9 \pm 0.1^{i-l}$	$6.5 \pm 0.1^{n-q}$	$6.1 \pm 0.1^{r-t}$	5.9 ± 0.1^{tu}	6.89^{b}
A4A7	8.5 ± 0.2^{a}	7.7 ± 0.1^{de}	$7.3 \pm 0.1^{f-i}$	$7.0 \pm 0.1^{h-k}$	$6.6 \pm 0.1^{l-p}$	$6.4 \pm 0.1^{p-s}$	$6.2 \pm 0.1^{q-t}$	7.10^{a}
A3A4A7	8.5 ± 0.1^{a}	7.8 ± 0.2^{cd}	$7.4 \pm 0.1^{d-g}$	$7.1 \pm 0.1^{g-j}$	$6.9 \pm 0.1^{i-m}$	$6.5 \pm 0.1^{o-r}$	$6.2 \pm 0.2^{q-t}$	7.21^{a}
Mean (Cd)	7.80^{a}	7.17^{b}	6.78^{c}	6.48^{d}	6.10^{e}	5.75^{f}	5.52^{g}	

Values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

The interaction effects of bacteria and Cd on weights of shoots and roots of Jujuncao generally indicate that combinations of bacteria culture recorded significantly higher values than single strains inoculations which were also always significantly higher than the CK at the various Cd stress levels. As concentrations of metals increased from 25 mg/kg onwards to 200 mg/kg, the influence of the bacteria on growths reduced

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progressively suggesting that a threshold exists beyond which the bacteria may not alleviate toxicity of the metals in plants. Similarly, as Cd concentration increased towards 200 mg/kg, the effect of the various bacteria treatments on plant growth dramatically decreased. The negative effect of the metal on plants grown in pots without PGPB was so severe that the yellowing of leaves and drying of leaf tips were profound at 200 mg/kg Cd concentration.

Plants inoculated with PGPB accumulated more Cd in their tissues

Cadmium concentrations in the different plant parts grown in the contaminated soil are presented in *Tables 6 and 7*. The effects of the PGPB on shoot Cd indicate that there was no significant difference between either the triple or double consortia applications. However, significant difference exists between cocktail of strains inoculation and single inoculation. The CK recorded the lowest shoot Cd of 28.18 mg/kg which was significantly different from all the bacteria treatments. Cd accumulation in shoots had an interesting pattern; for all Cd treatments, the Cd contents progressively increased up to 50 mg/kg Cd, after which it had an inverse relation with the Cd concentrations in the soil. The maximum cadmium uptake in shoot, 55.1 mg/kg, was recorded in plants inoculated with A3A4A7 at 50 mg/kg Cd concentration. The quantities of Cd in the shoot of plants treated with 0 to 50 mg/kg Cd was in the range of 0.01 to 55.1 mg/kg whereas treatments of 75 to 200 mg/kg Cd were in the range of 25.3 mg/kg (at 200 mg/kg Cd with no bacteria) to 46.0 mg/kg (at 75 mg/kg Cd with A3A4A7 bacteria inoculation). The pattern for shoot Cd content in terms of the PGPB influence was: cocktails > single > no bacteria (*Table 6*).

Table 6. *Concentrations of Cd in shoots of P. giganteum grown in different concentrations of Cd contaminated soil, under the application of different bacteria strains and their cocktails*

Treatments	**Shoots Cd (mg/kg)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	0.01 ± 0.0^{x}	26.1 ± 1.6^{vw}	$43.1 \pm 1.2^{f\text{-}k}$	$36.7 \pm 1.8^{o\text{-}r}$	$36.7 \pm 1.3^{o\text{-}r}$	29.1 ± 1.8^{uv}	25.3 ± 1.0^{w}	28.18^{c}
A3	0.01 ± 0.0^{x}	$39.2 \pm 1.4^{m\text{-}p}$	48.7 ± 1.4^{cd}	$40.3 \pm 1.0^{k\text{-}n}$	$40.5 \pm 1.0^{k\text{-}n}$	$39.7 \pm 0.9^{l\text{-}o}$	31.1 ± 0.8^{tu}	34.26^{b}
A4	0.02 ± 0.0^{x}	$39.1 \pm 1.6^{m\text{-}q}$	52.3 ± 1.0^{ab}	$40.6 \pm 0.6^{k\text{-}n}$	$41.7 \pm 1.0^{h\text{-}m}$	$40.7 \pm 1.3^{j\text{-}n}$	32.0 ± 0.8^{tu}	35.22^{b}
A7	0.01 ± 0.0^{x}	$37.6 \pm 1.9^{n\text{-}r}$	49.8 ± 0.9^{bc}	$42.6 \pm 0.8^{g\text{-}l}$	$40.9 \pm 1.3^{j\text{-}m}$	$39.2 \pm 1.7^{m\text{-}p}$	32.5 ± 0.5^{st}	34.70^{b}
A3A4	0.03 ± 0.0^{x}	$44.6 \pm 2.1^{e\text{-}h}$	53.7 ± 0.8^{a}	$44.3 \pm 1.5^{e\text{-}i}$	$44.6 \pm 0.8^{e\text{-}h}$	$43.3 \pm 1.3^{f\text{-}k}$	35.1 ± 1.0^{rs}	37.97^{a}
A3A7	0.02 ± 0.0^{x}	$41.4 \pm 1.1^{i\text{-}m}$	52.4 ± 0.6^{ab}	$45.3 \pm 0.5^{e\text{-}g}$	$45.3 \pm 1.1^{e\text{-}g}$	$44.5 \pm 1.1^{e\text{-}i}$	$36.5 \pm 0.8^{p\text{-}r}$	37.96^{a}
A4A7	0.03 ± 0.0^{x}	46.8 ± 1.6^{cd}	54.5 ± 0.6^{a}	$45.1 \pm 0.4^{e\text{-}g}$	$43.8 \pm 0.7^{e\text{-}j}$	$45.5 \pm 1.0^{e\text{-}g}$	36.0 ± 0.9^{qr}	38.83^{a}
A3A4A7	0.04 ± 0.0^{x}	46.4 ± 0.8^{de}	55.1 ± 1.4^{a}	$46.0 \pm 0.6^{d\text{-}f}$	$45.4 \pm 1.0^{e\text{-}g}$	$44.8 \pm 0.4^{e\text{-}g}$	35.8 ± 0.5^{r}	39.10^{a}
Mean (Cd)	0.02^{e}	40.20^{c}	51.23^{a}	42.64^{b}	42.39^{b}	40.88^{c}	33.076^{d}	

Values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

The effects of the bacteria strains on root Cd uptake showed that the inoculation of triple A3A4A7 and double A4A7 were significantly higher than all other bacterial treatments. The effects of the single strain inoculations were lower and significantly different from the combination treatments. A CK value of 42.07 was significantly lower than all other bacterial treatments. Generally, the pattern of the root Cd content in terms of the bacterial application was as follows: cocktails > single > no bacteria. On the other

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hand, the highest mean root Cd value of 78.83 mg/kg was obtained at 200 mg/kg whereas the lowest mean root Cd value of 0.045 mg/kg was recorded at 0 mg/kg Cd concentration. Maximum cadmium uptake in root of 83.5 mg/kg was recorded at the interaction of 200 mg/kg Cd and the consortium of the triple bacteria inoculation (*Table 7*).

Table 7. *Concentrations of Cd in roots of P. giganteum grown in different concentrations of Cd contaminated soil, under the application of different bacteria strains and their cocktails*

Treatments	**Root Cd (mg/kg)**							
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	0.02 ± 0.0^{a}	30.0 ± 1.1^{z}	48.5 ± 0.8^{u}	48.6 ± 1.3^{u}	50.1 ± 1.4^{st}	55.3 ± 0.8^{u}	$61.8 \pm 0.7^{k-n}$	42.07^{e}
A3	0.02 ± 0.0^{a}	37.6 ± 0.9^{y}	49.7 ± 3.4^{u}	$56.1 \pm 1.4^{q-t}$	69.9 ± 1.3^{g}	69.7 ± 2.6^{h}	$78.8 \pm 0.7^{c-e}$	51.16^{d}
A4	0.04 ± 0.0^{a}	38.9 ± 0.4^{xy}	$55.4 \pm 0.9^{r-t}$	$58.4 \pm 0.9^{o-q}$	62.5 ± 1.1^{fg}	$72.2 \pm 0.9^{j-m}$	$81.0 \pm 1.0^{a-c}$	52.64^{c}
A7	0.03 ± 0.0^{a}	36.5 ± 0.7^{y}	54.2 ± 0.5^{t}	$57.2 \pm 1.2^{p-s}$	65.1 ± 0.4^{hi}	$65.2 \pm 0.5^{h-j}$	$80.6 \pm 1.6^{b-d}$	51.28^{d}
A3A4	0.06 ± 0.0^{a}	41.7 ± 0.9^{w}	$59.4 \pm 0.6^{n-p}$	$63.0 \pm 0.2^{i-l}$	71.4 ± 0.2^{f}	73.8 ± 0.6^{fg}	$80.6 \pm 0.6^{b-d}$	55.72^{b}
A3A7	0.05 ± 0.0^{a}	41.2 ± 1.2^{wx}	$58.1 \pm 1.1^{o-r}$	$61.9 \pm 0.2^{k-n}$	73.0 ± 0.1^{e}	77.1 ± 0.1^{f}	$81.0 \pm 0.2^{a-c}$	56.07^{b}
A4A7	0.06 ± 0.0^{a}	44.4 ± 1.1^{v}	$60.4 \pm 0.6^{l-o}$	$64.2 \pm 0.3^{h-k}$	70.1 ± 0.3^{de}	77.9 ± 0.4^{g}	83.1 ± 0.5^{ab}	57.21^{a}
A3A4A7	0.07 ± 0.0^{a}	45.5 ± 0.1^{v}	$60.0 \pm 0.3^{m-o}$	$64.8 \pm 0.1^{h-j}$	73.1 ± 0.3^{de}	78.1 ± 0.1^{f}	83.5 ± 0.2^{a}	57.89^{a}
Mean (Cd)	0.045^{g}	39.504^{f}	55.746^{e}	59.313^{d}	71.196^{b}	66.412^{c}	78.83^{a}	

Values in each column represent the mean ± standard error of three replicates. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

P. giganteum chlorophyll content responses against Cd-induced oxidative stress in different bacteria inoculation

The chlorophyll content of plants under triple strain inoculation was significantly higher (32.52) than all other bacterial treatments. There was no significant difference in the chlorophyll contents of the double strain inoculated plants which were however significantly higher than the single strain inoculated plants. The CK showed a significantly least value of 23.21. On the other hand, the chlorophyll contents of the plants had an inverse relationship with the concentration of Cd with the highest chlorophyll content of 46.76 being recorded at 0 mg/kg Cd concentration and the lowest value of 16.63 recorded at 200 mg/kg Cd concentration. The chlorophyll content significantly differed from each other at the various Cd concentrations. The interactions of the bacteria strains and the Cd revealed the maximum mean chlorophyll content was 55.1 recorded in plants inoculated with A3A4A7 strains at 0 Cd concentration, whereas the minimum chlorophyll content of 15.2 was recorded from plants at 200 mg/kg Cd supplied with no bacteria application (*Table 8*).

Effect of cadmium on bioaccumulation and translocation factors

Bioaccumulation factor (BAF) and Translocation factor (TF) are widely used to determine the uptake and translocations of heavy metals into growing plant tissues. BAF is used to measure the efficiency of plant species in accumulating heavy metals from soil environment into its tissues. On the other hand, TF is a measurement of the efficiency of a plant's ability to translocate metals accumulated from its roots to its shoots (Ladislas et al., 2012).

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The BAF for shoot at 25 mg/kg Cd were all above 1.0, whereas the BAF values for shoot at 50 mg/kg were all above 1.0 with the exceptions of the CK and A3 inoculations. Above 50 mg/kg Cd treatments, the BAF values progressively decreased below 1.0 as Cd concentration increased. Overall, shoot BAF values were in the range of 0.13 (CK, 200 mg/kg) to 1.87 (A4A7, 25 mg/kg) (*Figure 1a*).

Table 8. *Chlorophyll content response of P. giganteum plants under different concentrations of Cd (mg/kg soil) under the application of different bacteria strains and their cocktails*

Treatments								
Conc. (mg/kg)	**0**	**25**	**50**	**75**	**100**	**150**	**200**	**Mean (Bacteria)**
CK	35.6 ± 0.99^{g}	30.1 ± 1.71^{hi}	$23.3 \pm 0.69^{n-q}$	$21.7 \pm 0.47^{p-s}$	$19.8 \pm 0.78^{r-u}$	16.4 ± 0.84^{wx}	15.2 ± 0.58^{x}	23.21^{e}
A3	41.8 ± 1.48^{f}	36.3 ± 1.14^{g}	26.7 ± 0.91^{kl}	$23.6 \pm 1.05^{m-p}$	$22.3 \pm 0.66^{o-r}$	$19.3 \pm 0.81^{s-v}$	16.1 ± 0.40^{wx}	26.60^{d}
A4	42.4 ± 1.24^{f}	40.0 ± 1.79^{f}	26.8 ± 0.52^{kl}	$24.4 \pm 0.73^{l-o}$	$23.1 \pm 0.35^{o-q}$	$20.3 \pm 0.47^{r-t}$	16.5 ± 0.44^{wx}	27.69^{c}
A7	41.2 ± 1.53^{f}	35.4 ± 1.10^{g}	$25.9 \pm 0.41^{k-m}$	$23.2 \pm 0.49^{n-q}$	$22.0 \pm 0.29^{o-r}$	$18.4 \pm 0.21^{t-w}$	16.5 ± 0.69^{wx}	26.12^{d}
A3A4	52.5 ± 1.24^{bc}	47.4 ± 1.04^{e}	30.2 ± 0.79^{hi}	$25.9 \pm 0.44^{k-m}$	$24.4 \pm 0.23^{l-o}$	$22.0 \pm 0.95^{o-r}$	$17.0 \pm 0.27^{v-x}$	30.99^{b}
A3A7	51.3 ± 1.18^{c}	48.4 ± 1.19^{de}	$29.3 \pm 0.64^{h-j}$	$25.7 \pm 0.27^{k-n}$	$23.5 \pm 0.55^{m-p}$	$21.3 \pm 0.67^{p-s}$	$17.1 \pm 0.19^{v-x}$	31.40^{b}
A4A7	54.0 ± 0.78^{ab}	47.5 ± 1.15^{e}	$29.8 \pm 1.32^{h-j}$	27.4 ± 1.19^{jk}	$23.0 \pm 0.67^{o-q}$	$21.0 \pm 0.58^{q-s}$	$17.0 \pm 0.58^{v-x}$	31.43^{b}
A3A4A7	55.1 ± 1.15^{a}	50.6 ± 0.99^{cd}	30.8 ± 1.45^{h}	$28.1 \pm 0.82^{i-k}$	$23.7 \pm 0.59^{m-p}$	$21.9 \pm 0.74^{p-r}$	$17.4 \pm 0.35^{u-x}$	32.52^{a}
Mean (Cd)	46.76^{a}	41.98^{b}	27.90^{c}	25.04^{d}	22.77^{e}	20.13^{f}	16.63^{g}	

Values in each column represent the mean ± standard error of three readings. Those marked with different superscripts in each of the column show significant difference at $P \leq 0.05$ as analyzed by LSD. The mean Cd analysis is within the row

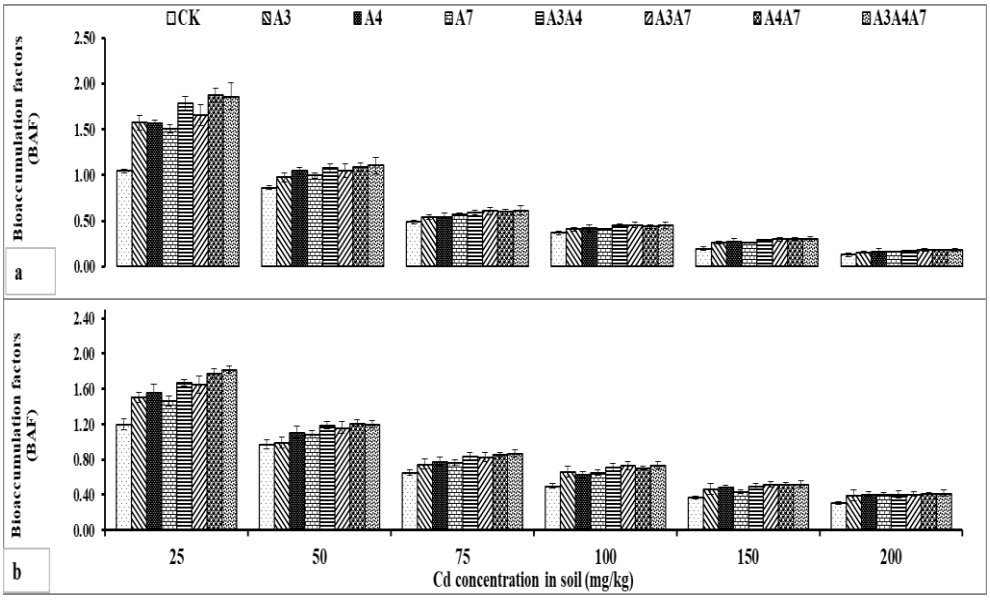

Figure 1. *Bioaccumulation factors (BAF) of Cd for (a) shoot and (b) root of P. giganteum plants grown on metal contaminated soils and under different bacteria strains and their cocktails with standard error shown as error bars*

The root BAF presented in *Fig. 1b* followed a similar pattern as the shoot BAF, decreasing with increasing Cd concentration. However, the root BAF values at the corresponding Cd treatments were slightly higher than the shoot. All treatments up to

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50 mg/kg had values 1.0 and above except the CK (no bacteria application) at 50 mg/kg. Overall, root BAF values were in the range of 0.31 (CK, 200 mg/kg) to 1.82 (A4A7, 25 mg/kg). BAF values for the treatments with no bacteria inoculation were lowest for all Cd treatments for both shoot and root.

The highest TF values for each of the bacteria treatment was recorded at 25 mg/kg Cd, however, above 25 mg/kg Cd, it then decreased progressively with increasing Cd content in the soil. Overall, the values of TF were in the range of 0.30 (CK, 0 Cd supplied) to 1.07 (A3A4, 25 mg/kg Cd supplied) (*Figure 2*).

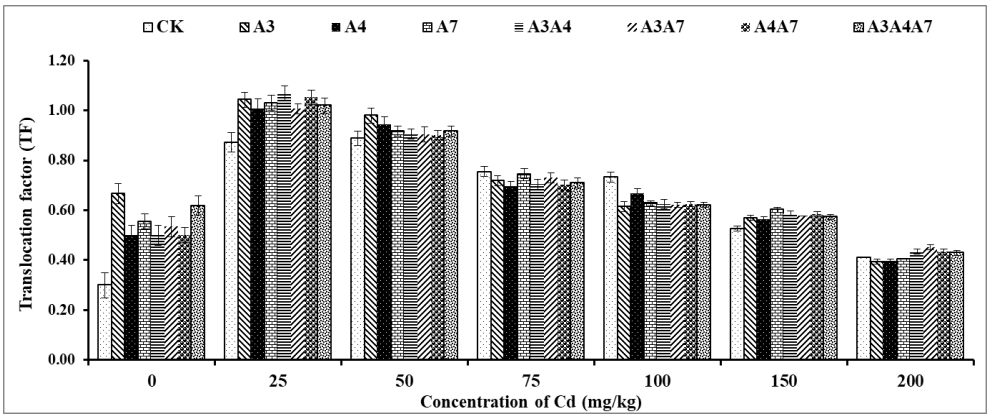

Figure 2. *Translocation factor (TF) of Cd for P. giganteum plants grown on metal contaminated soils and under different bacteria strains and their cocktails with standard error shown as error bars*

Discussion

Several studies have enumerated the beneficial effect of PGP rhizobacteria in promoting plant growths under various abiotic stresses whereby the bacteria act as biosorption for soil toxins and assist in the removal of pollutants from the soil (Glick et al., 2007; Glick, 2012; Saxena et al., 2019).

Although single strain inoculations promoted biomass production, the levels were not as high as in double or triple inoculations. As a result, double and triple could be the preferred combinations in *P. giganteum* growth promotion whereby A4A7 and A3A4A7 had the highest values. It could therefore be inferred that the combined traits from each of the bacterium played a considerable role in growth promotion as explained by the additive hypothesis (Bashan and Holguin, 1997). The additive hypothesis explains the synergistic mechanism of PGPB in achieving higher growth and phytoremediation abilities of consortium bacteria application over single strain application. Several studies have shown that *Klebsiella pneumonia* and *Klebsiella sp* produce high quantities of plant growth-promoting phytohormones such as IAA and ACC deaminase which may have been responsible for the observed high biomass (Qin et al., 2014).

The exposure of plants to excess levels of Cd could have inhibited physiologically active enzymes (Gadd, 2007), inactivated photosystems (Ghori et al., 2019; Zhu et al., 2019), affected mineral absorption and metabolism (Janas et al., 2010). Cd toxicity could have severely damaged various metabolic activities in plants leading to reduced height and weight, and leaf chlorosis (Chowdhury et al., 2018). Leaves of plants exposed to moderate to high Cd concentration without PGPB assistance turned yellow

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whereas plants under the 0 mg/kg Cd (no Cd added) and soil supplied with low metal concentrations were healthy. This is because, plant Cd uptake is largely influenced by the availability of metals, explaining why higher quantities of metals are absorbed into the root tissues of the plants at higher Cd supplied when the plants were growing well under the assistance of PGPB. However, the content of metals in shoots initially increased then starts decreasing as the concentration of Cd supplied continued to increase. This is partly because the excess Cd supplied affected the photosynthetic ability of the plant which in turn reduced the quantity of metals translocated to the shoot of the plant. Plant heights and weights significantly increased at low concentrations of Cd, and then progressively reduced as the quantity of Cd increased. This observation is in line with the findings of Jadia and Fulekar (2008) and Yang et al. (2018), who showed that low quantities of applied Cd elongated the root and shoot of sunflower but at higher concentrations, significantly reduced germination percentage and plant growth especially root and shoot elongation. Many authors, (Tewari et al., 2002; Zhou and Qiu, 2005; Gajewska and Skłodowska, 2007; Alaboudi et al., 2018) observed that different plant species grown in high Cd contaminated soils showed a visible effect on growth and metabolism and described symptoms including stunted growth, reduced biomass, and yellowing of leaves. Such characteristic effects were observed in the *P. giganteum* plants grown under the high Cd concentrations without PGPB.

The extraction of heavy metals from soil by plants is usually a slow process. In order to speed up the process, studies have suggested environmentally friendly approaches such as the use of PGPB and their synergistic effects. From our results, the average concentrations of Cd in shoot of plants inoculated with double strain at 0, 25, and 200 mg/kg Cd were significantly higher than those inoculated with single strain by 51.9%, 12.9%, and 11%, respectively. Similarly, in roots, the average concentrations of Cd in the double strain inoculated plants over the single strain inoculated plants were 53.3%, 11.3%, and 1.7% higher, respectively for 0, 25, and 200 mg/kg Cd. This indicates that a cocktail of the bacterial mixture is better than using single strain culture and their synergistic effect is key in Cd remediation whereby they boost the growth and Cd absorption by host plants. This is because each strain possesses unique characteristics such as Cd resistance, the synthesis of ACC deaminase enzymes, IAA, and siderophore productions and it could be inferred that the combined effects of these traits produced the observed positive results. For instance, *Klebsiella pneumonia* produce antioxidants and exhibit strong Cd tolerance abilities which enable high survival in Cd stressed environment and promote plant growth through the secretion of IAA, ACC deaminase, and other PGP physiological traits (Pramanik et al., 2017). Results from our study correlate the findings of Kang et al. (2016), who reported that the synergistic effect of *Viridibacillusarenosi* B-21, *Sporosarcina soli* B-22, *Enterobacter cloacae* KJ-46, and *E. cloacae* KJ-47 had better resistance and efficiency in the remediation of Pb, Cd, and Cu compared with using single strain culture after 48 h.

Heavy metals such as Cd are known to inhibit the transport of electron in photosynthetic pathway (Monni et al., 2001). The synthesis of chlorophyll in the plant is also directly inhibited by Cd through a misstepping of an enzymatic process or by reducing the efficiency of an essential nutrient (Duan et al., 2018). There was significant reduction in chlorophyll content in the leaves of plants in our study as Cd concentrations increased above 25 mg/kg. At all Cd concentrations, chlorophyll contents were high in treatments with cocktail bacteria inoculations. Numerous studies have demonstrated that the decrease in photosynthetic rate with an increasing amount of

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Cd may be a result of inhibition of the chlorophyll biosynthesis and the photochemical reactions (Song et al., 2019) as well as the disturbance in the activity of enzymes involved in CO_2 fixation (Krantev et al., 2008). However, as evidenced by this study and by other numerous studies (Tak, 2015; Pan et al., 2016; Chiboub et al., 2018), PGPB can improve oxidative stress thereby improving the synthesis of chlorophyll, improve other physiological and biochemical stresses imposed by heavy metals, and improve the adaptability of remediation plants to heavy metal pollution.

According to McGrath and Zhao (2003), the efficiency at which a plant can extract pollutants from the soil is determined by two key factors: biomass production and metal hyperaccumulating capacity. The high biomass production of the *P. giganteum* plant is noted by Zhanxi and Zhanhua (2001) as high as 300 tons (green) material annual yield per hectare is achieved. In terms of metal hyperaccumulating capacity, our study characterized the plant based on the understanding that firstly, for a plant to qualify as a hyperaccumulator of a particular metal, its BAF and TF values should be greater than 1 (Mirza et al., 2010). BAF for both shoot and roots were all > 1 at 25 mg/kg Cd supplied even without the contribution of bacteria, indicating that *P. giganteum* could be a suitable hyperaccumulator of Cd at levels 25 mg/kg. However, to sustain this hyperaccumulative ability up to 50 mg/kg, the plants required the concerted effort of PGPB.

According to our study, TF values at 25 mg/kg were > 1 in plants assisted with PGPB. At same Cd concentration of 25 mg/kg, the TF values in plants without PGPB were less than 1, showing the role the bacteria are playing in the plant's hyperaccumulative characteristics. At same Cd concentrations, root BAF were generally higher than shoot BAF indicating that the mobilization and storage of the metal in aerial plant parts is reduced. This observation was also noted by Xue et al. (2013) who also found that Cd accumulated more in the roots of soybean than in the shoots. Xu et al. (2018) observed similar findings and asserted that in higher plants, roots are the first organs with contact to Cd, and hence, the roots strongly retain more of the Cd with just about 2 % of the accumulated Cd translocated to leaves. It is however necessary to note that, the uptake and translocation of metal from roots to shoots is strongly linked to the speciation of the metal in question, the soil pH, and other factors. Since the goal of the phytoremediation process is to reduce heavy metal concentrations in contaminated soil to acceptable levels within a reasonable time frame, high root BAF values are appreciated. Our results agree with the view that plants possessing greater shoot biomass compensate for lower ability to concentrate metals in shoots in phytoextraction techniques (Ebbs et al., 1997).

Overall, even without PGPB assistance, *P. giganteum* is a hyperaccumulator at lower concentrations of Cd. At moderate concentrations, BAF and TF values were greater than 1 only with the assistance of PGPB, and at high concentrations, the toxicity effects of the metals on both the plants and the bacteria reduced its metal accumulative capacity. Thus, BAF and TF increase as a function of Cd supplied up to 25 mg/kg, after which the factors start decreasing reaching a low of 0.31 (root) and 0.13 (shoot) for BAF and 0.39 for TF, all at 200 mg/kg. These relationships, as seen in *Figures 1 and 2*, were also observed by Sabeen et al. (2013) who found the translocation and bioaccumulation factors of *Arundo donax* L. increased with increasing concentration of Cd up to 500 μg/g, after which it decreased at higher concentration of Cd in soil.

Biological approaches at remediating polluted lands are catching up with a green-conscious society that is trying hard to reduce the excessive use of chemicals in its

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agricultural and environmental clean up activities. This study is a contribution to knowledge in this direction that seeks to reduce pollution and could be further utilized in the production of biofertilizer for the enhancement of plant growth and remediation of Cd polluted lands.

Conclusion

The study employed the use of *P. giganteum* in Cd remediation at different concentrations with the help of three bacteria strains and their combinations. As Cd concentrations progressively increased, plant growth parameters, including plant height, chlorophyll contents, and fresh and dry weights of shoots and roots significantly reduced. However, when the plants were inoculated with PGPB combinations, the measured growth parameters were enhanced. Although significant differences were observed between single and consortium bacteria application, there was no significant difference in growth parameters when either a double or triple consortium was applied. The study also revealed that, as the concentration of the metal increased, the effect differences of either a single, double, or triple strain application became similar. Overall, it has been asserted that high plant biomass can substitute for a relatively low metal accumulation capacity, resulting in the eventual accumulation of a large amount of heavy metal. Therefore, it is recommended that the synergistic abilities of these strains could be utilized in association with *P. giganteum* for Cd remediation. Many different heavy metals pollute our land and multiple contaminations are often common. A study to understand how Jujuncao in association with bacteria could remediate other heavy metals or their multiple contaminations needs to be undertaken.

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FACTORS IN CULTURE MEDIA AFFECTING THE GROWTH, AND PIGMENT CONTENTS OF ALGA *TRENTEPOHLIA MONILIA*

SARAPHOL, S. – VAJRODAYA, S. – WONGKANTRAKORN, N. – SANEVAS, N.*

Department of Botany, Faculty of Science, Kasetsart University, 50, Ngam Wong Wan Road, Lat Yao, Chatuchak, Bangkok 10900, Thailand
(phone: +66-2562-5555 ext. 646301; fax: +66-2940-5627)

**Corresponding author*
e-mail: fscintsv@ku.ac.th; phone: +66-2562-5555 ext. 646329; fax: +66-2940-5627

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Abstract. A genus *Trentepohlia* is the major type of filamentous subaerial green alga that grows in tropical zones. Its species have a yellow to red colour because of a large amount of total carotenoids. *Trentepohlia monilia*, a dominant *Trentepohlia* species, was investigated during the winter season at 720 metres above sea level in the Chiang Dao Wildlife Sanctuary, Chiang Mai Province, in northern Thailand. Growth of the species in different liquid culture media was measured to find the best such medium, and various factors in the media were measured to determine which ones resulted in the highest amount of total carotenoids. The results showed that the enriched seawater medium (ESM) liquid culture produced the maximum growth of the species in week 9 of the study and also produced the highest total carotenoid content. A pH of 7.0, with an added peptone of 1%, culture strength of 50%, nitrogen source of –0.50 times and vitamin B_{12} source of –0.50 times the usual concentration in the ESM generated the optimum conditions for growing *T. monilia* and also produced a total carotenoid content that was higher than the total chlorophyll content. This level of growth could make the species a future source of carotenoids for industrial products.
Keywords: *Chiang Dao Wildlife Sanctuary, subaerial green algae, enriched seawater medium, chlorophyll content, total carotenoid content*

Introduction

The genus *Trentepohlia* Martius, a subaerial or terrestrial alga, is the largest genus in the Trentepohliaceae family, belonging to the Trentepohliales order, Ulvophyceae class and Chlorophyta division (John, 2002; López-Bautista et al., 2002; Rindi, 2007; Rindi et al., 2009; Guiry and Guiry, 2015; Lemes-da-Silva et al., 2017). The alga grows on a wide range of substrata, from soils, rocks, bark, stems, and the leaves of trees to various manmade constructions (John, 2002, 2003; López-Bautista et al., 2002; Rindi, 2007; Rindi et al., 2009, 2018; Guiry and Guiry, 2015; Kharkongor and Ramanujam, 2017; Binoy et al., 2019). It is the most diverse and dominant of the subaerial algae that are abundant in tropical and subtropical regions (John, 2002; López-Bautista et al., 2002; Rindi, 2007; Rindi et al., 2009, 2018; Allali, 2011; Binoy et al., 2019). In addition, all *Trentepohlia* species produce a large amount of total carotenoids, which protects them from ultraviolet light or high irradiance. The pigment of the carotenoids gives the algae a yellow, orange or red color that is easily recognizable in natural habitats (López-Bautista, 2008; Rindi et al., 2018). Of the carotenoids, β-carotene is the one most abundantly found in this genus (Czeczuga and Maximov, 1996; Abe et al., 1998, 1999; Mukherjee et al., 2010; Aburai et al., 2013; Chen et al., 2015; Rindi et al., 2018; Binoy et al., 2019). Many scientists have suggested, therefore, that these algae could be used as a rich source of foods or supplementary foods for humans, natural food colorants, animal feeds, cosmetics and medicines (e.g., as a source of vitamin A, an enhancer of the immune response, an antioxidant or an anticancer agent) (Mortensen, 2006; Rao and Rao, 2007; Cazzonelli, 2011; Guedes et al., 2011; Takaichi, 2011; Priyadarshani and Rath,

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2012; Eldahshan and Singab, 2013; Kharkongor and Ramanujam, 2017). Many scientists around the world have been sampling *Trentepohlia* species to culture and isolate their total carotenoid content (López-Bautista, 2008; Rindi et al., 2009; Aburai et al., 2013; Chen et al., 2015), but no one has done it yet in Thailand. Therefore we collected *Trentepohlia monilia* De Wildeman, a dominant species, from the Chiang Dao Wildlife Sanctuary, Chiang Mai Province, in northern Thailand, which has the highest limestone mountain in the country and the third highest overall.

Our study aimed to examine the optimum factors that would enhance the growth of *T. monilia* by collecting samples of the alga from natural sites. Samples were taken to the laboratory, and various liquid culture media were used for growing the algae. We measured the growth of the algae to find which liquid culture medium was the best one. When we found the best medium, we attempted to determine which of its factors were involved in producing the highest total carotenoid content. This study of *T. monilia* could be the first step in gathering data that could stimulate the development of algal carotenoids as a marketable product in Thailand.

Materials and methods

Sampling area

The filamentous subaerial green alga *T. monilia* was collected from a curved steel barrier at points along the Ban Yang Thung Pong and Sop Hui Pha Tang Na Lao trails in the Chiang Dao Wildlife Sanctuary, Chiang Mai Province, northern Thailand, at 720 metres above sea level (latitude 19° 22.588′ North and longitude 98° 45.080′ East). Most of the samples were found in a dry evergreen forest. The algae were collected during the winter (dry season).

Algae materials

The algal samples were collected by following Saraphol et al. (2020), with a sterile scraper and placed into plastic boxes for further identification and culturing. Some environmental factors, such as the type of substrate on which the algae were found and their colour and preliminary morphologic characteristics, were observed and noted to help with identifying the samples correctly.

Algae identification

The algal samples were freeze-dried at –4 °C in the laboratory. Identification of *T. monilia* was done under the Olympus SZ30 stereomicroscope, Olympus CH30 light compound microscope (both from the Olympus Corp., Tokyo, Japan) and scanning electron microscope (Quanta 450 FEI, Thermo Fisher Scientific, Inc., Hillsboro, Oregon, United States) using the floristic keys and algae base website of López-Bautista et al. (2002), John (2002, 2003) and Guiry and Guiry (2015).

Algae sampling and culture

Once the algal samples had been collected from their habitats, they were kept in plastic boxes until used for further study. They were then cleaned by submersion in 70% ethanol and 1.2% sodium hypochlorite for 5 minutes. Each sample was transferred to a 1.5 ml Eppendorf tube, which contained one of seven liquid media: enriched seawater medium (ESM), BG-11 medium, Jaworski's medium (JM), Bold's basal (BB) medium with

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$NaNO_3$ or NH_4Cl source, Bristol medium (BM), high-salt medium (HSM), and tris-acetate-phosphate (TAP) medium in the field. Upon arrival at the Department of Botany, Kasetsart University, each sample was transferred from its Eppendorf tube to a sterile 250 ml Erlenmeyer flask containing one of the seven media.

The subaerial green algal cells of *T. monilia* were grown on a shelf at room temperature (25 °C) under continuous illumination by cool-white fluorescent lamps (3,000 lux) with a light-to-dark ratio of 12:12 for 3 months. During this time, the algae were subcultured for purification to an axenic culture. The cells grew and increased by large amounts. The subaerial green algal cells were identified by microscope again as being *T. monilia*.

Measurement of algae growth

T. monilia cells grown for six months were shaken, and then 10 mL of a minimum of about 0.1 g algal fresh weight were transferred to a sterile 100 ml Erlenmeyer flask, which contained a liquid medium on the shaker. The optical density of algal growth was set as 0.1 at 550 nm. The growing cells were shaken at 100 rpm, at 25 °C, under continuous illumination by cool-white fluorescent lamps (3,000 lux) with a light-to-dark ratio of 12:12. The growth of cells in each 10 ml was measured every week from week 1 to week 11. The photosynthetic pigments in the total chlorophyll (a and b) and the total carotenoid content were measured with the spectrophotometer methods of Pompelli et al. (2013) and Chen et al. (2015, 2016). The pigment contents were calculated as follows:

$$Chlorophyll\ a\ content\ (g/l) = (12.19A665)–(3.45A649) \quad \text{(Eq.1)}$$

$$Chlorophyll\ b\ content\ (g/l) = (21.99A649)–(5.32A665) \quad \text{(Eq.2)}$$

$$Total\ carotenoid\ content\ (g/l) = \frac{[(1000A480)–(2.14\ Chlorophyll\ a\ –(70.16\ Chlorophyll\ b)]}{220} \quad \text{(Eq.3)}$$

$$Car/Chl\ ratio = \frac{Total\ carotenoid\ content}{Chlorophyll\ a\ content+Chlorophyll\ b\ content} \quad \text{(Eq.4)}$$

The algae in different media were analyzed to find the best medium for optimal growth so this medium could be used in the next experiment.

Analysis of various liquid culture factors

The best strain of algae in the best liquid culture medium was chosen based on previous studies to evaluate the various liquid culture factors (*Table 1*) that might have affected it.

The first *T. monilia* cells were evaluated every week from week 1 to week 6 and the total chlorophyll (a and b) and total carotenoid contents were measured with the spectrophotometer methods from the above method.

Table 1. *The summaries of liquid culture factors using in the experiment*

pH	Medium strength	$NaNO_3$ or nitrogen source	Vitamin B_{12} or thiamine HCl solution
3.0	25%	–0.50 times	–0.50 times
5.0	50%	–0.25 times	–0.25 times
7.0	75%	0 times	0 times
9.0	100%	0.25 times	0.25 times
11.0	200%	0.50 times	0.50 times

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Statistical analysis

These measurements were carried out with three replicates, and the results presented were the means of the three replicated experiments. Duncan's new multiple range test (DMRT) (ANOVA, α=0.05) was used to determine the significant differences. The Jamovi statistic programming version 21.0 (The jamovi project, Sydney, Australia) was performed.

Results

Morphologic characteristics of the algae

The colonies of *T. monilia* that were sampled were crustose algae in thallus form found on a steel barrier along a trail in a dry evergreen forest at 700 metres above sea level. They had dark-green to yellow-greenish filaments. The thallus consisted of a dense mat with marked separations between the dense prostrate parts and slightly erect parts. The prostrate parts had a spreading form and produced a pseudoparenchymatous layer, which consisted of several layers of globular cells. The erect part had short filaments arising from the upper prostrate parts; they were 37.47 to 58.68 µm tall, with a thin cell wall. The cells of the erect filaments had a globular, swollen or inflated shape and were 2.67 to 3.59 µm wide and 4.21 to 3.60 µm long. The lateral filaments branching in the central region and the apical cells were often slightly pointed, usually with a small pectic cap at the tip. Neither zoosporangia nor gametangia were observed in this habitat (*Fig. 1*).

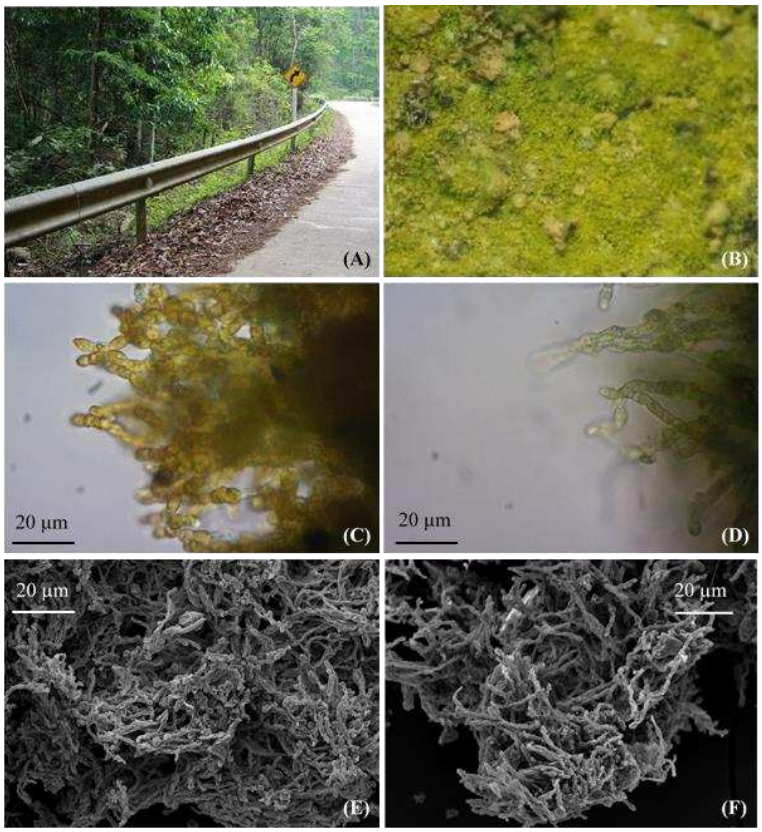

Figure 1. *Characteristics of T. monilia. (A) Habitat from which algae were taken. (B) Cell morphologic characteristics as seen by the naked eye, (C to D) under the light compound microscope and (E to F) by scanning electron microscopy*

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Growth of algae

T. monilia samples from the Chiang Dao Wildlife Sanctuary were successfully grown and isolated only in ESM for 3 months (90 days) (*Fig. 2*). The ESM was used in culture for the analysis of the growth. After the cells had been placed in a fresh ESM culture for 3 months, the growth of *T. monilia* was measured by photosynthetic pigment methods to confirm the growth curves.

Figure 2. *T. monilia cells culture at week 6 in 7 liquid media (liquid enrich seawater medium (ESM), liquid BG-11 medium, liquid Jawoski's medium (JM), liquid Bold's basal (BB) medium with $NaNO_3$ or NH_4Cl source, liquid Bristol medium (BM), liquid high salt medium (HSM) and liquid Tris acetate-phosphate (TAP) medium, respectively)*

The growth of the *T. monilia* cells was measured by the photosynthetic pigments of the total chlorophyll (a and b) and total carotenoid content. In the media, the growth curve was the same growth pattern from week 1 to week 11, but we found that in the ESM, the growth was the highest in week 9 (p-value≤0.05) (*Table A1*). We found that the amounts of total chlorophyll were highest in week 9, at 0.1793±0.0852 g/l, but the total carotenoid content was highest in week 11, at 0.0530±0.0005 g/l. The ratio of total carotenoids to total chlorophyll did not vary much over the time of the study; it was 0.1800±0.0088 to 0.4660±0.0852 (see *Fig. 3* and *Fig. 4*).

Total carotenoid content of algae in various culture conditions

The influence of different features of the cultures on the total carotenoid content accumulation of *T. monilia* varied. The effect of the pH on *T. monilia* growth, as shown in *Fig. 5 (A)*, and on the total carotenoid content was similar and changed quite a lot from week 1 to week 6. The total carotenoid content at a pH of 5.0, 7.0, 9.0 or 11.0 were un-pattern changed in all of the weeks, except in the final week, when it was very high (*Table A2*). It was highest at a pH of 7.0, at 0.0273±0.0140 g/l in week 6. The effect of peptone on the *T. monilia* growth is shown in *Fig. 5 (C)*. The total carotenoid content with various amounts of peptone was similar in pattern and was also quite high from week 1 to week 6 (*Table A3*). The total carotenoid content with peptone of 0.5% and 1.0% changed the most from week 5 to week 6, and peptone of 1.0% caused the most accumulation of total carotenoids (0.0301±0.0000 g/l). The effect of the culture strength

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on *T. monilia* growth was shown in *Table A4* and *Fig. 6 (A)*. The total carotenoid content with various culture strengths had a similar pattern and changed quite a bit from week 1 to week 6, similar to the peptone growth curve. At a culture strength of 50%, the total carotenoid content had a positive pattern of accumulation from week 1 to week 6. It was highest in week 6, at 0.0256±0.0000 g/l. The effect of the nitrogen source on *T. monilia* growth was statistically significant (*Table A5*) and shown in *Fig. 6 (C)*. The total carotenoid content with various nitrogen sources had quite a similar pattern and was quite changed from week 1 to week 6, just like the peptone growth and culture strength curves. The total carotenoid content had a positive pattern of accumulation from week 1 to week 6 when only $NaNO_3$ was used at –0.50 and 0.50 times. $NaNO_3$ of –0.50 times led to the highest accumulation of total carotenoids in the final week, at 0.0484±0.0004 g/l. Finally, the effect of vitamin B_{12} on the *T. monilia* growth was statistically significant (*Table A6*) and shown in *Fig. 7 (A)*. The total carotenoid content with various vitamin B_{12} sources was quite similar and quite changed from week 1 to week 6, like the peptone growth, culture strength and nitrogen source curves. With vitamin B_{12} of –0.50 times, the total carotenoid content was found to be high from week 1 and highest in week 6, at 0.0423±0.0000 g/l.

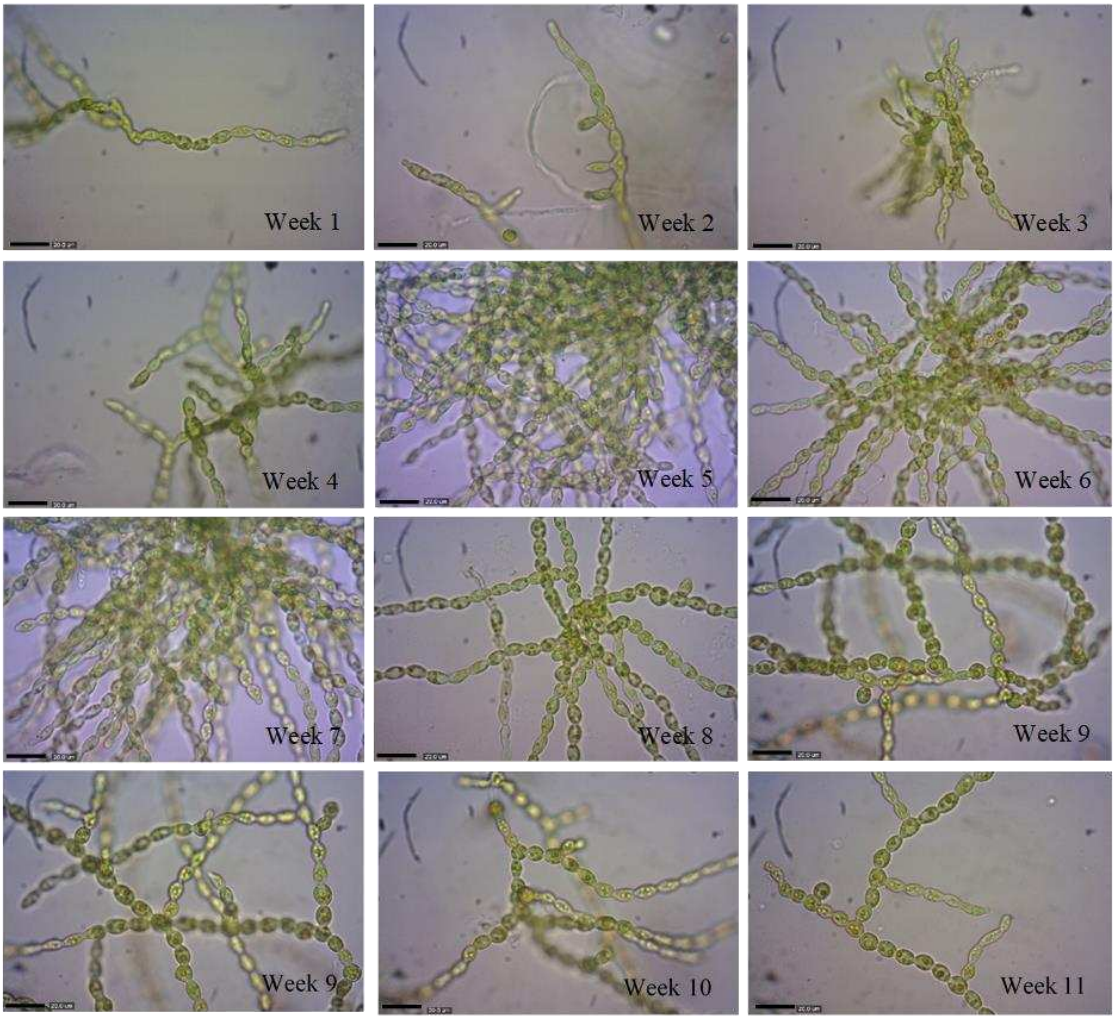

Figure 3. *Characteristic cells of T. monilia in liquid ESM from week 1 to week 11 (scale bars=20 µm)*

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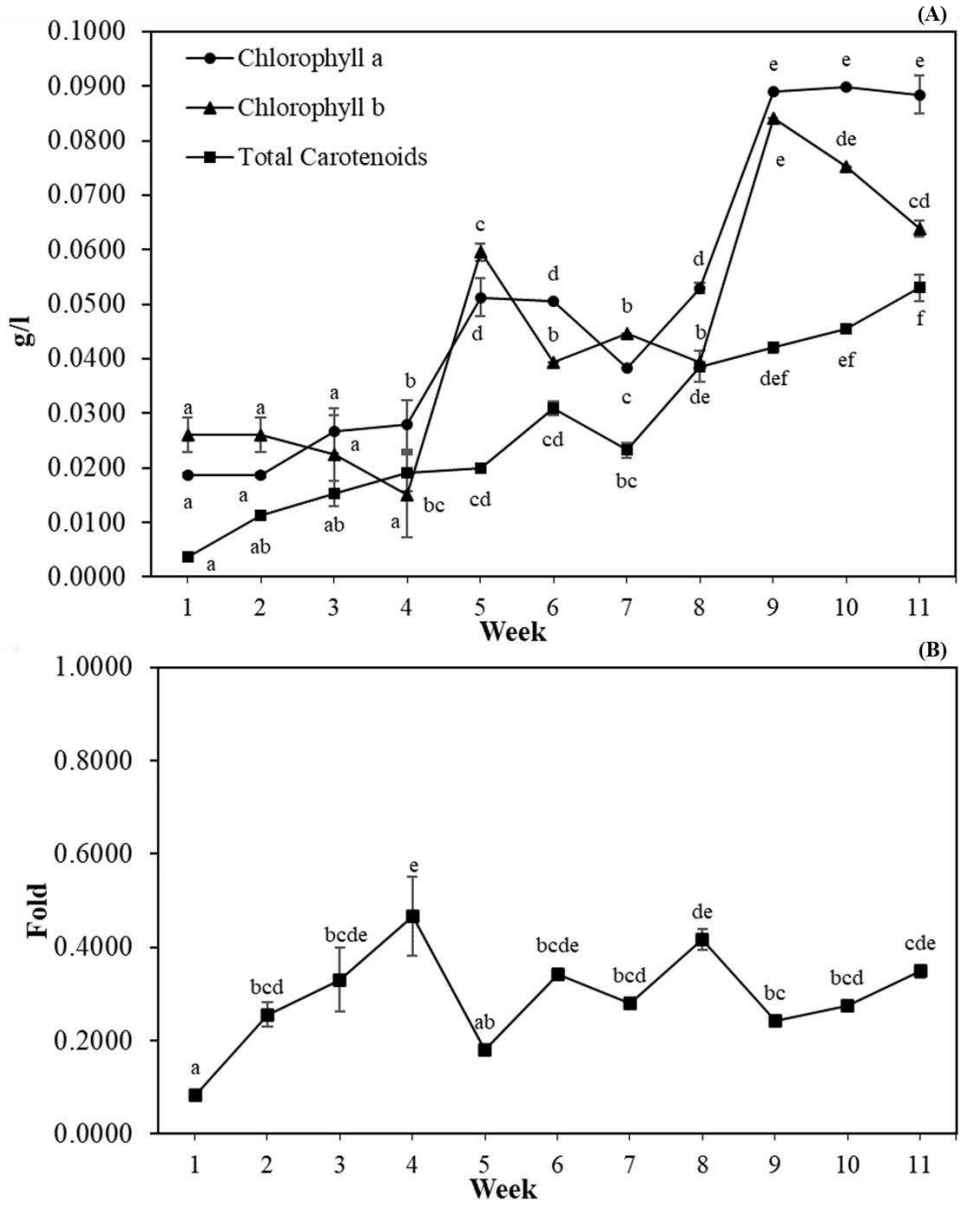

Figure 4. *The growth of T. monilia in liquid ESM as measured by photosynthetic pigment methods. (A) Pigment accumulation and (B) total carotenoids/chlorophyll content ratios. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences ($p \leq 0.05$) at each time by DMRT*

All of the measurements of the total carotenoids/total chlorophyll ratios for all of the culture factors are shown in *Fig. 5 (B, D)*, *Fig. 6 (B, D)* and *Fig. 7 (B)*. The results were clear because the ratios changed in all of the weeks from week 1 to week 6.

In the condition of pH7, added peptone 1%, culture strength 50%, nitrogen source -0.50 times and vitamin B_{12} source -0.50 times which nitrogen source -0.50 times from general concentration in enriched seawater medium (ESM) were the optimum of *T. monilia* growth to produce the increased of total carotenoids content more than total chlorophyll content accumulation in algae.

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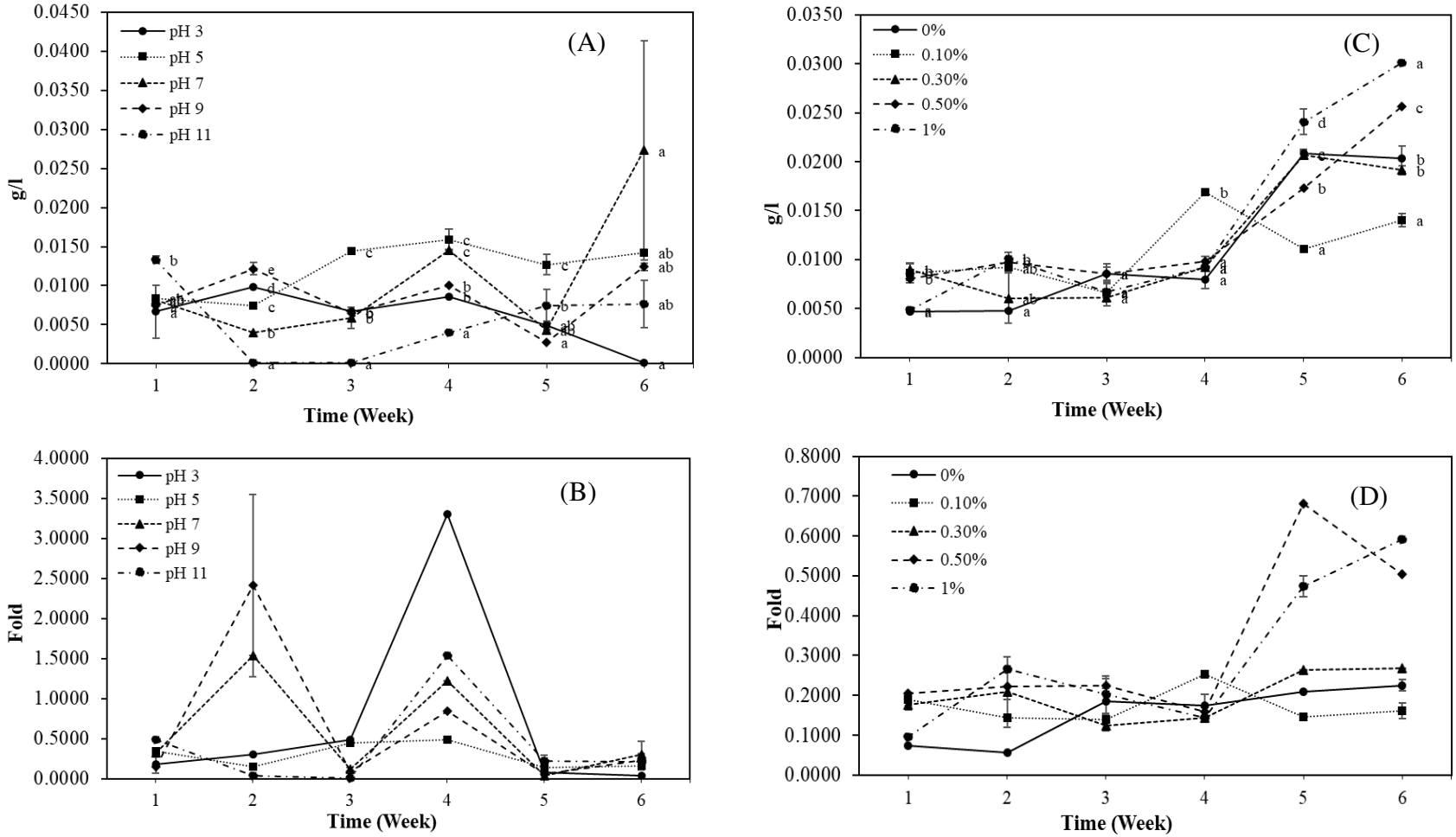

Figure 5. *Evolution over time of the total carotenoid content and total carotenoid/total chlorophyll content of T. monilia in liquid ESM with various culture factors: (A and B) pH, and (C and D) peptone. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences (p≤0.05) at each time by DMRT*

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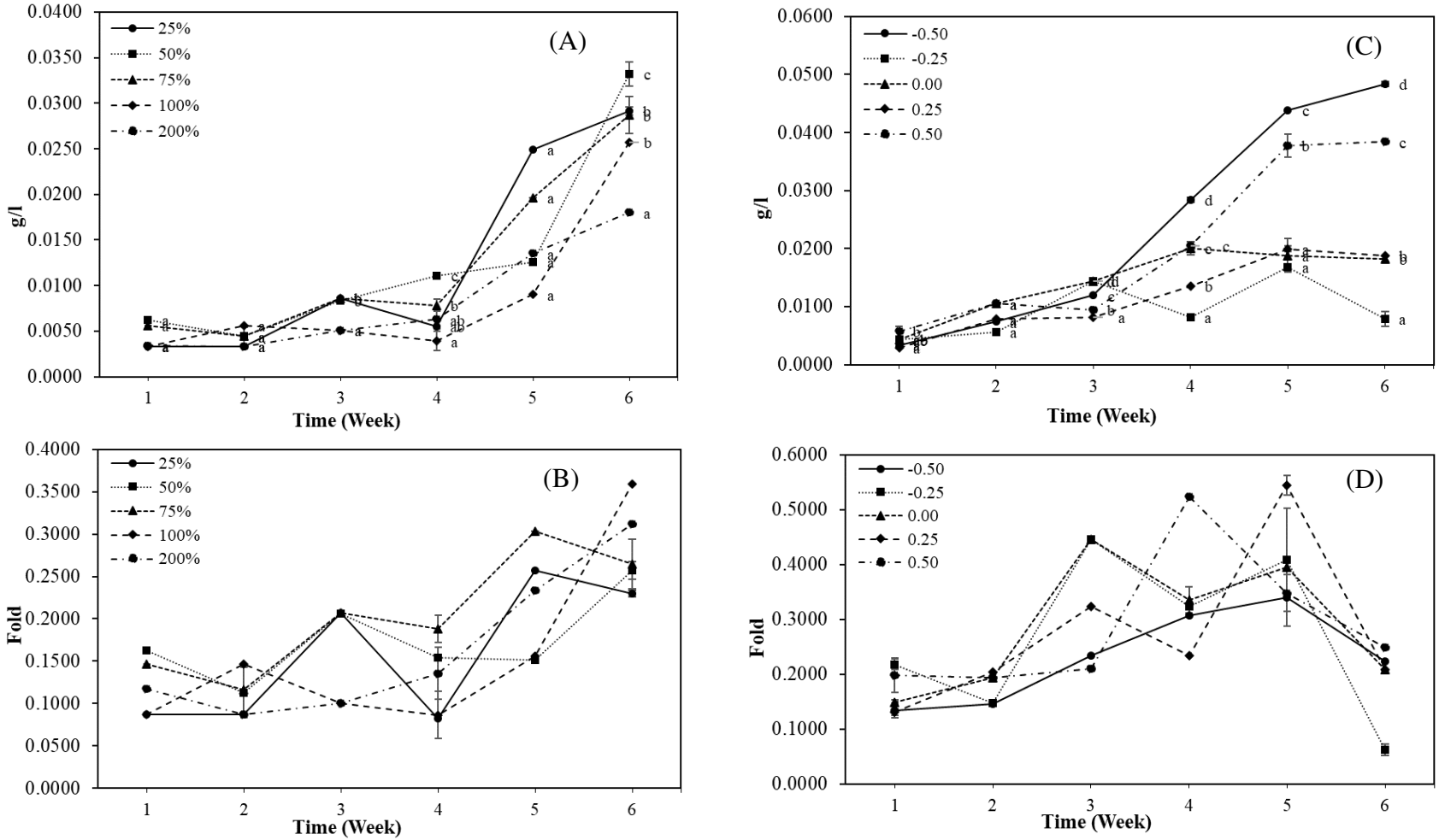

Figure 6. *Evolution over time of the total carotenoid content and total carotenoid/total chlorophyll content of T. monilia in liquid ESM with various culture factors: (A and B) culture strength, and (C and D) nitrogen source. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences (p≤0.05) at each time by DMRT*

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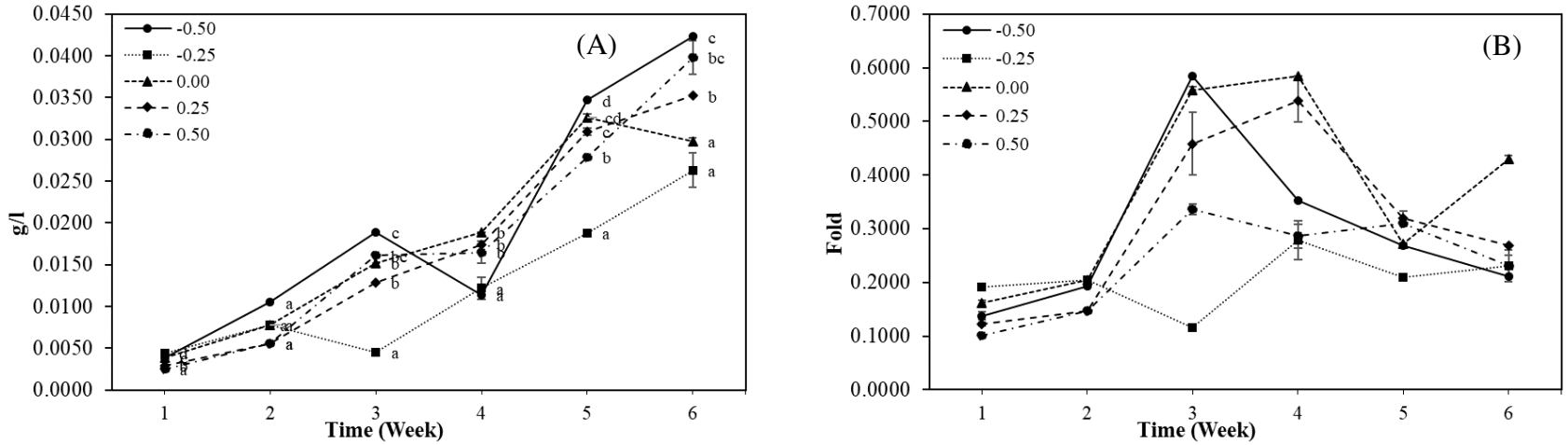

Figure 7. *Evolution over time of the total carotenoid content and total carotenoid/total chlorophyll content of T. monilia in liquid ESM with various culture factors: (A and B) vitamin B_{12} source. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences ($p \leq 0.05$) at each time by DMRT*

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Discussion

Trentepohlia spp. grow mainly in tropical and subtropical zones of the world. They grow on tree barks, soils and rocks and also on manmade structures exposed to full sunlight (John, 2002; López-Bautista et al., 2002; Kharkongor and Ramanujam, 2017; Rindi et al., 2018). These algae are thought to have a high tolerance for and adaptability to extreme conditions such as desiccation and high temperatures (Rindi, 2007; Rindi et al., 2009; Bartoli et al., 2019; Binoy et al., 2019). As a source of useful substances, *T. monilia* has the negative characteristics of a longer lag phase and a lower growth rate than other microalgae grown in liquid cultures. This study showed that *T. monilia* grew the most and produced the most total carotenoid content only in ESM. According to Abe et al. (1998), who studied *Trentepohlia aurea*, the best growth also occurred in liquid ESM with 3,000 lux. The ESM has nutrients and properties of soil extraction similar to those of the natural habitats of *Trentepohlia* spp.

In terms of various growth factors, a pH of 6.0 to 8.0 is optimal for the growth of *Trentepohlia* spp. (Abe et al., 1999; Lemes-da-Silva et al., 2017). The optimum pH was 7.0 in a liquid culture, based on colony size, the relative abundance of new colonies formed and the dimensions of apical cells. The algae preferred a slightly alkaline (pH 7.5) environment, although they could be grown in a wide range of pH conditions. The preference for an alkaline environment is not surprising. In nature, the species colonizes whitewashed building walls, as well as painted surfaces or manmade constructions, where the pH is distinctly alkaline (Lee et al., 1990; Lemes-da-Silva et al., 2017; Bartoli et al., 2019; Binoy et al., 2019). In the presence of peptone, the growth rate was even greater. Peptone supplementation activated nitrogen metabolism in the cells of *T. monilia*, resulting in an acceleration of the algal growth rate (Abe et al., 1998). An earlier study had shown that the growth rate and total chlorophyll content increased markedly with the addition of peptone as a nitrogen source. The culture strength, at nutrient strengths of 75%, 100% and 200%, resulted in a normal-appearing growth rate and the cells remained green. Transferring the algae from a diluted 25% or 50% culture medium changed the color to yellow or orange (Lee et al., 1990), which was optimal for producing total carotenoids. With a nitrogen source of –0.50 times, the chlorophyll a content was the lowest. The carotenoid content was higher than the chlorophyll a content with the lowest nitrogen source (Chen et al., 2016). Nitrogen deficiency had a significantly positive effect on carotenoid accumulation in *Trentepohlia arborum* and other species in the same genus (Tan et al., 1993; Abe et al., 1998); this might have depended on high expression levels of enzymes involved in β-carotene synthesis, resembling that for *Haematococcus pluvialis,* when grown under nitrogen deficiency (Recht et al., 2014). The effect of a vitamin B_{12} source or thiamine was negligible. It has been reported that vitamins are necessary for the growth of various groups of algae, but the growth of *T. monilia* in their presence was slow and have high total carotenoid content accumulation (Lee et al., 1990).

In the present study, the growth and accumulation of total carotenoids in the subaerial green alga *T. monilia* in liquid ESM were shown. It was thus possible to demonstrate the simultaneous production of useful materials such as β-carotene by *T. monilia*. The algae could be used to provide a rich source of foods or supplementary foods, natural food colourants, animal feeds, cosmetics and medicines. *T. monilia* could also be utilized as a biofunctional material in the future. This report provides new information on the nature of different carotenoids biosynthesized by *T. monilia* collected from natural sources without culturing the alga in an artificial medium.

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Conclusion

T. monilia grows the best in liquid ESM culture. The maximum growth occurred in week 9 of the study. The total carotenoids accumulated at the highest rate when the species was being grown in this culture. In ESM, the best liquid culture, the optimal conditions were a pH of 7.0, peptone of 1%, culture strength of 50%, nitrogen source -0.50 times and vitamin B_{12} source –0.50 times the normal concentration. This culture produced the optimum growth of *T. monilia*, the highest amount of total carotenoids and situations in which the total carotenoids were higher than the total chlorophyll in a liquid culture. In further studies, we will focus on modifying more specialized ESM mediums to optimize for a short period to cultivate *T. monilia* less than three weeks, which should be promising for future carotenoid-producing industries.

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APPENDIX

Table A1. *ANOVA analysis results of growth of T. monilia in liquid ESM medium which measured by chlorophyll-a, chlorophyll-b and total carotenoids content from week 1 to week 11*

Source	df	Mean square	F	Sig.
Intercept	1	0.083	3612.965	.000
Chlorophyll a	10	0.002	100.561	.000
Error	22	0.00002302		
Total	33			
Intercept	1	0.067	1322.438	.000
Chlorophyll b	10	0.002	30.756	.000
Error	22	0.00005071		
Total	33			
Intercept	1	0.027	499.133	.000
Total Carotenoids	10	0.001	13.330	.000
Error	22	0.00005392		
Total	33			

Table A2. *ANOVA analysis results of total carotenoids content in T. monilia on ESM medium with a different pH on week 1 to week 6*

Source	df	Mean square	F	Sig.
Intercept	1	0.001	126.811	.000
pH (Week 1)	4	0.00002067	2.253	.136
Error	10	0.000009174		
Total	15			
Intercept	1	0.001	1285.735	.000
pH (Week 2)	4	0.00006813	130.342	.000
Error	10	0.0000005227		
Total	15			
Intercept	1	0.001	464.237	.000
pH (Week 3)	4	0.00007769	53.456	.000
Error	10	0.000001453		
Total	15			
Intercept	1	0.002	1248.444	.000
pH (Week 4)	4	0.00006841	50.678	.000
Error	10	0.000001350		
Total	15			
Intercept	1	0.001	129.948	.000
pH (Week 5)	4	0.00004526	9.453	.002
Error	10	0.000004787		
Total	15			
Intercept	1	0.002	13.860	.004
pH (Week 6)	4	0.000	1.818	.202
Error	10	0.000		
Total	15			

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Table A3. *ANOVA analysis results of total carotenoids content in T. monilia on ESM medium with a different peptone (%) on week 1 to week 6*

Source	df	Mean square	F	Sig.
Intercept	1	0.001	576.697	.000
peptone (Week 1)	4	0.00001314	10.214	.001
Error	10	0.000001287		
Total	15			
Intercept	1	0.001	156.244	.000
peptone (Week 2)	4	0.00001722	2.821	.084
Error	10	0.000006103		
Total	15			
Intercept	1	0.001	326.392	.000
peptone (Week 3)	4	0.000004094	1.666	.233
Error	10	0.0000024658		
Total	15			
Intercept	1	0.002	1901.323	.000
peptone (Week 4)	4	0.00003839	43.197	.000
Error	10	0.0000008887		
Total	15			
Intercept	1	0.005	3062.149	.000
peptone (Week 5)	4	0.00007277	42.029	.000
Error	10	0.000001731		
Total	15			
Intercept	1	0.007	3751.864	.000
peptone (Week 6)	4	0.000	60.454	.000
Error	10	0.000001905		
Total	15			

Table A4. *ANOVA analysis results of total carotenoids content in T. monilia on ESM medium with a different culture strength (%) on week 1 to week 6*

Source	df	Mean square	F	Sig.
Intercept	1	0.000	.	.
Culture strength (Week 1)	4	0.000006069	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.000	163.030	.000
Culture strength (Week 2)	4	0.000002766	1.693	.227
Error	10	0.000001633		
Total	15			
Intercept	1	0.001	17956.000	.000
Culture strength (Week 3)	4	0.00001051	246.273	.000
Error	10	0.00000004267		
Total	15			
Intercept	1	0.001	344.008	.000
Culture strength (Week 4)	4	0.00002219	10.650	.001
Error	10	0.000002084		
Total	15			
Intercept	1	0.004	.	.
Culture strength (Week 5)	4	0.000	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.011	2274.012	.000
Culture strength (Week 6)	4	0.00009633	20.122	.000
Error	10	0.000004787		
Total	15			

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Table A5. *ANOVA analysis results of total carotenoids content in T. monilia on ESM medium with a different nitrogen source (times) on week 1 to week 6*

Source	df	Mean square	F	Sig.
Intercept	1	0.000	339.381	0.000
Nitrogen source (Week 1)	4	0.000003656	4.857	0.019
Error	10	0.0000007527		
Total	15			
Intercept	1	0.001	.	.
Nitrogen source (Week 2)	4	0.00001351	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.002	37765.444	.000
Nitrogen source (Week 3)	4	0.00002411	446.556	.000
Error	10	0.00000005400		
Total	15			
Intercept	1	0.005	3975.497	.000
Nitrogen source (Week 4)	4	0.000	141.990	.000
Error	10	0.000001241		
Total	15			
Intercept	1	0.011	1111.761	0.000
Nitrogen source (Week 5)	4	0.000	45.605	0.000
Error	10	0.00001015		
Total	15			
Intercept	1	0.010	6013.979	.000
Nitrogen source (Week 6)	4	0.001	474.511	.000
Error	10	0.000001731		
Total	15			

Table A6. *ANOVA analysis results of total carotenoids content in T. monilia on ESM medium with a different vitamin B_{12} source (times) on week 1 to week 6*

Source	df	Mean square	F	Sig.
Intercept	1	0.000	3555.696	0.000
vitamin B_{12} source (Week 1)	4	0.000001882	35.728	0.000
Error	10	0.00000005267		
Total	15			
Intercept	1	0.001	.	.
vitamin B_{12} source (Week 2)	4	0.00001229	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.003	838.516	.000
vitamin B_{12} source (Week 3)	4	0.00009016	27.599	.000
Error	10	0.000003267		
Total	15			
Intercept	1	0.004	864.735	.000
vitamin B_{12} source (Week 4)	4	0.00003272	8.079	.004
Error	10	0.000004050		
Total	15			
Intercept	1	0.013	8075.653	0.000
vitamin B_{12} source (Week 5)	4	0.000	74.217	0.000
Error	10	0.000001561		
Total	15			
Intercept	1	0.018	2626.223	.000
vitamin B_{12} source (Week 6)	4	0.000	19.499	.000
Error	10	0.000006875		
Total	15			

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ENTOMOPATHOGENIC NEMATODES AS BIOINSECTICIDES – A REVIEW

SIKANDAR, A.[1] – YUAN, R. H.[2] – LIAN, X. L.[2] – ZHEN, M. A.[2] – ZHAO, P.[3] – LI, F.[4] – LU, X. P.[5] – WANG, Y. Y.[1.2*]

[1]*Nematology Institute of Northern China, Shenyang Agricultural University, Shenyang 110866, Liaoning, China*

[2]*College of Biosciences and Biotechnology, Shenyang Agricultural University, Shenyang 110866, Liaoning, China*

[3]*College of Plant Protection, Shenyang Agricultural University, Liaoning, People Republic of China*

[4]*Chifeng Academy of Agricultural and Animal Husbandry Sciences, Chifeng, Inner Mongolia, People Republic of China*

[5]*Liaoning Agricultural Development Service Center, Shenyang, Liaoning, People Republic of China*

[*]*Corresponding author*
e-mail: wyuanyuan1225@syau.edu.cn; phone: +86-138-9818-2096

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Abstract. Entomopathogenic nematodes (EPNs) belonging to the *Heterorhabditis* and *Steinernema* genera provide effective bio-control of insect pests. They are extremely lethal to such pests due to mutualistic association with the genera of bacteria *Photorhabdus* and *Xenorhabdus* and are safe for beneficial insects. They are used as biopesticides because they are eco-friendly with no harmful effects on human wellbeing. EPNs are well-suited with the number of agrochemicals and a cost-effective substitute for chemical insecticides. This review assesses the use of EPNs under agroclimatic conditions, the influence of abiotic factors, life cycle, trapping, production, storage, mechanisms, biopesticides and their suitable application. However, a previous study on EPNs and their role as biopesticides have been surveyed for their possible usage as biological control agents.
Keywords: *Heterorhabditis, Steinernema, Photorhabdus, Xenorhabdus, agrochemicals, biological control agent*

Introduction

Nematodes are roundworms, colourless microorganisms, possessing almost all schemes of higher organisms except circulatory or respiratory systems (Sikandar et al., 2019, 2020b). They may be parasitic, predaceous, or free-living and have an abundance of various associations from useful to harmful (Ferris et al., 2012). Their association can be divided into four basic groups; facultative parasitism, obligate parasitism, necromenic and phoretic (Askary et al., 2018). Entomopathogenic nematodes (EPNs) can be obligate or facultative parasites on harmful insects. They have been recorded on all continents except Antarctica (Abate et al., 2017). Entomopathogenic nematodes have a wide host range and can easily find their suitable host. Biological control is a safe way to control pests and pathogens (Sikandar et al., 2020a). They have huge consideration in the field of biological control (Davari and Parker, 2018; Trdan et al., 2020).

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Entomopathogenic nematodes are harmless or safe for non-targeted organisms (Dutka et al., 2015) and yet pose no threat to beneficial insects (Akhurst and Smith, 2002). They are eco-friendly and non-toxic to humans and can easily apply with pesticide equipment (Shapiro-Ilan et al., 2006). Steiner in 1923 described the first entomopathogenic nematode (EPNs) (Dillman, 2013).

Twenty-three families of nematodes have been recorded as a parasite of insects but the species of seven families have more potency to control insects (Shapiro-Ilan et al., 2012). Families Heterorhabditiae and Steinernematidae have been more frequently and effectively used in pest biological control (Bal and Grewal, 2017). Usually, species of genera *Steinernema* and *Herorhabditis* (Rhabditida) are used as biological control agents in the domain of plant protection (Laznik and Trdan, 2014). Species of genus *Oscheius* are entomopathogenic because they are also a parasite of insect pest meanwhile Steinernematidae and Heterorhabditidae received more attention as effective biocontrol agents (Dillman et al., 2012b). The symbiosis of EPNs with bacteria like Photorhabdus and Xenorhabdus can effectively control Coleopteran, Dipteran and Lepidopteran pests (Mohan, 2015).

Entomopathogenic nematodes have been divided into two categories according to their host searching behaviour including cruisers and ambushers. Cruisers such as *Heterorhabditis bacterophora* and *Steinernema glaseri* are subterranean, more active to find suitable host, while ambushers like *Steinernema carpocapsae* usually wait to attack suitable host in the upper surface of the soil (Mohan, 2015). They can search their host in different ways like vibration, carbon dioxide, or other chemicals (Lortkipanidze et al., 2016). They showed significant potential as natural pest control agents in the soil environment (De Brida et al., 2017). After finding a suitable host and penetrating it, they can kill the host within 1-4 days. Their killing capacity depends upon the host and nematode species.

More than 90% of insects have their life stages in the soil, so are easily exposed to EPNs (Radová, 2010). They can suppress a huge variety of commercially targeted important pests (Lacey and Georgis, 2012). Entomopathogenic nematodes can control a variety of insect species i.e. *Alissonolurn impressicalla, Arbela dea, Blitoportha pallidipennis Reitter, Holotrichia parallela, Odoiporus longicollis, Otiorhynchus sulcatus, Pachraetus litus, Paranthrene tubaniformis, Phylloreta striolut,* etc in China (Mahmoud, 2016). Substantial progress in the application, production and research of entomopathogenic nematodes has been made in the last decade (Lacey et al., 2015).

Influence of abiotic and biotic factors

Entomopathogenic nematodes can survive under a variety of environments but some abiotic factors influence their activity such as infectious juveniles (IJs) can survive in the value of pH of soil between 4-8 but their activities are minimized at a pH value of 10. Moisture and pH also influenced their occurrences (Stuart et al., 2015). During a survey on EPNs in North China, the environmental conditions imposed harmful effects on their virulence, survival, and reproduction (Ma et al., 2010). High salinity also affected the activity of EPNs (Kergunteuil et al., 2016), but it increased the rate of tolerance toward high temperatures (Hussaini, 2017).

They are distributed throughout the world and they can tolerate overwintering conditions, as reported from extreme cold Heilongjiang Province of China (Chunjie et al., 2011). Their recovery depends upon the texture and characterization of soil

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(Noosidum et al., 2010). They found usually less in clay soil due to the low oxygen value and matter contents in it. They are more frequently recovered in sandy soil (Banu, 2017). Entomopathogenic nematodes were isolated from sandy soil in South China and Beijing areas (Griffin et al., 2000).

The effects of biotic factors (like species of EPNs, soil fauna, age of targeted insects) and abiotic factors (moisture, temperature, soil type and aeration) have been reported by several researchers (Shapiro-Ilan et al., 2012). Researchers have mainly focused on their potential as inductively applied augmentative biocontrol agents (Grewal et al., 2005; Laznik et al., 2010; Laznik et al., 2011). That's why they have needed to adapt environmental conditions on application sites to become more effective biocontrol agents (Del Pino et al., 2018).

Trapping of entomopathogenic nematodes

Galleria soil trap method

The soil trap method is usually used for the extraction of EPNs. 200/250g soil was added into the plastic pots having approximately 90 cm diameter (Razia and Sivaramakrishnan, 2014). Five instar larvae of the greater wax moth, *Galleria mellonella* L. were placed into each pot and covered for incubation of five to seven days at 27-30ºC. From the second day onwards, dead larvae were examined and removed regularly. The dead larvae were rinsed with water and dissected into Ringer's solution to collect the entomopathogenic nematodes.

White trap method

The white trap method is used to collect IJs from cadaver insects. Following this method, dead insects were placed into the white trap for three to four weeks until all juveniles emerged out from the cadaver. Usually, a plastic container was used for this method and filled with distilled water up to 1cm. The bottom of the inverted Petri dish was placed in the container and the juveniles that emerged out were collected (White, 1927).

Life cycle

Heterorhabditis and *Steinernema* have similar life stages. These nematodes have a high reproduction rate and can easily culture in lab conditions. They have a chemoreceptor that can detect their host easily and kill them quickly. They are parasitic completely in all stages outside the host except the dauer stage (Onstad et al., 2006). Dauer juvenile is a special developmental stage of all rhabditids. The term dauer is a German word meaning enduring (Fuchs, 1915). It is an infective of these nematodes (Susurluk and Ehlers, 2008). The free-living infective stage is only one stage of their cycle that can exist outside of the host (Spence et al., 2011). Once they find their suitable host they can easily enter into it (Andaló et al., 2017).

Infectious juveniles penetrate the host through the opening including the anus, breathing pore, mouth, spiracles, and pores in cuticles (De Siqueira Sabino et al., 2014) while *S. glaseri* entered into the host through body openings (Hoctor et al., 2012). Mostly their penetration into the host through breathing pore (Fujimoto et al., 2007).

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The relationship between entomopathogenic nematode and entomopathogenic bacteria is highly specific; bacteria *Photorhabdus* spp. and *Xenorhabdus* spp. are associated with *Heterorhabditis* and *Steinernema*, respectively (Ferreira and Malan, 2014). Entomopathogenic nematodes with their symbiotic bacteria can efficiently suppress insect pests in cryptic and soil habitats (Divya and Sankar, 2009). These bacteria entirely depend upon nematodes as a vector from one host to another as well as nematodes immune system of the host (Lewis and Clarke, 2012). The bacteria are rapidly reproduced within the insect and kill it within 24-48 hours, and nematodes are feed on it and complete their life stages (Adams and Nguyen, 2002).

In *Heterorhabditis* infectious juveniles become hermaphroditic adults but their next-generation is produced by males and females while in *Steinernema* all generations are produced by males and females (Grewal et al., 2005). After continuously feeding upon cadaver of insect the second stage juveniles develop into third stage juveniles at that time they leave the insect cadaver for searching new living hosts (Jagdale et al., 2005). The insect cadaver turns into tan or brown if Steinernematids killed it whereas it becomes red when Heterorhabditids killed it, these pigmentations depend upon the mutualistic bacteria (Yadav, 2012).

Production

Entomopathogenic nematodes are produced by a different method *in vitro* or *in vivo* by liquid and solid culture (Shapiro-Ilan et al., 2012). *In vitro* production, the dauer juveniles are the only stage of entomopathogenic nematode that is used commercially. Culturing is based upon introducing nematodes in a nutrition medium with a pure culture of their symbiotic bacteria. Bedding three-dimensional productions have been the most successful method in solid culture for the production of *Heterorhabditis* and *Steinernema* (Shapiro-Ilan et al., 2012). Large fermentation units are used for the production of these nematodes in large quantities for commercial use.

The liquid culture method is a more cost-efficient process than solid culture for the commercial market. However, it also demands a high level of technical expertise and capital investment (Shapiro-Ilan et al., 2012). Advancement in liquid culture is necessary to improve efficiency and quality of production through different processes like optimizing bioprocess and media kinetics (Chavarría-Hernández et al., 2010), improving inoculum and its timing and density of bacterial cells (Hirao and Ehlers, 2010), improving useful traits such as desiccation and heat tolerance in *Heterorhabditis* and downstream processing (Anbesse et al., 2013).

In *in vitro* liquid culture, EPNs have produced continuously at a high level of efficacy with improvement in media, bioreactor design, and other parameters (Chavarría-Hernández et al., 2010). In *in vivo* production, culturing of EPNs in hosts is a simple process because it requires less technology. *Galleria mellonella* (L.) is the most common insect used for commercial and laboratory EPN cultures whereas *Tenebrio molitor* L. was also used for EPNs production (Shapiro-Ilan et al., 2002). Other hosts have been studied for culturing them, including beet armyworm *Spodoptera exigua* (Hübner), cabbage looper *Trichoplusia ni* (Hübner), corn earworm *Helicoverpa zea* (Boddie), house cricket *Acheta domesticus* (L.), gypsy moth *Lymantria dispar* (L.), orange worm *Ameylois transitella* (Walker), pink bollworm *Pectinophora gossypiella* (Saunders), tobacco budworm *Heliothis virescens* (F.) and various battles (Shapiro-Ilan et al., 2012).

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White trap method is used for natural escape of EPNs from a host cadaver. This method is ideal for laboratory studies or small markets because of cost-effective production (Shapiro-Ilan et al., 2002). *In vivo* production is a two-dimensional system based on production in shelves and trays (Ehlers and Shapiro-Ilan, 2005). Its yields depend upon host density and nematode dosage (Boff et al., 2000). In vitro production quality may vary from batch to batch (Cottrell et al., 2011). Whereas in vivo production depends upon the source of production (Gaugler et al., 2000). Now research is being focused on bioreactor design and media optimization, which expected to lead to benefits such as reduced cost and higher yields (Shapiro-Ilan et al., 2014).

Storage

Entomopathogenic nematodes can be stored in different ways like water-dispersible granules, autoclaved polyether polyurethane foam, alginate gel, vermiculite and baits. EPNs used less energy because they have no fully dormant resting stage, but juveniles can store a little bit of carbohydrate, protein and lipid in them (Andaló et al., 2009). Their quality depends upon the ratio of viable to non-viable, age, virulence and viability assay (Grewal et al., 2005). Low temperatures up to 2-5ºC can increase shelf life and reduced metabolic activity of nematodes except for *H. indica* and *S. riobrave* which cannot survive below 10ºC (Grewal, 2002).

Application

The EPNs can successfully be applied against soil-inhabiting insect pests through soil application and above-ground insects (foliar spray) in cryptic habitats (Shapiro-Ilan et al., 2006). They can be applied through electrostatic sprayers, mist blowers and pressurized sprayers (Shapiro-Ilan et al., 2006), or by mixing them with water dispersal polymers and particular surfactants (Shapiro-Ilan et al., 2010).

Entomopathogenic nematodes as bio-insecticides

Chemical insecticides are usually used to control pests of fruits, vegetables and crops in China, their extensive use may cause environmental pollution and hamper the export of products (Jianguang et al., 2008). A high frequency of chemical insecticide application may lead to developing resistance in pests (Feng et al., 2000). Chemical insecticides are carcinogenic and cause environmental pollutions because these are not easily degradable.

Entomopathogenic nematodes are used as insecticides (Ulu et al., 2015). They are eco-friendly and safe for human health. Their associations with bacteria have no harmful impacts on other mammals or plants (Ehlers, 2003). The entomopathogenic nematodes were extensively used as pest control agents in various parts of the world (Kaya et al., 2006; Trdan et al., 2008). As compared with chemical insecticides EPNs are too costly for the average grower in China (Yan et al., 2012). Chemical pesticides may cause problems to EPNs if used arbitrarily (Negrisoli Jr et al., 2010). Entomopathogenic nematodes are utilized as augmentative, classical and conservational biological control agents (Lacey and Georgis, 2012). Entomopathogenic nematodes can effectively control the insect pests listed in *Table 1*.

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Table 1. *Insect-pests controlled by entomopathogenic nematodes*

Scientific name	Common name	ENP	Reference
Agrotis ipsilon	Black cut worm	*H. amazonensis*	(de Oliviera Giannasi et al., 2018)
Alissonotum impressicolle	Banana borer	*H. indica*	(Anh et al., 2017)
Anomala graueri	White grub	*S. longicaudum*	(Kajuga et al., 2018)
Anoplophora glabripennis	Long-horn beetle	*S. carpocapsae*	(Solter et al., 2001)
Arbela dea	Litchi beetle	*S. carpocapsae*	(Saleh, 2017)
Bactrocera tryoni	Queensland fruit fly	*H. bacteriophora*	(Langford et al., 2014)
Bactrocera zonata	Peach fruit fly	*H. marelatus*	(Saleh et al., 2018)
Bradysia odoriphaga	Chive maggot	*H. bacteriophora*	(Bai et al., 2016)
Carposina nipponensis	Apple fruit moth	*S. carpocapsae*	(Yang et al., 2000)
Cephus cinctus	Wheat stem sawfly	*S. kraussei*	(Portman et al., 2016)
Chilo infuscatellus	Sugarcane borer	*S. feltiae*	(Karunakar et al., 2002)
Chironomus plumosus	Buzzer midge	*S. kraussei*	(Edmunds et al., 2017)
Coptotermes formosanus	Termite	*S. karii*	(Wagutu, 2017)
Curculio elephas	Chestnut weevil	*S. weiseri*	(Demir et al., 2015)
Cydia pomonella	Codling moth	*S. jeffreyense*	(Odendaal et al., 2016)
Earis vittella	Spotted bollworm	*S. mushtaqi*	(Pervez and Ali, 2011)
Ectomyelois ceratoniae	Carob moth	*S. carpocapsae*	(Memari et al., 2016)
Eriosoma lanigerum	Wooly apple aphid	*H. megidis*	(Berkvens et al., 2014)
Heliothis virescens	Tobacco budworm	*H. bacteriophora*	(Gulzar et al., 2020)
Holcocercus insularis	Tree borer moth	*S. carpocapsa*	(Kaya et al., 2006)
Holotrichia consanguinea	Sugarcane beetle	*S. abbasi*	(Patil et al., 2016)
Holotrichia oblita	White grub	*S. longicaudum*	(Guo et al., 2015)
Holotrichia parallela	Peanut grubs	*H. beicherriana*	(Li et al., 2021)
Hylobius abietis	Large pine weevil	*S. downesi*	(Kapranas et al., 2017)
Leucinodes orbonalis	Brinjal fruit borer	*S. siamkayai*	(Adiroubane et al., 2010)
Maconellicoccus hirsutus	Mango mealybug	*H. amazonensis*	(Fuenmayor et al., 2020)
Macrotermes bellicosus	Termite	*H. sonorensis*	(Zadji et al., 2014)
Mamestra brassicae	Cabbage moth	*S. carpocapsae*	(Beck et al., 2014)
Musca domestica	Housefly	*H. indica*	(Bream et al., 2018)
Mythimna separate	Armyworm	*H. indica*	(Acharya et al., 2020)
Odoiporus longicollis	Banana borer	*S. carpocapsae*	(Belien, 2018)
Otiorhynchus ligustici	Alfalfa snout beetle	*H. bacteriophora*	(Shields et al., 2009)
Paranthrene diaphana	Clearwing moth	*S. carpocapsae*	(Azarnia et al., 2018)
Periplaneta americana	Cockroach	*S. carpocapsae*	(Maketon et al., 2010)
Phyllotreta cruciferae	Crucifer flea beetle	*S. carpocapsae*	(Reddy et al., 2014)
Phyllotreta striolata	Striped flea beetle	*S. feltiae*	(Xu et al., 2010)
Planococcus ficus	Vine mealybug	*S. yirgalemense*	(Platt et al., 2018)
Plutella xylostella	Diamondblack moth	*S. carpocapsae*	(Sunanda et al., 2014)
Polyphylla fullo	Pine chafer	*S. glaseri*	(Demir et al., 2015)
Rhynchophorus ferrugineus	Red palm weevil	*S. carpocapsae*	(Dembilio et al., 2010)
Scirpophaga incertulas	Rice stem borer	*H. bacteriophora*	(Devi, 2020)
Spodoptera littoralis	Cotton leaf worm	*S. monticolum*	(Sobhy et al., 2020)
Spodoptera litura	Armyworm	*S. glaseri*	(Safdar et al., 2018)
Stomoxys calcitrans	Stable fly	*H. baujardi*	(de Souza Leal et al., 2017)
Tuta absoluta	Tomato leaf miner	*H. bacteriophora*	(Damme et al., 2016)
Zeuzera pyrina	Leopard moth	*H. bacteriophora*	(Salari et al., 2015)

Whereas; ENP (entomopathogenic nematodes). List of insect pests currently controlled by applications of entomopathogenic nematodes

Mechanisms of EPNs

Entomopathogenic nematodes are deadly insect parasites that generate and discharge toxins into their host's body. Main venom proteins include both immune-modulating and tissue-damaging proteins, that these parasites have both a general and a specialized group of effectors, and a modified collection that is more unique to the hosts they invade (Chang et al., 2019). They are used as templates for host-parasite relationships such as host searching behaviour (Lewis et al., 2006), triggering of the parasite (Alonso et al., 2018),

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the function of secreted-products in parasitism (Lu et al., 2017), and ecology (Hodson et al., 2012). Numerous experiments revealed that juveniles of *S. feltiae* use their cuticle to block the immunity of the host (Brivio et al., 2004). Helminthes are commonly known as modulating the host's immune system and inducing pathology primarily by the secretion of small molecules and proteins that interfere with the host's cells (Brivio et al., 2004).

Secretion of lethal venom

Entomopathogenic nematodes have been widely believed to serve mostly as a vector for pathogenic-bacterial symbiosis. Besides, when these bacteria enter the host, they start to multiply and feed on the tissues of the host, which is responsible for the death of the host (Karthik et al., 2014). There is, however, an increasing body of investigation identifying nematode as a key asset to pathogenesis and in certain instances such as *S. scapterisci* play the role of the key agent of virulence (Lewis and Clarke, 2012). Apart from acting as a conduit for the bacteria they bring, it is evident that EPNs aid pathogenesis in two different ways such as; they actively destroy the tissue and impede the defense of the host, allowing more energy for themselves and the bacteria they contain to conquer and abolish the host. Previous investigates have revealed that axenic juveniles of *S. carpocapsae* can multiply within the host and destroy the host (Han et al., 2000; Sicard et al., 2003). Specific Steinernematidae effector molecules have been described and shown to participate in tissue damage and immune suppression of host (Toubarro et al., 2013a,b). Chang et al. (2019) revealed that both *S. carpocapsae* and *S. feltiae* possess a greater concentration of excreted/secreted proteins (ESPs) which are either Ig-like, Von Willebrand, or Ig (immunoglobulin) and FAR (fatty acid/retinol binding-protein). Excreted/secreted proteins (ESPs) are the primary link between hosts and parasites and therefore impact the protection of the parasites and their toxicity of the hosts (Cuesta-Astroz et al., 2017). However, FAR proteins were believed to influence immune signaling (Kennedy et al., 2013). These secretions of EPNs were found to be a complicated mixture comprising several proteins and together, it is poisonous to insects (Chang et al., 2019).

Nematode defenses and the immune system of insect

Nematode parasites and their related bacteria are ideal pathogenic agents for anti-bacterial and anti-nematode immune responses to insect host searches (Kenney and Eleftherianos, 2016). The nematode immune-modulation mechanism demonstrates the variety of modulatory strategies developed for suppression of host immune response by various types of parasitic nematodes (Cooper and Eleftherianos, 2016). In a study, Toubarro et al. (2013b) reported that *S. carpocapsae* displayed destructive approaches for host immunity through proteolytic secretion which inhibits host immunological defenses.

The nematodes and bacteria collaborate to suppress the immune response of the host, allowing vegetative replication of the bacteria (Dowds and Peters, 2002). *Xenorhabdus nematophila* and *S. carpocapsae* can inhibit the antibacterial peptide immune reaction of insects (Binda-Rossetti et al., 2016). The molecular studies of various insect hosts showed that both *Heterorhabditis* and *Steinernema* have a wide variety of results. Such as, *Manduca sexta* and *S. exigua*, impedes the transcription genes of insect those encoding for antimicrobial peptides (AMPs); in *X. nematophila* and *S. exigua*, cells were capable to impede the development of nodules by inhibiting the biosynthetic pathway of eicosanoid while in *P. luminescens* and *M. sexta*, cells released an anti-phagocytic factor that enabled the bacterial cells to disrupt their phagocytosis (Silva et al., 2002; Park et al., 2003, 2007).

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Both bacteria *Photorhabdus* and *Xenorhabdus* have also been displayed similar lifestyles but have different molecular defensive mechanisms (Goodrich-Blair and Clarke, 2007). The symbiotic *Xenorhabdus* bacterium inhibits the host's immune system by producing a variety of toxins and carrying type III effector molecules that may interfere with the actin cytoskeleton and prevent phagocytosis (Dillman et al., 2012a). *Photorhabdus* used lipopolysaccharide (LPS) modification to resist the action of the host-derived AMPs (Eleftherianos et al., 2006), while *Xenorhabdus* prevents induction of insect AMP expression altogether (Istkhar et al., 2019). In insects, the pathogenic impacts of bacteria and the anti-bacterial resistance mechanisms have been well described, however, nematode-associated defenses are nowadays primarily the focus of research. The EPN-pest interaction is illustrated in *Fig. 1*.

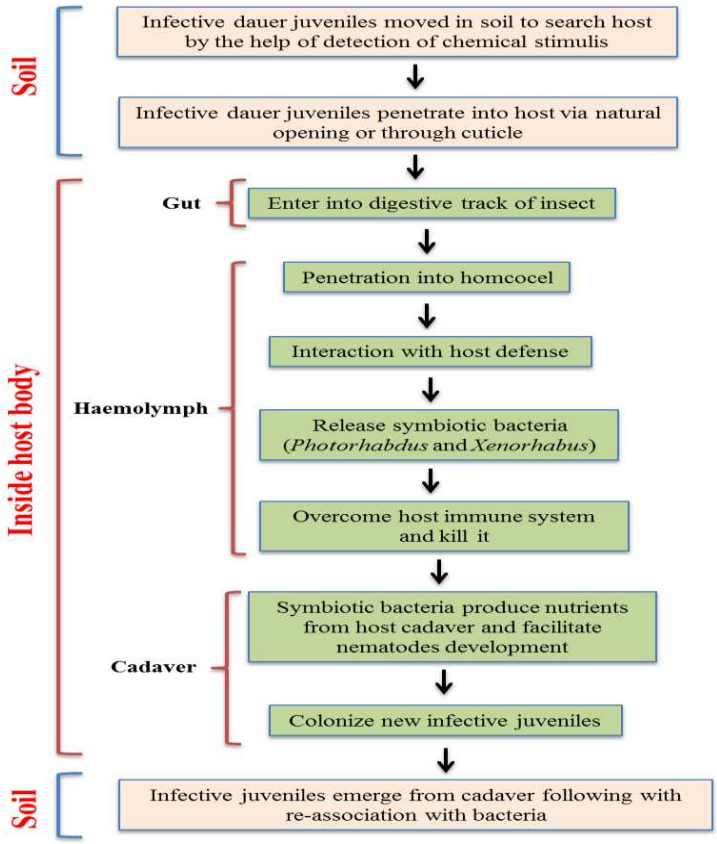

Figure 2. *The interaction between entomopathogenic nematodes and pests*

Commercially available entomopathogenic nematodes worldwide

In recent years, *Heterorhabditis and Steinernema* products have successfully been manufactured and commercialized by various companies around the world. The most popular species that have been successfully manufactured and utilized as commercial products are *H. bacteriophora*, *H. downesi*, *H. indica*, *H. megidis*, *S. carpocapsae*, *S. feltiae*, *S. kraussei*, *S. kushidai*, *S. riobrave* and *S. scapterisci* (*Table 2*). The EPN products are marketed under various brands, in developed and developing countries of the world, such as Austria, Australia, Belgium, Canada, Germany, India, Japan, Kenya, Lietuva, Netherlands, Newzealand, Poland, the United Kingdom and the United State of America.

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Table 2. *Commercial products of entomopathogenic nematodes (EPNs) prepared by different countries*

EPN	Commercial product	Manufacturer
H. bacteriophora	Larvanem	Koppert Biological System, Berkel en Rodenrijs, NL
	NemaShield-HB	BioWorks Inc. Victor, New York State, US
	Nematop	BioForce Limited, Drury, NZL
	NemaTrident-C	Bionema Limited, Swansea, UK
	Nema-green	e-nema, Schwentinental, Germany
	Otinem	Bioenterprises PTY Limited, Roseville, NSW
	Optinem-H	Agronovos, Ąžuolo g. 25, Alionių II k, Lietuva
	E-Nema Gmbh	e-nema, Schwentinental, Germany
H. downesi	NemaTrident-CT	Bionema Limited, Swansea, UK
H. indica	Calterm	Kisan Manch, IN
	Grub Terminator	Benzer Crop Science, Sirsi, IN
	GrubStake-Hi	Integrated Biocontrol System Inc., Greendale IN
	Soldier	Swami Samarth Agro Biotech LLP, Pune, IN
H. megidis	LarvaNema	Koppert B.V., Berkel en Rodenngs, NL
	Nemasys H	MicroBto, Cambndge, UK
S. carpocapsae	BioSafe	SDS Biotech, Minato-Ku, Tokyo, Japan
	Biosafe-N	Thermo Triology, Corp., Columbia, MD
	BioVector	Thermo Triology, Corp., Columbia, MD
	Bouncer	Swami Samarth Agro Biotech LLP, Pune, IN
	Boden-Niitzlinge	Rhone-Poulenc, Celaflor, Germany
	Capsanem	Koppert Biological System, Berkel en Rodenrijs, NL
	Carpocapsae-System	Biobest Sustainable Crop Management, Westerlo, BEL
	Exhibitline SC	Bioline AgroSciences Ltd. Camarillo, US
	Helix	Novartts, Misnssauga, Canada
	Optinem-C	Agronovos, Ąžuolo g. 25, Alionių II k, Lietuva
	Mioplant	Novartts, Vienna, Austria
	NemaGard	Purely Organic Products LLC, Portsmouth, US
	Nemastar	BioForce Limited, Drury, NZL
	NemaTrident-T	Bionema Limited, Swansea, UK
	Nemasys C	BASF Corporation, Ludwigshafen, Germany
	Palma-Life	Biobest Sustainable Crop Management, Westerlo, BEL
	Vector TL	Lesco, Lansing, MI
	X-GNAT	E C Geiger, Harleysvtlle, PA
S. feltiae	Entonem	Koppert Biological System, Berkel en Rodenrijs, NL
	Exhibit	Novartts, Basel, Switzerland
	Magnat	Amycel-Spawn Mate, Watsonvllle, CA
	Nemasys	MicroBto, Cambndge, UK
	NemaShield	BioWorks Inc. Victor, New York State, US
	NemaTrident-F	Bionema Limited, Swansea, UK
	Nemapom	e-nema, Schwentinental, Germany
	Nemaplus	BioForce Limited, Drury, NZL
	Nemaflor	e-nema, Schwentinental, Germany
	Nemasys F	BASF Corporation, Ludwigshafen, Germany
	Nematech-S SP	Dudutech, Naivasha, Kenya
	NemaTrident-S	Bionema Limited, Swansea, UK
	Nemax-F	Serbios, Badia Polesine, Italy
	Nemycel	e-nema, Schwentinental, Germany
	Optinem-F	Agronovos, Ąžuolo g. 25, Alionių II k, Lietuva
	Owinema SC	Owiplant Ltd. Horticultural Enterprise, Poznań, PL
	Stealth	Novartis, Macclesfield, Chester, UK
S. kraussei	Exhibitline Sk	Bioline AgroSciences Ltd. Camarillo, US
S. kushidai	SDS biotech	SDS Biotech K.K, Tsukuba, Japan
	Kraussei-System	Biobest Sustainable Crop Management, Westerlo, BEL
S. riobrave	Biovector	Thermo Triology, Corp., Columbia, MD
	Vector MC	Lesco, Lansing, MI
S. scapterisci	Proactant Ss	BtoControl, Gamesvtlle, FL

The representative trade names are those displayed on the respective company websites

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Conclusion

In the end, it is concluded that EPNs are suggested to be used as biopesticides due to huge host range, safety and compatibility within a variety of environmental conditions. Entomopathogenic nematodes are excellent biological control agents for soil-dwelling insects. Its mutual association with bacteria can easily kill insect pests so that they can be applied easily. Better understanding and development of EPNs are necessary for a suitable replacement of synthetic pesticides. More research is needed in China and all over the world to evaluate the biopesticides potential of EPNs as an effective tool for integrated pest management (IPM). Nowadays, the immune system of insects is being studied on a wide scale but their associations with EPNs are still less studied. The analysis of insect defenses and EPNs offenses would give a more precise description for effective control of insect pests as a future prospect. A better understanding of insect defense mechanisms against the EPNs and decreasing them by some adjuvants or nematode species with complex pathogenicity and powerful immune-suppressive abilities may be useful for potential pest management programs in the future.

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PHYTOCHEMICAL ANALYSIS AND ANTIFUNGAL ACTIVITY OF GYMNOSPERM AGAINST *FUSARIUM* WILT OF BANANA

AIN, Q. U.[1*] – ASAD, S.[2] – JAMAL, A.[2] – ARIF, M.[3] – MAHMOOD-UL-HASSAN, M.[4]

[1]*PARC Institute of Advanced Studies in Agriculture, National Agricultural Research Center, Islamabad, Pakistan*

[2]*Crop Diseases Research Institute, National Agricultural Research Centre, Islamabad, Pakistan*

[3]*Plant Pathology Department, The University of Agriculture, Peshawar, Pakistan*

[4]*Land Resources Research Institute, National Agricultural Research Centre, Islamabad, Pakistan*

[*]*Corresponding author*
e-mail: quratulainsatti@yahoo.com; phone: +92-344-810-7441

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Abstract. Fusarium vascular wilt caused by hyphomycete i.e. *Fusarium oxysporum* f. sp. *cubense* (Foc) exterminated the banana industry of Central America due to its dependence on monoculture. In this investigation, six gymnosperm plants i.e. *Cupressus sempervirens, Thuja orientalis, Cedrus deodara, Pinus wallachiana, Picea smithiana* and *Pinus roxburghii* were evaluated for their ability to control Foc; Tropical race 4 (TR4). Leaf and cone extracts (5-10%) of all six plants were screened using poisoned food technique. Extracts recording higher percent inhibition (≥60%) were further evaluated, an *in vitro* and a pot experiment. Highest percent inhibition in the *in vitro* assay and lowest disease severity index (DSI) in the pot experiment were recorded for ethanol leaf extract of *P. wallachiana* (Pw_{EL}), followed by *T. orientalis* ethanol leaf extract (To_{EL}). The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of both extracts was performed and the results indicated detection of seven compounds. Three of the compounds *viz* cis-9-Hexadecenal, 1-Naphthalenepentanoic acid and 1-Naphthalenecarboxylic acid were found in both extracts whereas Vitamin C, Hexatriacontyl trifluoroacetate, Oleic acid, delta-Cadinene were found in Pw_{EL} while Cedrol, Podocarp-7-en-3-one, 1-Phenanthrenecarboxylic acid and Pimaric acid were identified in To_{EL}. It is hypothesized that these phytocompounds might be the possible reason of Foc inhibition in current studies.

Keywords: *Fusarium oxysporum f. sp. cubense, poisoned food technique, disease severity index, Gas Chromatography-Mass Spectrometry, phytocompounds*

Introduction

Banana belongs to *Musa* genus and Asia is its center of origin (Ploetz et al., 2007). Trade quality banana are parthenocarpic and triploids that are vegetatively propagated (Simmonds, 1986). Even though hundreds of banana cultivars exist globally, majority of the production is based on few cultivars (Perrier et al., 2011). Panama epidemic devastated banana industry of Central America that was exclusively growing susceptible variety Gros Michel (Drenth and Guest, 2016). There was a major substitution of susceptible banana with resistant Cavendish cultivars (Ghag et al., 2015). Due to dissemination of diseased rhizomes and suckers intercontinental spread of the fusarium wilt occurred. Fusarium wilt is reported in most of the banana growing regions of the world e.g. Australia, Asia, Tropical Americas and Africa. The disease is induced by a hyphomycete named *Fusarium oxysporum* f. sp. *cubense* (Ploetz, 2000, 2006). External disease symptoms consist of leaf yellowing of older leaves, pseudostem splitting followed

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by leaf buckling and wilting (Yin et al., 2011). Internal symptoms consist of vascular discoloration blocking water conducting xylem vessels (Mackesy and Sullivan, 2015).

There are four recognized races of Foc separated on the basis of host susceptibility. Race 4 attacks Cavendish cultivars as well as those varieties that are attacked by race 1 and 2. Race 4 has two recognized strains i.e. TR4 (tropical race 4) and SR4 (subtropical race 4). The SR4 attacks banana that are growing suboptimally while TR4 attack Cavendish banana under any growing conditions (Ploetz, 2015; Drenth and Guest, 2016). Intercontinental spread of infected rhizome bits and suckers has led to the transmission of Foc TR4 from Taiwan to China, Indonesia, Philippines, Malaysia, Myanmar, Australia, Laos, India, Pakistan, Oman, Lebanon, Israel, Jordan and Mozambique. Dissemination of TR4 is much quicker (Drenth and Guest, 2016; Dita et al., 2018).

In Pakistan Fusarium wilt was reported in small farm, Thattha district in 2012-13 (Syed et al., 2015). Pakistan banana industry upto 95% of the area is based on Dwarf Cavendish (Basrai) cultivation. Presence of Foc TR4 is an alarming situation that if not controlled could lead to devastation as observed in past. Cultural practices i.e. flood-fallowing, suppressive soil, soil solarization and crop rotation are proposed (Stover, 1962) but have limited control (Gnanasekaran et al., 2015; Pegg et al., 2019). Development of resistant cultivars from diploids that are seed bearing is very expensive and time consuming (Ortiz and Swennen, 2014).

Different control options have been seeked in the recent decades for Foc management using chemicals, antagonistic microbes and botanicals against Foc. These management studies reported that control comes either by directly affecting the Foc morphology or by stimulating the host response. That is achieved from chemical compounds that might be the fungicide or the metabolites of plants and microbes. Many research investigations comprising on the efficacy of botanicals indicated that the secondary metabolites of higher plants possess such phytochemicals that had antimicrobial activity against phytopathogens (Doughari et al., 2009; Saravanakumar et al., 2015). Gymnosperms have never been tested against Foc although their antimicrobial activity is well known. This study addressed wilt problem using gymnosperm botanicals as control option for substituting conventional fungicide use. Six gymnosperm plants were selected including *Cupressus sempervirens*, *Cedrus deodara, Picea smithiana, Pinus wallachiana, Pinus roxburghii* and *Thuja orientalis*.

Materials and methods

Acquisition, revival and confirmation of fungal culture

Fusarium oxysporum f. sp. *cubense* (Foc; TR4) was graciously provided by the Tissue culture lab at National Agricultural Research Centre (NARC), Islamabad. Its molecular confirmation tests had already been performed by tissue culture department that isolated Foc from the diseased banana rhizomes collected from Thatta district of Sindh, Pakistan (Muhammad et al., 2017). The Foc culture was revived on Potato Dextrose Agar (PDA) and its morphological characters were analysed. Microscopic studies showed typical conidial morphology of Foc TR4. Microconidia are kidney shaped, hyaline, produced on false heads, mostly without septation. Macroconidia were sickle-shaped, hyaline, pointed at both ends, mostly 3-5 septate, borne on single phialides.

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Collection and drying of samples

Fresh leaves and cones of selected botanicals were collected from Murree (33°54′15″N 73°23′25″E with 2291 m altitude) and Swat (35°12′N 72°29′E with 980 m altitude) in the year 2014 and brought to fungal pathology lab at Crop Diseases Research Institute (CDRI), NARC, Islamabad, Pakistan. The samples were thoroughly washed and disinfected with 5% Clorox. Collected samples were kept under shade for one month. Dried samples (leaf and cone) were ground in an electric grinder and stored in labeled air tight containers till further use.

Extraction of samples

The n-hexane, ethanol and methanol were used as solvents for extraction. Two concentrations (5% and 10%) for each sample were prepared (Monteiro et al., 2013). Powdered samples were individually mixed with 100 mL solvent in flasks (Erlenmeyer) and shaken at 60 rpm (revolution per minute) for 48 hours. Extract in each flask was filtered and filtrate's solvent was removed by rotary evaporator (Sati and Joshi, 2010). These extracts were enclosed in labeled vials (Bajpai and Kang, 2010).

Evaluation of antifungal potential of the plant extracts

The evaluation of the antifungal potential of solvent extracts was done using food poisoning technique (Nene and Thapilyal, 2000; Monteiro et al., 2013). Each solvent extracts were amended with autoclaved PDA media and then poured into sterilized 90-mm petri plates. Disk of Foc (6 mm) was placed aseptically in the center of each poisoned petri plate. Plates having PDA with only 5% solvent in it, served as the control. Each treatment comprised of five replicates and plates were incubated (25±2 ˚C). Radial mycelia growth (RMG) of Foc was recorded from second day after inoculation till that day on which plates of control treatment were completely filled with mycelial growth of Foc. For each treatment, percent inhibition was calculated by using formula:

$$\text{Percent Inhibition} = \frac{\text{RMG of Control} - \text{RMG of Treatment}}{\text{RMG of Control} \times 100} \quad \text{(Eq.1)}$$

The in vitro assay and pot experiment of selected solvent extracts

Selected solvent extracts were further investigated in the *in vitro* assay using 50% concentration to determine the most effective botanical. Only 5% solvents amended with PDA, served as a solvent control. The PDA plates having only Foc disk, without any solvent and extract, served as positive control whereas the Propiconazole fungicide (Tilt) served as negative control (100 µg/mL). Assay was performed in the same manner as described earlier and percent inhibition of each treatment was calculated.

For pot experiment, four months old tissue cultured banana plants belonging to Dwarf Cavendish were grown in pots (20 cm x 25 cm) with peat moss and soil in 2:1 ratios were used as potting mix and freshly grown five day old Foc TR4 culture was used for inoculation. The inoculation of Foc was done using soil impregnation with spore suspension at 10^6 conc. (Huang et al., 2012). Four type of treatments were used in the pot assay *viz* positive control, solvent controls, negative control and solvent extracts

1) Simple control (positive control): Banana plants without any treatment.

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2) Solvent controls: n-hexane, ethanol and methanol (5% conc.) without any plant extract.
3) Fungicide treatment (negative control): Propiconazole (Tilt=100 µg/mL) was used as fungicide.
4) Botanical treatments: 50% conc. of selected solvent extract.

The banana plant roots were first dipped in the treatment (botanicals/ 5% respective solvents/ fungicide) for 35 minutes and were then sown in the potting mixture of the pots already impregnated with fungal spore suspension. Three replications for each treatment were used. Scale of external symptoms (Vicente et al., 2014) for Fusarium wilt was used for disease evaluation.

For each treatment, DSI was calculated (Huang et al., 2012) using formula:

$$\text{DSI} = \frac{\sum(\text{Class} \times \text{No.of plants in that class})}{\text{Total no.of assessed plants} \times 5} \times 100 \quad \text{(Eq.2)}$$

When the clear disease symptoms appeared on the banana plants after inoculation, visual wilt symptom assessment was done after every 2 months. Plant growth parameters i.e. leaf length, pseudostem length and leaf width were also recorded on termination of pot experiment.

Statistical analysis of experimental data

Experimental results were analyzed using the Statistix (ver. 8.1.) software. The ANOVA (analysis of variance) was performed for each experiment and the results were compared through Least Significant Difference (LSD) between means at $p<0.05$.

Gas Chromatography and Mass Spectrometry (GC-MS)

Botanicals were prepared with 100 mg/mL in 100% pure ethanol and 0.45 µm membrane filter was used to filter the botanicals. GC-MS analysis was done according to Karpagasundari and Kulothungan (2014) methodology with slight modifications. The GC-MS analysis of the most effective botanicals was executed using Shimadzu (GCMS-QP2010 Ultra) comprising an auto sampler (AOC-20i) and gas chromatograph that is interfaced to mass spectrometer (MS) instrument employed with as following specifications:

Column	DB 5 Ms (30 m × 0.25 mm × 0.25 µm)
Injection volume	3 µL (10:1split ratio)
Ion-source temperature	200ºC
Carrier gas	Helium (99.99%) at the constant flow of 1.73 mL/minute
Injector temperature	260ºC
Oven temperature	40°C (constant temperature for two minutes), with an increase of 8°C/minutes, to 150°C (constant temperature for two minutes), then 8°C/minutes to 250°C (constant temperature for two minutes), ending with a 20 minutes isothermal at 280°C
GC running time	56 minutes
Mass spectra	Taken at 70 eV, fragments from 10-1000 Da and a scan interval of 0.5 seconds

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TurboMass (Ver 5.2.0) Software was adopted to handle chromatograms and mass spectra. Database of NIST (National Institute Standard and Technology) was used to conduct interpretation on GCMS and relative peak area percentages of each component were calculated by comparing its average peak area to total areas.

Results

Screening of gymnosperm solvent extracts

Twelve gymnosperm solvent extracts recorded percent inhibition that was approximately ≥60% including 6 n-hexane extracts, 4 ethanol extracts and 2 methanol extracts (*Table 1*). Statistical analysis of solvent extract at 5 and 10% concentrations presented differences in percent inhibition values among treatments ($p < 0.05$, *Table S1*). Most of the plant solvent extracts significantly inhibited mycelial growth of Foc compared to the control treatments ($p < 0.05$, *Table S2*). The *P. wallachiana* methanol leaf extract (66.67%) and *P. wallachiana* n-hexane cone extract (66.66%) recorded highest inhibition against Foc. The n-hexane cone extracts from *Cedrus deodara* (66.07%), *P. smithiana* (66.07%), *P. roxburghii* (62.97%), *T. orientalis* (60.59%) also recorded higher inhibition against Foc. Moreover, methanol cone extract of *P. smithiana* (65.47%), ethanol leaf (62.38%) and cone (65.83%) extracts from *T. orientalis*, *P. wallachiana* ethanol leaf extract (60.12%) and *T. orientalis* n-hexane leaf extract (59.64%) also recorded higher percent inhibition values.

Table 1. *Percent inhibition of Foc mycelia growth recorded for 5% and 10% of gymnosperm extracts prepared in n-hexane, ethanol and methanol solvents (Data presented as Mean±SE)*

Treatments	n-Hexane (extracts)		Ethanol (extracts)		Methanol (extracts)	
	5%	10%	5%	10%	5%	10%
Control	0^{n}	0^{n}	0^{n}	0^{n}	0^{n}	0^{n}
***C. sempervirens* leaf**	27.498 ± 0.98^{def}	33.69 ± 1.07^{ab}	23.45 ± 1.10^{ghi}	39.40 ± 1.86^{WX}	33.81 ± 0.74^{a}	49.64 ± 1.44^{MNO}
***C. sempervirens* cone**	32.26 ± 2.14^{abc}	51.43 ± 0.61^{KLM}	28.93 ± 0.77^{cde}	43.09 ± 2.19^{STUV}	41.19 ± 0.58^{UVWX}	54.17 ± 0.90^{IJK}
***T. orientalis* leaf**	46.90 ± 0.76^{NOPQR}	59.64 ± 0.87^{DEFG}	47.74 ± 0.35^{NOPQ}	62.38 ± 1.27^{CD}	38.45 ± 0.794^{XY}	46.08 ± 2.14^{PQRS}
***T. orientalis* cone**	55.95 ± 1.67^{HIJ}	60.59 ± 1.79^{DE}	50.24 ± 0.97^{LMN}	65.83 ± 0.35^{AB}	44.17 ± 0.51^{RSTU}	56.07 ± 1.18^{HIJ}
***C. deodara* leaf**	12.498 ± 1.22^{m}	28.81 ± 0.55^{de}	22.50 ± 1.09^{hij}	38.21 ± 0.58^{XYZ}	30.35 ± 1.23^{bcd}	39.17 ± 1.61^{WX}
***C. deodara* cone**	58.81 ± 0.47^{EFGH}	66.07 ± 1.54^{AB}	48.81 ± 0.63^{MNOP}	60.47 ± 0.55^{DE}	46.31 ± 0.44^{OPQRS}	57.74 ± 0.65^{EFGH}
***P. wallachiana* leaf**	17.26 ± 0.75^{l}	25.83 ± 4.58^{efgh}	49.17 ± 0.44^{MNOP}	60.12 ± 0.73^{DEF}	53.34 ± 0.31^{JKL}	66.67 ± 0.53^{A}
***P. wallachiana* cone**	56.31 ± 1.17^{GHIJ}	66.66 ± 1.25^{A}	18.81 ± 1.49^{kl}	34.76 ± 1.56^{a}	33.93 ± 0.33^{a}	44.64 ± 0.86^{QRST}
***P. smithiana* leaf**	26.19 ± 1.42^{efg}	34.40 ± 0.87^{a}	20.95 ± 0.98^{ijk}	35.24 ± 1.10^{YZa}	24.28 ± 2.192^{fghi}	30.35 ± 1.38^{bcd}
***P. smithiana* cone**	56.55 ± 0.94^{GHIJ}	66.07 ± 2.32^{AB}	42.02 ± 0.97^{TUVW}	57.74 ± 1.11^{EFGH}	51.78 ± 0.42^{KLM}	65.47 ± 0.37^{ABC}
***P. roxburghii* leaf**	42.26 ± 0.997^{TUVW}	51.78 ± 1.06^{KLM}	26.19 ± 0.90^{efg}	34.88 ± 0.81^{Za}	19.76 ± 0.87^{jkl}	40.47 ± 0.33^{VWX}
***P. roxburghii* cone**	52.14 ± 1.80^{KLM}	62.97 ± 0.93^{BCD}	1.55 ± 1.12^{n}	17.02 ± 1.36^{l}	46.31 ± 0.87^{OPQRS}	56.90 ± 1.04^{FGHI}

Mean values of treatments indicated by capital letter superscripts are significantly different from those of small letter superscripts and higher mean values are indicated by capital letter superscripts i.e. A, B,….Z while lower mean value are indicated by small letters i.e. a, b,….z. Values having same capital letters or small letters superscript do no differ statistically and the common letters (either capital or small letters) sharing between the treatments indicate non-significant difference (LSD = 3.38)

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The in vitro assay and pot experiment of selected solvent extracts

a) In vitro assay

Selected solvent extracts recorded significant mycelial growth inhibition as compared to the positive control and solvent controls ($p < 0.05$, *Table S3*). The fungicide control (propiconazole) and ethanol extact of *P. wallachiana* leaf recorded comparable mycelial inhibition. The *P. wallachiana* ethanol leaf extract (Pw_{EL}=99.3%) followed by ethanol leaf extract of *T. orientalis* (To_{EL}=85.9%) were found best treatments against Foc mycelial growth using 50% conc. *P. wallachiana* methanol leaf extract (Pw_{ML}=82.8%), *P. smithiana* methanol cone extract (Ps_{MC}=77.4%), *P. wallachiana* n-hexane cone extract (Pw_{HC}=75.9%) and *T. orientalis* ethanol cone extract (To_{EC}=74.6%) also recorded significant inhibitory activity against Foc (*Table 2*). Only Pw_{EL} treatment was close to the inhibitory activity of propiconazole fungicide while rest of the treatments had lower inhibition values against Foc as compared to the fungicide treatment.

Table 2. *Percent inhibition of Foc mycelia growth using 50% concentrations of the selected solvent extracts and growth parameter values and severity scoring recorded at the end of greenhouse assay (Data presented as Mean±SE)*

Treatment	Percent Inhibition (LSD = 4.28)	Length of Pseudostem in cm (LSD = 3.6)	Leaf length in cm (LSD = 4.4)	Leaf width in cm (LSD = 1.9)	Severity scores (LSD = 0.5)
Positive control	0^{I}	27.69 ± 1.2^{I}	23.83 ± 1.4^{F}	9.91 ± 0.3^{F}	5 ± 0.0^{A}
Hexane control	0^{I}	27.18 ± 1.1^{I}	26.42 ± 1.1^{EF}	12.45 ± 0.5^{E}	5 ± 0.0^{A}
Ethanol control	0^{I}	29.21 ± 0.7^{HI}	27.69 ± 0.39^{DEF}	13.12 ± 0.1^{DE}	5 ± 0.0^{A}
Methanol control	0^{I}	29.72 ± 0.9^{GHI}	27.69 ± 0.9^{DEF}	12.78 ± 0.4^{DE}	5 ± 0.0^{A}
Fungicide control	100 ± 0.0^{A}	33.02 ± 0.7^{EFG}	35.14 ± 1.8^{AB}	16.00 ± 1.0^{BC}	3 ± 0.0^{EF}
Hexane cone extract of *P. wallachiana* (Pw_{HC})	75.9 ± 1.4^{C}	37.42 ± 0.6^{BCD}	31.33 ± 0.4^{BCD}	14.65 ± 0.4^{CD}	3 ± 0.0^{EF}
Methanol leaf extract of *P. wallachiana* (Pw_{ML})	82.8 ± 1.2^{B}	39.79 ± 0.4^{ABC}	34.04 ± 1.2^{ABC}	16.17 ± 0.4^{BC}	3 ± 0.0^{EF}
Hexane cone extract of *P. smithiana* (Ps_{HC})	55.9 ± 1.2^{H}	38.9 ± 1.1^{ABCD}	33.44 ± 1.8^{BC}	16.26 ± 0.8^{ABC}	3.67 ± 0.3^{CD}
Hexane cone extract of *C. Deodara* (Cd_{HC})	66.7 ± 0.7^{EF}	39.37 ± 0.0^{ABC}	33.44 ± 1.1^{BC}	15.66 ± 0.5^{BC}	3 ± 0.0^{EF}
Ethanol cone extract of *T. orientalis* (To_{EC})	74.6 ± 1.9^{CD}	38.35 ± 1.0^{ABCD}	32.60 ± 1.1^{BC}	16.34 ± 0.7^{ABC}	3 ± 0.0^{EF}
Methanol cone extract of *P. Smithiana* (Ps_{MC})	77.4 ± 0.9^{C}	31.37 ± 3.9^{GH}	29.63 ± 3.7^{CDE}	14.73 ± 1.6^{CD}	3 ± 0.0^{EF}
Hexane cone extract of *P. roxburghi* (Pr_{HC})	70.8 ± 0.8^{DE}	38.10 ± 0.0^{ABCD}	34.63 ± 1.4^{AB}	17.36 ± 0.5^{AB}	3.67 ± 0.3^{CD}
Ethanol leaf extract of *T. orientalis* (To_{EL})	85.9 ± 0.8^{B}	40.22 ± 0.8^{AB}	33.27 ± 0.7^{BC}	16.00 ± 0.4^{BC}	2.67 ± 0.3^{FG}
Hexane cone extract of *T. orientalis* (To_{HC})	63.2 ± 0.9^{FG}	$31.75\pm0.7F^{GH}$	31.33 ± 2.2^{BCD}	15.15 ± 0.4^{C}	4 ± 0.0^{BC}
Ethanol cone extract of *C. Deodara* (Cd_{EC})	62.9 ± 1.9^{FG}	35.31 ± 0.9^{DEF}	31.50 ± 1.7^{BCD}	15.15 ± 0.7^{C}	3.33 ± 0.3^{DE}
Ethanol leaf extract of *P. wallachiana* (Pw_{EL})	99.3 ± 0.5^{A}	41.66 ± 1.2^{A}	38.27 ± 0.7^{A}	18.20 ± 0.8^{A}	2.33 ± 0.3^{G}
Hexane leaf extract of *T. orientalis* (To_{HL})	59.9 ± 4.8^{GH}	36.41 ± 1.4^{CDE}	30.06 ± 1.1^{CDE}	15.15 ± 0.7^{C}	4.33 ± 0.3^{B}

Values with same superscript letters within an individual column do no differ statistically. Common letter sharing indicate non-significant difference between the treatments

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b) Pot experiment

Banana plants in control treatments recorded visual symptoms of disease earlier compared to the majority of the treatments of solvent extract. Except fungicide control, disease severity index (DSI) of the other controls were higher compared to extract treatments. At the end of experiment, all the controls excluding fungicide control had 100% DSI and highest (5) severity scores. Minimum DSI was recorded for ethanol leaf extract of *P. wallachiana* (Pw_{EL}) i.e. 46.6%, followed by ethanol leaf extract of *T. orientalis* (To_{EL}) i.e. 53.3% DSI (*Fig. 1*). Statistical analysis presented differences in the values of severity scores ($p < 0.05$, *Table S4*), pseudostem length ($p < 0.05$, *Table S5*), leaf width ($p < 0.05$, *Table S6*) and leaf length ($p < 0.05$, *Table S7*) among treatments. Lowest severity scoring (2.33) and highest pseudostem length (41.66 cm), leaf width (18.2 cm) and leaf length (38.27 cm) had also been recorded for Pw_{EL} (*Table 2*).

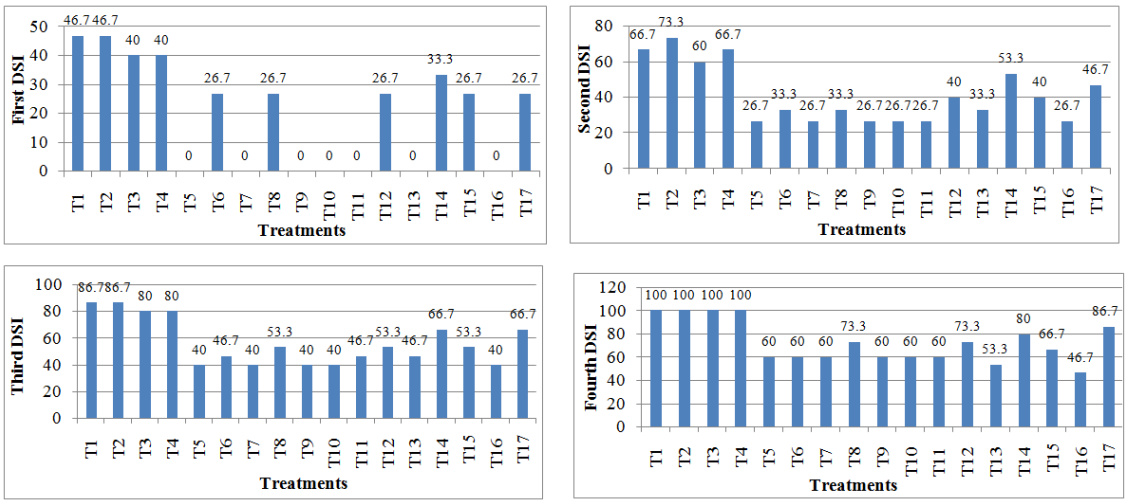

Figure 1. *Progression in the Disease severity index (DSI) of different root dipping treatments, including positive control (T_1); solvent controls (T_2-T_4); negative control (T_5) and solvent extract treatments (T_6-T_{17}), recorded during pot experiment. (T_1: Positive control, T_2: n-Hexane control, T_3: Ethanol control, T_4: Methanol control, T_5: Negative (Fungicide) control, T_6: n-Hexane cone extract of P. wallachiana, T_7: Methanol leaf extract of P. wallachiana, T_8: n-Hexane cone extract of P. smithiana, T_9: n-Hexane cone extract of C. deodara, T_{10}: Ethanol cone extract of T. orientalis, T_{11}: Methanol cone extract of P. smithiana, T_{12}: n-Hexane cone extract of P. roxburghi, T_{13}: Ethanol leaf extract of T. orientalis, T_{14}: n-Hexane cone extract of T. orientalis, T_{15}: Ethanol cone extract of C. deodara, T_{16}: Ethanol leaf extract of P. wallachiana, T_{17}: n-Hexane leaf extract of T. orientalis)*

GC-MS analysis for Pw_{EL} and To_{EL}

The total ion chromatogram (TIC) of Pw_{EL} detected seven compounds having retention time ranging between 19.1 to 41.6 minutes (*Fig. 2*). The delta-Cadinene, Vitamin C, cis-9-Hexadecenal, Oleic acid, 1-Naphthalenecarboxylic acid, 1- Naphthalenepentanoic acid and Hexatriacontyl trifluoroacetate were present in Pw_{EL}. The 1-Naphthalenecarboxylic acid, Vitamin C and Hexatriacontyl trifluoroacetate were detected with high peak area percent (*Table 3*). Total ion chromatogram of To_{EL} also detected seven compounds having retention time ranging between 21.06 to 36.2 minutes (*Fig. 2*). The Cedrol, cis-9-Hexadecenal, Podocarp-7-en, Pimaric acid,

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1-Phenanthrenecarboxylic acid, 1-Naphthalenepentanoic acid and 1-Naphthalenecarboxylic acid were detected in To_{EL}. The 1-Naphthalenecarboxylic acid (Hardwickiic acid), Cedrol and 1-Naphthalenepentanoic acid were found with high percentage of peak area (*Table 3*). The 1-Naphthalenepentanoic acid, 1-Naphthalenecarboxylic acid and cis-9-Hexadecenal were noticed in the two extracts (*Fig. 3*).

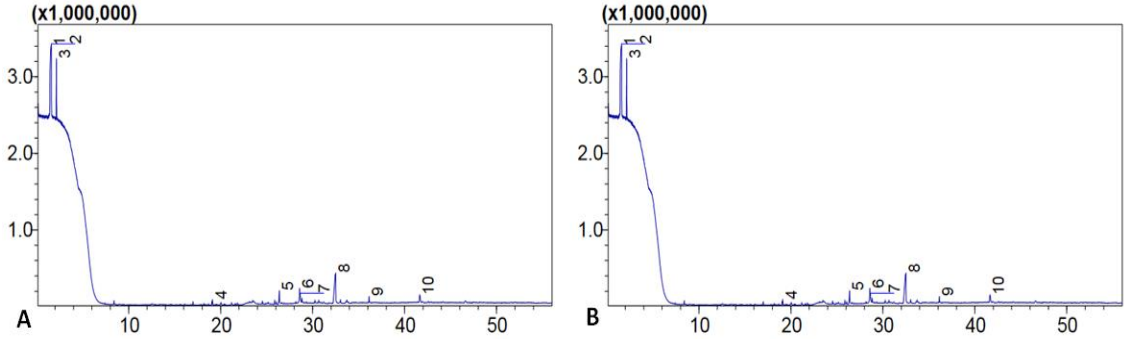

Figure 2. *Total ion chromatogram of Pw_{EL} (A) and To_{EL} (B) obtained through GCMS analysis*

Table 3. *Compounds detected in Pw_{EL} and To_{EL} with GC-MS analysis*

Peak	Compounds in Pw_{EL}				Compounds in To_{EL}			
	R_T	Area%	Name/ Formula/ Molecular weight	Chemical group	R_T	Area%	Name/ Formula/ Molecular weight	Chemical group
1	1.55	41.8	Ethanol/ C_2H_6O/ 46	Solvent	1.59	55.0	Ethanol/ C_2H_6O/ 46	Solvent
2	1.59	11.4	Ethanol/ C_2H_6O/ 46	Solvent	2.14	11.0	Chloroform/ $CHCl_3$/ 118	Solvent
3	2.14	10.3	Chloroform/ $CHCl_3$/ 118	Solvent	21.06	3.9	Cedrol/ $C_{15}H_{26}O$/ 222	Sesquiterpene alcohol
4	19.11	1.0	delta-Cadinene/ $C_{15}H_{24}$/ 204	Cadinene family of sesquiterpines	28.60	2.9	cis-9-Hexadecenal/ $C_{16}H_{30}O$/ 238	Palmitole aldehyde
5	26.39	3.1	Vitamin C / $C_{38}H_{68}O_8$/ 652	Ester	28.66	1.1	Podocarp-7-en-3-one, 13.beta.-methyl-1/ $C_{20}H_{30}O$/ 286	Diterpenoid/ meroterpene natural phenol
6	28.59	3.7	cis-9-Hexadecenal/ $C_{16}H_{30}O$/ 238	Palmitole Aldehyde	30.61	1.7	Podocarp-7-en-3-one, 13.beta.-methyl-1/ $C_{20}H_{30}O$/ 286	Diterpenoid/ meroterpene natural phenol
7	28.64	1.4	Oleic acid/ $C_{18}H_{34}O_2$/ 282	Monounsaturated (omega-9) fatty acid	31.26	2.3	Pimaric acid/ $C_{20}H_{30}O_2$/ 302	Carboxylic acid from the resin acid group
8	2.51	22.6	1-Naphthalenecarboxylic acid / C20H28O3/ 316	Clerodane diterpenoid	32.00	3.3	1-Phenanthrenecarboxylic acid / $C_{21}H_{32}O_2$/ 316	Methyl ester/ Neoabietic acid
9	36.14	1.5	1-Naphthalenepentanoic acid/ $C_{22}H_{36}O_4$/ 364	Methyl ester	32.47	8.9	1-Naphthalenecarboxylic acid /$C_{20}H_{28}O_3$/ 316	Clerodane diterpenoid
10	41.64	3.1	Hexatriacontyl trifluoroacetate/ $C_{38}H_{73}F_3O_2$/ 618	Alcohol acetate	36.21	9.8	1-Naphthalenepentanoic acid/ $C_{22}H_{36}O_4$/ 364	Methyl ester

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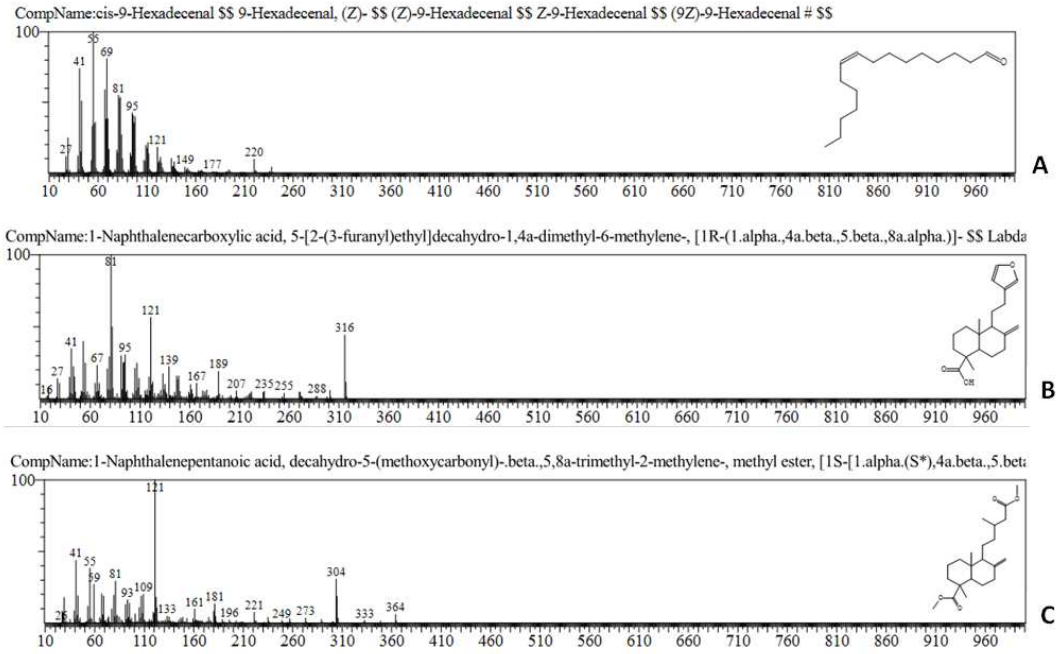

Figure 3. *Standard molecular spectra of cis-9-Hexadecenal (A), 1-Naphthalenecarboxylic acid (B) and 1-Naphthalenepentanoic acid (C) detected in Pw_{EL} and To_{EL}*

Discussion

Modern science is using huge array of plants because of their antimicrobial traits, which are attributed to compounds synthesized in the secondary metabolism of the plant (Nascimento, 2000). Terpenoids, tannins, ligans, steroids, alkaloids, phenols, glycosides, and sugar derivatives are noted as secondary metabolites in the gymnosperms (Harborne and Baxter, 2001). Plant secondary metabolites structure has been optimized during evolutionary process so that they can interfere with microbial molecular targets thus acting as a defense mechanism (Wink et al., 2012). Plant extracts disrupts the normal cell functioning of microbes, thus affecting important steps in the pathogenic process (Gupta and Birdi, 2017). Botanicals comprises such phytocompounds that cause morphological alterations in fungal hyphae which results to shriveling of hyphae, protoplast leakage and vacuolations coagulation (Soylu et al., 2006). The modes of action of phytoproducts on cells of fungi are thought to be membrane rupture in cytoplasm, granulation of cytoplasm, inhibition of extracellular and intracellular synthesis of enzymes (Cowan, 1999). Result of preliminary *in vitro* screening for gymnosperm solvent extracts recorded significant inhibition of Foc compared to the control treatments that clearly indicates antifungal efficacy of the tested gymnosperm plants. Joshi and Sati (2012) described antimicrobial efficacy of botanicals from many plant species belonging to various families of gymnosperms and current study is in accordance with their findings. Although, antimicrobial potential of gymnosperm botanicals has been reported against various tested pathogens but their antifungal efficacy against Foc is never been tested. This study first time reports the efficacy of solvent extracts of selected gymnosprem plants against Foc. It was noted that some extracts had similar inhibition activity with all three solvent types while some extracts recorded different inhibition potential using three solvents. This might be because of the variable content of phytochemicals in each plant extract and

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because of different extraction potentials of each solvent. Khoddami et al. (2013) described that type of plant/plant part and extraction solvent are reason for the variation of phytochemical formation in various extracts. Altemimi et al. (2017) noticed solvent effects on the phytochemical constituent profiles and antioxidant activities of different plant extracts.

Twelve solvent extracts selected through antifungal screening and further evaluated in the *in vitro* assay and green house assay, reported efficacy of Pw_{EL} comparable to fungicide (propiconazole) treatment. Highest mycelial inhibition against Foc indicated that Pw_{EL} was the most efficient solvent extract, suggesting presence of comparatively higher amount of effective phytochemicals in their composition, which effectively restricted and inhibited Foc mycelial growth. Sharma et al. (2018) recorded insecticidal and antimicrobial activities of *P. wallachiana* leaf extracts. Sharma et al. (2015) also noted antioxidant and antibacterial activities of alcoholic extracts of *P. wallachiana* leaf. Highest values in growth parameters and minimum DSI were recorded for Pw_{EL} followed by To_{EL} compared to the control treatments in green house assay. Huang et al. (2012) and Gopi and Thangavelu (2014) noted that botanical treatments effectively suppressed Fusarium wilt in green house experiments.

GCMS analysis of Pw_{EL} and To_{EL} detected seven compounds in each extract. Some reported compounds have well-known antimicrobial efficacies i.e. Oleic acid, 1-Naphthalenepentanoic acid, cis-9-Hexadecenal, delta-Cadinene, 1-Naphthalenecarboxylic acid (Hardwickiic acid), Cedrol and 1-Phenanthrene carboxylic acid. Dar et al. (2012) reported delta-Cadiene as one of the chemical constituent detected from the essential oil of *P. wallachiana* needle. Joshi et al. (2016) described thirty-eight compounds in the *P. wallachiana* methanolic leaf extracts including Vitamin C. Anburaj et al. (2016) reported Vitamin C as antioxidant, immunomodulator and anticancer. Jang et al. (2016) noted antimicrobial activity from *Eleutherococcus senticosus* essential oil containing delta-Cadinene as one of its compositional compounds. The cis-9-Hexadecenal has been reported for its antimicrobial activity (Mujeeb et al., 2014). Awa et al. (2012) noticed antibacterial activity of Oleic acid. McChesney et al. (1991) described antimicrobial potential of 1-Naphthalenecarboxylic acid (Hardwickiic acid) isolated from the *Croton sonderianus*. Similarly, Kuete et al. (2007) also revealed antimicrobial potential of compounds (including Hardwickiic acid) extracted from *Irvingia gabonensis* stem bark.

Khubeiz et al. (2016) and Moawad and Amin (2019) noted sesquiterpenoids and monoterpenes as chemical components from leaf essential oil of *T. orientalis* containing Cedrol as one of the major component recording significant antibacterial activity. Madhumitha et al. (2012) described insecticidal efficacy of aqueous extract fruit peel of *Annona squamosa* against parasites (blood feeding). Its constitutional chemical compounds included Podocarp-7-en. Naikwadi et al. (2017) revealed antibacterial potential of *Vetiveria zizanioides* root extracts containing 1-Phenanthrene carboxylic acid, Phenanthrene carboxylic acid and Naphthalene pentanoic acid as major bioactive constituents. Manimegalai et al. (2011) isolated compounds from *T. orientalis* bark that were antibacterial and separation by TLC and GCMS disclosed presence of Phenanthrene carboxylic acid in it. Ali et al. (2011) reported anti-inflammatory and antibacterial potency of Pimaric acid.

Compounds that were detected through GCMS in Pw_{EL} and To_{EL} might be the probable phytoconstituents for Foc control in the *in vitro* and pot assays. These phytochemicals either through stimulating host's defence response by activating PR-proteins or directly

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toxifying Foc, inhibited mycelial amelioration in the pot and *in vitro* experiments respectively. Zhang et al. (2013) described that volatiles derived from plant source displayed higher inhibition potential on Foc and the compound i.e. 2-methyl-2 pentenal, completely inhibited mycelial growth of Foc. Gopi and Thangavelu (2014) reported lipid compound in botanical treatment as effective constituent that significantly suppressed Panama disease under greenhouse assay. Guimaraes et al. (2011) and Siripornvisal (2010) also noted antifungal potential of volatile compounds against Foc inhibiting spore germination and mycelial growth. Three compounds i.e. 1-Naphthalenepentanoic acid, 1-Naphthalenecarboxylic acid and cis-9-Hexadecenal, were revealed in both extracts that might be the potential active components of Pw_{EL} and To_{EL}. According to our knowledge, cis-9-Hexadecenal, 1-Naphthalenecarboxylic acid (Hardwickiic acid), Oleic acid, Hexatriacontyl trifluoroacetate and 1-Naphthalenepentanoic acid are noticed in Pw_{EL}, for the first time, through current study. Moreover, Podocarp-7-en, cis-9-Hexadecenal, 1-Phenanthrenecarboxylic acid, Pimaric acid, 1-Naphthalenepentanoic acid and 1-Naphthalenecarboxylic acid are also reported first time in To_{EL} as most of the phytochemical studies on *Thuja orientalis* are focused on its essential oil which only reports cedrol presence.

Conclusion

A baseline study that for the first time addresses Fusarium wilt dilemma using botanicals and reports antifungal efficacy of selected gymnosperms against Foc. Even though variability was found in the inhibitory activity of different gymnosperm solvent extracts, However, few solvent extracts recorded good efficacy against Foc both in the *in vitro* and pot experiments. The ethanolic leaf extract of *P. wallachiana* (Pw_{EL}) and the ethanolic leaf extract of *T. orientalis* (To_{EL}) were evidenced through GCMS to have bountiful sources of such valuable phytochemicals that can replace conventional chemicals and fungicides that are used for the management of Panama wilt disease. It is therefore, strongly recommended that the efficient gymnosperm extract should be further explored through spectrophotometric and chromatographic analysis. Moreover, compound detected through GCMS should be individually evaluated against Foc using *in vitro* and green house experiments.

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APPENDIX

Supplementary Information

Table S1. *Analysis of variance (factorial 3-way) applied for percent inhibition of Foc mycelia growth recorded for 5% and 10% of gymnosperm extracts prepared in n-hexane, ethanol and methanol solvents ($p < 0.05$)*

Source	DF	SS	MS	F	P
Replicate	4	10	2.4		
Treatment	12	84953	7079.5	956.93	0.0000
Solvent	2	3019	1509.4	204.03	0.0000
Conc	1	12178	12178.4	1646.15	0.0000
Treatment*Solvent	24	32780	1365.8	184.62	0.0000
Treatment*Conc	12	1235	102.9	13.91	0.0000
Solvent*Conc	2	200	99.8	13.48	0.0000
Treatment*Solvent*Conc	24	831	34.6	4.68	0.0000
Error	308	2279	7.4		
Total	389	137485			

Grand Mean 39.656, CV 6.86

Table S2. *Pairwise comparisons test (Treatment*Solvent*Concentration) of percent inhibition of Foc mycelia growth recorded for 5% and 10% of gymnosperm extracts prepared in n-hexane, ethanol and methanol solvents*

Treatment	Solvent	Conc	Mean	Homogeneous Groups
8	3	2	66.666	A
9	1	2	66.664	A
7	1	2	66.074	AB
11	1	2	66.070	AB
5	2	2	65.832	AB
11	3	2	65.474	ABC
13	1	2	62.974	BCD
4	2	2	62.384	CD
5	1	2	60.592	DE
7	2	2	60.474	DE
8	2	2	60.116	DEF
4	1	2	59.642	DEFG
7	1	1	58.808	EFGH
11	2	2	57.738	EFGH
7	3	2	57.736	EFGH
13	3	2	56.902	FGHI
11	1	1	56.546	GHIJ
9	1	1	56.306	GHIJ

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Treatment	Solvent	Conc	Mean	Homogeneous Groups
5	3	2	56.068	HIJ
5	1	1	55.952	HIJ
3	3	2	54.172	IJK
8	3	1	53.338	JKL
13	1	1	52.138	KLM
11	3	1	51.784	KLM
12	1	2	51.780	KLM
3	1	2	51.428	KLM
5	2	1	50.238	LMN
2	3	2	49.644	MNO
8	2	1	49.166	MNOP
7	2	1	48.810	MNOP
4	2	1	47.736	NOPQ
4	1	1	46.902	NOPQR
7	3	1	46.308	OPQRS
13	3	1	46.308	OPQRS
4	3	2	46.078	PQRS
9	3	2	44.642	QRST
5	3	1	44.166	RSTU
3	2	2	43.094	STUV
12	1	1	42.260	TUVW
11	2	1	42.020	TUVW
3	3	1	41.186	UVWX
12	3	2	40.474	VWX
2	2	2	39.404	WX
6	3	2	39.168	WX
4	3	1	38.448	XY
6	2	2	38.212	XYZ
10	2	2	35.240	YZa
12	2	2	34.880	Za
9	2	2	34.764	a
10	1	2	34.404	a
9	3	1	33.928	a
2	3	1	33.808	a
2	1	2	33.692	ab
3	1	1	32.260	abc
6	3	1	30.354	bcd
10	3	2	30.354	bcd
3	2	1	28.926	cde
6	1	2	28.810	de
2	1	1	27.498	def
10	1	1	26.188	efg
12	2	1	26.188	efg
8	1	2	25.834	efgh
10	3	1	24.284	fghi
2	2	1	23.452	ghi
6	2	1	22.498	hij
10	2	1	20.952	ijk
12	3	1	19.762	jkl
9	2	1	18.808	kl
8	1	1	17.260	l
13	2	2	17.022	l
6	1	1	12.498	m
13	2	1	1.5480	n
1	1	1	0.0000	n
1	1	2	0.0000	n
1	2	1	0.0000	n
1	2	2	0.0000	n
1	3	1	0.0000	n
1	3	2	0.0000	n

Alpha 0.05 Standard Error for Comparison 1.7202, Critical T Value 1.968, Critical Value for Comparison 3.3849, Error term used: Replicate*TREATMENT*Solvent*Conc., 308 DF, there are 40 groups (A, B, etc.) in which the means are not significantly different from one another

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Table S3. *Completely randomized analysis of variance for percent inhibition of Foc mycelia growth using 50% concentrations of the selected solvent extracts ($p < 0.05$)*

Source	DF	SS	MS	F	P
Treatment	16	97956.4	6122.28	532	0.0000
Error	68	781.9	11.50		
Total	84	98738.4			

Grand Mean 57.387, CV 5.91

Table S4. *Completely randomized analysis of variance for severity scoring of banana plants treated with different solvent extracts in green house experiment ($p < 0.05$)*

Source	DF	SS	MS	F	P
Treatment	16	39.6471	2.47794	21.1	0.0000
Error	34	4.0000	0.11765		
Total	50	43.6471			

Grand Mean 3.6471, CV 9.40

Table S5. *Completely randomized analysis of variance for measurement of banana pseudostem length measured in different treatments during greenhouse experiment ($p < 0.05$)*

Source	DF	SS	MS	F	P
Treatment	16	1085.16	67.8225	13.	0.0000
Error	34	167.47	4.9257		
Total	50	1252.63			

Grand Mean 35.030, CV 6.34

Table S6. *Completely randomized analysis of variance for leaf width of banana plants measured in different treatments during greenhouse experiment ($p < 0.05$)*

Source	DF	SS	MS	F	P
Treatment	16	192.539	12.0337	8.65	0.0000
Error	34	47.312	1.3915		
Total	50	239.850			

Grand Mean 15.006, CV 7.86

Table S7. *Completely randomized analysis of variance for leaf length of banana plants measured in different treatments during greenhouse experiment ($p < 0.05$)*

Source	DF	SS	MS	F	P
Treatment	16	619.498	38.7186	5.35	0.0000
Error	34	246.141	7.2394		
Total	50	865.639			

Grand Mean 31.429, CV 8.56

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OPTIMAL PLANTING DENSITY FOR ACHIEVEING HIGHER YIELD AND FIBER QUALITY OF TWO TYPES OF COTTON (*GOSSYPIUM HIRSUTUM* L.)

YE, S.[1] – JIANG, C.[2] – LIU, F.[1] – TIAN, C.[1] – LI, C.[1] – HAN, W.[1] – WU, D.[3*]

[1]*Cotton Research Institute, Anhui Academy of Agricultural Sciences, Hefei 230001, China*

[2]*Tobacco Research Institute, Anhui Academy of Agricultural Sciences, Hefei 230001, China*

[3]*College of Agronomy, Anhui Agriculture University, Hefei 230036, China*

**Corresponding author*
e-mail: deixiangwu198@163.com; phone/fax: +86-551-6282-7187

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Abstract. Planting density is important for a high yield and quality of cotton, but still little is known about its effects on seed yield and fiber quality in Anhui province, China. A field experiment was carried out with two varieties (Wankemian-1 and Wanmian-191), and five planting densities (D1, D2, D3, D4, D5). Results showed that the boll number per plant and boll weight decreased significantly with increasing planting density. The highest yields of Wanmian-191 and Wankemian-1 were 3591.0 and 3504.5 kg ha^{-1} in D2 and D4, respectively. It was estimated that the planting densities in Wanmian-191 and Wankemian-1 for the maximum seed yield were 79000 and 115000 plants ha^{-1}, and the corresponding yields were 3610.3 and 3513.2 kg ha^{-1}, respectively. Suitable planting density increased the seed yield mainly by regulating the boll number per unit area and boll weight. Although the fiber quality was better for Wanmian-191 and Wankemian-1 under the planting density of 90000 and 79000 plants ha^{-1}, in order to obtain high seed yield, quality and economic benefits, the optimal planting densities are 79000 and 115000 plants ha^{-1}, respectively. There results will provide guideline for planting density to maximize yield and quality of cotton production in Anhui, China.
Keywords: *cotton variety, fruit branch, fiber quality, lint yield, micronaire value*

Introduction

Cotton (*Gossypium hirsutum* L.) is an important cash crop all over the world (Constable and Bange, 2015). Planting density is an important controllable factor in cotton production and has been paid more attention recently (Li et al., 2015; Zhi et al., 2015, 2016; Khan et al., 2017). There were many studies on the effect of density on cotton yield. Within a certain density range, plant height of cotton was increased with the increase of density (Dai et al., 2014). However, under a high density, the number of fruit branches, boll quality and lint percentage per plant were decreased, and the number of buds and bolls per plant were also decreased, and thus caused a decrease in the economic yield of cotton (Lou et al., 2010; Wang et al., 2012; Li et al., 2015). An appropriate planting density can offer a better ecological environment for the plant growth and development of cotton (Yang et al., 2014), to coordinate the contradiction between crop groups and individuals, and also to guarantee a certain number of groups. Therefore, the total number of the cotton plant, the boll number per plant, boll quality can be coordinated to produce higher seed cotton yield (Dong et al., 2012; Li et al., 2015). However, the suitable planting density was always different for different cotton varieties under different ecological conditions, so the optimum planting density must be determined by field experiments for each variety.

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The suitable planting density of cotton is mainly determined by the planting area, variety, pruning mode and soil fertility level (Chen et al., 2014; Li et al., 2015; Qi et al., 2020). The fruit branch types of cotton are mainly divided into finite fruit branch (compact) and infinite fruit branch (loose), and the degree of compact or loose of the plant has a decisive effect on the suitable planting density (Chen et al., 2014; Qi et al., 2020). Cotton varieties with limited fruit branches have reasonable plant type, good ventilation and light transmittance in the field, which can make full use of light energy, and are suitable for planting with high fertilizer and high density, which is conducive to improving the seed cotton yield (Chen et al., 2014). Generally, different planting areas also have large differences in planting densities, due to the large differences in cultivation techniques and soil fertility. For example, a high planting density was suitable for achieving a high yield in Xinjiang cotton region (Zhao et al., 2003; Zhu et al., 2020), and a reasonable dense planting was beneficial to increase yield in the Yellow River Basin (Zhou et al., 2018), however, a proper sparse planting was always used for obtaining higher weed cotton yield in the Yangtze River Basin in China (Wang et al., 2006).

Anhui is one of the major cotton planting provinces in China, with an annual cotton planting area of 350000 hm^2 (Zang et al., 2019). It is great important to study the optimal planting density of two cotton plant types (finite and infinite fruit branches) to increase the seed cotton yield and economic benefits in Anhui province. Therefore, this study explored the role of planting density on the plant growth characteristics, seed cotton yield and fiber quality of two cotton varieties (finite and infinite fruit branches), and achieved the suitable planting density for the two cotton varieties. There results will provide management guidelines to cotton growers for obtaining a high yield and economic benefits.

Materials and methods

Experiment site and growth conditions

The field experiment (*Fig. 1*) was conducted in 2016 on the experimental farm of the Cotton Research Institute, Anhui Academy of Agricultural Sciences, Anqing, Anhui, China (30°31' N, 117°06' E). The field has a sandy loam soil with a pH of 8.0, 11.9 g kg^{-1} organic matter, 57.5 mg kg^{-1} alkaline nitrogen (N), 34.3 mg kg^{-1} available phosphorus (P) and 102.4 mg kg^{-1} available potassium (K). The average temperatures from May to October was 22.3 °C, and the total rainfall was 1896 mm in 2016.

Experimental design and management

The experiment included two cotton varieties (Wankemian-1 and Wanmian-191, a finite and infinite fruit branch variety, respectively) (*Fig. 2*), and five planting densities [67500 (D1), 82500 (D2), 97500 (D3), 112500 (D4), 127500 (D5) plants ha^{-1}]. According to the fruit branch characteristics and the field growth of the varieties, the infinite fruit branch variety has more branches and bigger plant types, therefore, its planting density was relatively low, while the planting density of finite fruit branch variety was relatively higher. Therefore, D1, D2, D3, D4 were used for Wanmian-191, and D2, D3, D4, D5 for Wankemian-1. Each plot was 20 m^2 (3.6 m × 5.6 m) and contained 6 rows. The row spacing was 60 cm for each treatment. Plant spacing of D1, D2, D3, D4 and D5 was 25, 20, 17, 15 and 13 cm, respectively. The experiment was carried out with a randomized complete block design with three replicates. Fertilizers, at

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the rate (kg ha^{-1}) of 180 N, 90 P_2O_5 and 180 K_2O with urea (46% N), compound fertilizer (17% N, 17% P_2O_5, 17% K_2O) and potassium chloride (60% K_2O), were applied at early flowering (65 days after emergence). Other field managements were conducted according to local agronomic practices.

Figure 1. *The field experiment*

Figure 2. *The two cotton varieties (left: Wanmian-191, right: Wankemian-1)*

Sample collection and determination

Ten continue plants in the third row of each plot were selected to investigate the date of seedling emergence, squaring, first bloom and the opening (half of the plants were at

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first bloom) of the cotton plants. At peak boll stage (85 days after emergence), ten plants per plot were randomly selected to measure plant height, the first fruit node, and the number of fruit branch. Plants of each plot were manually harvested three times from September to October.

Seed cotton yield and lint yield were recorded three times from the manually harvested plants in each subplot. During each harvest, 50 bolls were randomly sampled per plot to determine boll weight and lint percentage. Number of bolls per plant and boll density were determined based on the three harvests (Zhi et al., 2016).

Fiber quality depends on fiber properties such as average fiber length, fiber strength, fiber uniformity, micronaire value (Bradow et al., 1997). Fiber quality parameters, namely upper half fiber length, fiber strength, fiber uniformity, fiber micronaire value and spinning consistence index, were analyzed according to the methods of Zhi et al. (2016) and Zhang et al. (2006).

Statistical analysis

Data were processed in Microsoft Excel 2013 and analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Means of the treatments were compared by the method of least significance difference at $P < 0.05$.

Results

Cotton plant growth stages and periods

As shown in *Table 1,* the planting density did not significantly affect the emergence time of the cotton. However, both the squaring time and first bloom time of the cotton were increased with the increasing in plant density. The first bloom time was significant early in the low planting density (D1 and D2) than in the highest planting density (D4 and D5). However, the opening time was longer in D1 than other treatments for Wanmian-191, and longer in D2 than D5 for Wankemian-1. The growth period was decreased with the increasing in plant density.

Table 1. *Effects of plant density on plant growth stages and periods of two cotton varieties*

Variety	Density	Growth stage (month-day)				Growth period (d)
		Emergence	Squaring	First bloom	Opening	
Wanmian-191	D1	05-28	07-05	08-01	09-16	111.7 a
	D2	05-28	07-06	08-01	09-14	110.0 b
	D3	05-28	07-07	08-02	09-14	109.7 b
	D4	05-28	07-08	08-04	09-14	110.0 b
Wankemian-1	D2	05-28	07-03	07-29	09-14	110.0 a
	D3	05-28	07-04	07-30	09-13	109.0 ab
	D4	05-28	07-04	08-01	09-13	109.3 a
	D5	05-28	07-05	08-02	09-12	108.0 b
Wanmian-191						110.3 a
Wankemian-1						109.1 b

Means within a column of the same cotton variety that have different letters are significantly different from each other at $P < 0.05$. The D1, D2, D3, D4 and D5 were respectively plant densities of 67500, 82500, 97500, 112500, 127500 plants ha^{-1}

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Cotton plant growth characteristics

As showed in *Table 2,* the first fruit node of the two cotton varieties decreased significantly with increasing plant density. Similarly, the number of fruit branch decreased significantly with increasing plant density in both Wanmian-191 and Wankemian-1. The plant height was highest at D3 of Wanmian-191, which was higher than that at the maximized density (D4). The plant height of Wankemian-1 decreased significantly with increasing plant density. The Wanmian-191 had higher first fruit node and plant height than the Wankemian-1, but lower number of fruit branch.

Table 2. *Effects of plant density on plant growth characteristics of two cotton varieties*

Variety	Density	First fruit node	Number of fruit branch	Plant height (cm)
Wanmian-191	D1	5.8 a	12.6 a	106.5 ab
	D2	5.7 ab	12.1 a	110.0 ab
	D3	5.7 ab	10.2 b	112.2 a
	D4	5.6 b	9.5 b	105.4 b
Wankemian-1	D2	5.3 a	14.2 a	105.5 a
	D3	5.2 ab	13.5 ab	102.2 ab
	D4	5.1 bc	12.8 b	100.3 ab
	D5	5.0 c	10.2 c	95.2 b
Wanmian-191		5.7 a	11.1 b	108.5 a
Wankemian-1		5.2 b	12.7 a	100.8 b

Means within a column of the same cotton variety that have different letters are significantly different from each other at $P < 0.05$. The D1, D2, D3, D4 and D5 are respectively plant densities of 67500, 82500, 97500, 112500, 127500 plants ha^{-1}

Yield and yield components

The effect of plant density on yield and yield components of the two cotton varieties were showed in *Table 3*. The boll number of per plant decreased significantly with increasing plant density, and the boll number in D4 was decreased by 36% compared with D1 in Wanmian-191. Similarly, the boll weight decreased with increasing plant density, the boll weight was maximized at the lowest density for both the Wankemian-1 and Wanmian-191. However, there was no significant differences in boll weight between D1 and D2 for Wanmian-191, and was also no significant differences for Wankemian-1 in the density of D1, D2 and D3. Conversely, the lint percentage increased as plant density increased in both Wanmian-191 and Wankemian-1. The seed cotton yield and lint yield were increased with increasing plant density and then decreased when plant density was over certain level. The highest seed cotton yield and lint yield of Wanmian-191 and Wankemian-1 was found in the density of D2 and D4, respectively. And no significant differences in the highest seed cotton yield between two cultivars were observed. The Wanmian-191 had higher boll number, boll density and lint percentage than the Wankemian-1. However, no significant differences in the boll weight, seed cotton yield and lint yield between the two cultivars were observed.

According to the relationship between yield (y) and density (x), a quadratic regression equation ($y = ax^3 + bx^2 + cx + d$) was established to describe the change of seed cotton yield with planting density (*Fig. 3*). As shown in *Table 4,* it was calculated that the planting density in Wanmian-191 and Wankemian-1 for the maximum seed

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cotton yield was 115000 and 79000 plants ha^{-1}, and the corresponding seed cotton yields were 3610.3 and 3513.2 kg ha^{-1}, respectively.

Table 3. *Effects of plant density on yield and yield components of two cotton varieties*

Variety	Density	Boll number (boll plant^{-1})	Boll density (boll m^{-2})	Boll weight (g boll^{-1})	Lint percentage (%)	Seed cotton yield (kg·ha^{-1})	Lint yield (kg·ha^{-1})
Wanmian-191	D1	15.6 a	105.3 b	5.2 a	40.1 c	3204.7 b	1285.1 b
	D2	14.2 b	117.2 ab	5.0 a	40.5 bc	3591.0 a	1454.4 a
	D3	12.1 c	118.0 a	4.6 b	40.8 ab	3170.2 b	1293.4 b
	D4	10.2 d	114.8 ab	4.4 b	41.0 a	3009.8 b	1234.0 b
Wankemian-1	D2	11.2 a	92.4 b	5.2 a	38.9 c	2889.1 c	1123.9 c
	D3	10.1 b	98.5 ab	5.1 a	39.2 bc	3178.8 b	1246.1 b
	D4	9.6 b	108.0 a	5.0 ab	39.8 ab	3504.5 a	1394.8 a
	D5	8.5 c	108.4 a	4.6 b	40.2 a	3146.7 b	1265.0 b
Wanmian-191		13.0 a	113.8 a	4.8 a	40.6 a	3243.8 a	1316.7 a
Wankemian-1		9.9 b	101.8 b	5.0 a	39.5 b	3179.6 a	1257.3 a

Means within a column of the same cotton variety that have different letters are significantly different from each other at $P < 0.05$. The D1, D2, D3, D4 and D5 were respectively plant densities of 67500, 82500, 97500, 112500, 127500 plants ha^{-1}

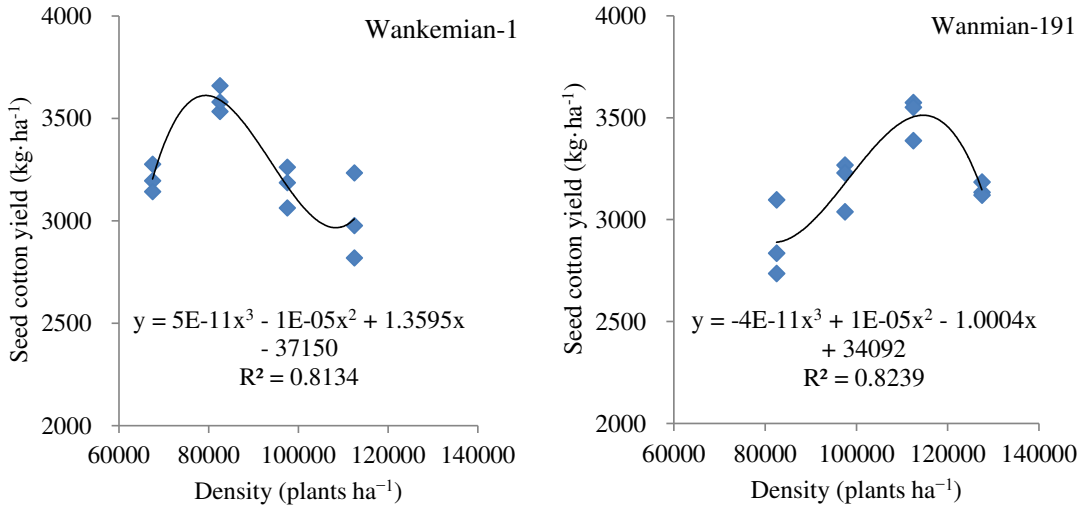

Figure 3. *Effect of planting density on seed cotton yield of two cotton varieties. Data were the means of twelve replicates*

Table 4. *Planting density for maximum seed cotton yield of two cotton varieties*

Variety	Plant density of maximum yield (plants ha^{-1})	Maximum seed cotton yield (kg·ha^{-1})
Wanmian-191	79000	3610.3
Wankemian-1	115000	3513.2

Fiber quality parameters

The effects of plant density on fiber quality parameters of two cotton varieties were showed in *Table 5*. The upper half fiber length of the Wankemian-1 and Wanmian-191 under different densities was between 29.6 and 31.7 mm, and no significant differences

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in the upper half fiber length were observed in Wankemian-1 under different planting densities. The fiber strength of the Wankemian-1 and Wanmian-191 was between 29.2 and 32.9 $cN \cdot tex^{-1}$, and the fiber strength was the highest in D2 for Wankemian-1, and in D3 for Wanmian-191. The micronaire value and uniformity index increased with increasing density in Wanmian-191, while the micronaire value was decreased with increasing density in Wankemian-1. The upper half fiber length, fiber strength and spinning consistence index were higher in Wankemian-1 than that in Wanmian-191. No significant differences in the micronaire value and uniformity index between the two cultivars were found. In all, most of the fiber quality parameters were higher in D3 and D1 for Wanmian-191 and Wankemian-1, respectively.

Table 5. *Effects of plant density on fiber quality parameters of two cotton varieties*

Variety	Density	Upper half fiber length (mm)	Fiber strength ($cN \cdot tex^{-1}$)	Micronaire value	Uniformity index (%)	Spinning consistence index (SCI)
Wanmian-191	D1	30.5 a	30.8 a	5.05 ab	84.9 c	135.0 b
	D2	29.6 b	29.5 b	4.83 b	85.8 b	136.0 b
	D3	30.7 a	30.9 a	5.20 ab	86.7 a	141.0 a
	D4	30.7 a	29.2 b	5.38 a	86.7 a	136.0 b
Wankemian-1	D2	31.5 a	32.9 a	5.30 a	86.4 ab	147.0 a
	D3	31.5 a	30.9 b	4.90 b	86.6 ab	147.0 a
	D4	31.7 a	29.9 b	4.82 b	86.2 b	145.0 a
	D5	30.9 a	30.8 b	4.86 b	87.0 a	148.0 a
Wanmian-191		30.4 b	30.1 b	5.12 a	86.0 a	137.0 b
Wankemian-1		31.4 a	31.1 a	4.97 a	86.6 a	146.8 a

Means within a column of the same cotton variety that have different letters are significantly different from each other at $P < 0.05$. The D1, D2, D3, D4 and D5 were plant densities of 67500, 82500, 97500, 112500, 127500 plants ha^{-1}, respectively

Discussion

Effects of plant density on the growth and yield of cotton

Planting density is one of the major control measures of cotton production, it can regulate the growth and development of cotton leaves and agronomic traits (Wang et al., 2020). Plant height, number of branches and stem diameter are important agronomic traits in cotton cultivation. Previous studies have shown that plant height increased with the increase of density (Li et al., 2011; Khan et al., 2017). However, Zhou et al. (2019) found that planting density had little influence on plant height. In this study, the plant height of the Wankemian-1 tended to decrease with the increase of the planting density, and the plant heigh of the Wanmian-191 also was lower in D4 than that in D3 (*Table 2*). This result is consistent with the research of Khan et al. (2017) who suggested that planting density has a certain influence on plant height, and plant height tended to decrease with the increase of density. The main reason for the difference of plant height between the two cotton varieties affected by planting density in this study may be due to that the Wankemian-1 is an infinite fruit branch with a large area per plant, so the increase of density significantly affects the plant growth, and the plant height decreases with the increase of density, while the Wanmian-191 belongs to a finite fruit branch,

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which each plant occupies a small area, so the plant growth was not significantly affected by the density. With the increase of planting density, the number of fruit branch of the two cotton varieties showed a decreasing trend, which was similar to the results of previous studies (Li et al., 2011). In general, due to plant type difference, the number of fruit branch of Wankemian-1 decreased more sharply with the increase of density, suggesting that Wankemian-1 is more suitable for low-density planting.

Planting density is the main means to regulate crop growth and dry matter accumulation, and has great regulatory effect on the growth and development, and the yield of cotton (Wang et al., 2020). Suitable density can coordinate the relationship between the population and the individual, make the individual grow robust without premature senescence, and ensure a suitable number of plant groups, the boll number and boll weight of cotton (Wang et al., 2020). Studies have shown that both the lint yield and seed cotton yield are not the highest under the high density or low density planting modes, however, a reasonable planting density is the main factor to obtain a high yield of cotton (Zhang et al., 2004). Therefore, suitable planting density has become the main way to increase cotton yield. In this study, it was found that the cotton yield increased first and then decreased with the increase of planting density. When the planting density was too high, the cotton yield decreased (*Table 3*). The optimal planting density was different in different cotton planting areas in China. It was reported that the suitable density of insect-resistant hybrid cotton was 30000 plants ha^{-1} in the lower reaches of the Yangtze River Basin (Liu et al., 2010). The suitable planting density of cotton was between 51000 to 87000 plants ha^{-1} in the North Plain of China (Zhang et al., 2016). And the density of high lint yield for cotton was about 180000 plants ha^{-1} in Xinjiang (Zhang et al., 2004). However, the most common plant density recommendation to optimize yield for cotton is about 81000 plants ha^{-1} in the United States (Adams et al., 2019). In this study, it was concluded that planting density of 82500 plants ha^{-1} (D2) for Wanmian-191 and 112500 plants ha^{-1} (D4) Wankemian-1 in Anhui cotton region produced the highest yields. The Wankemian-1 belongs to finite fruit branch, and its suitable cultivation density is larger than Wanmian-191, because the cotton variety of infinite fruit branch has reasonable plant type, good ventilation and light in the field, which can make full use of light energy, and is suitable for cultivation with high fertilizer and high density for increasing the yield per unit area of cotton (Chen et al., 2014). Some studies suggested that the planting density of cotton varieties with finite fruit branch was 75000-120000 plants ha^{-1}, which can effectively solve the contradiction between the individual and the population to achieve a high yield (Chen et al., 2014).

In this study, among the yield components, boll number per plant and boll weight decreased with the increase of planting density, which was consistent with the study of Zhi et al. (2016). This study revealed that the increase of cotton yield was mainly due to the increase of boll number per unit area. Previous study also showed that the total boll number per unit area increased with the increase of planting density (Zhou et al., 2019; Zhi et al., 2016). However, when the planting density is too high, the total boll number per unit area will not increase and boll weight will significantly decrease, which is consistent with the results of previous studies (Khan et al., 2017). Therefore, the yield will significantly decrease if the density is too high. In order to achieve the maximum yield, an appropriate planting density must be selected to obtain a higher boll weight per unit area and boll number per unit area. In this study, in order to obtain the highest yield, the planting densities of Wanmian-191 and Wankemian-1 should be selected as 79000 and 115000 plants ha^{-1}, respectively, according to a regression equation (*Fig. 3; Table 4*).

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Effects of plant density on the fiber quality of cotton

Fiber quality of cotton depends on fiber properties including average fiber length, fiber strength, fiber uniformity, micronaire value (Bradow et al., 1997). The quality of cotton fiber is mainly determined by genotype, and also influenced by the climatic and cultivation condition. Previous researches showed that planting density had no significant effect on fiber quality of the variety 'Ji 863', but had a significant influence on the fiber quality of 'Shikang126' except the upper half fiber length (Zhou et al., 2019). The different effects of planting density on fiber quality between the 'Ji 863' and 'Shikang126' may be due to the stability of the variety. In this study, the upper half fiber length, fiber strength and spinning consistence index were not significant affected by planting densities, while they were significant different between the two cotton varieties (*Table 5*). Therefore, our study suggested that the quality of fiber were affected by the variety rather than by the planting density. Zhang et al. (2019) also found that planting density had no significant effect on cotton fiber quality. Similar results were also obtained by Bridge et al. (1973), who found that fiber quality was not closely related to planting density. However, the results of Zhou et al. (2018) indicated that the micronaire value showed a trend of first decreasing and then increasing with the increase of planting density. Wang et al. (2010) also found that the increase of density slightly improved the fiber length and macronaire value. These results were evidenced in the present study of the infinite fruit branch variety (Wanmian-191), but not in the finite fruit branch variety (Wankemian-1). These results indicated that the appropriate planting density is an important way to optimize cotton population and improve fiber quality for the infinite fruit branch variety (Zhi et al., 2015, 2016; Zhang et al., 2019; Zhou et al., 2019). In general, cotton fiber quality was affected by the interaction between genotype and environment. Cultivation condition and environment have influence on the formation of cotton fiber quality in some varieties (Darawsheh et al., 2009), but not in other varieties. Bednarz et al. (2006) pointed out that reducing density can increase the souring-pool ratio at the filling stage of inner fruiting node and thus improve fiber quality, which was the same for the finite fruit branch variety Wankemian-1 in this study. The fiber quality was highest under planting density of 90000 plants ha^{-1}, which the yield was highest under density of 79000 plants ha^{-1} for Wanmian-191. The high quality of fiber for Wankemian-1 was 82500 plants ha^{-1}, but was lower than the highest yield density (115000 plants ha^{-1}).

Conclusion

This study explored the effect of planting density on the plant growth characteristics, seed cotton yield and fiber quality of two cotton varieties (finite fruit branch Wankemian-1 and infinite Wanmian-191). The highest yields of Wanmian-191 and Wankemian-1 were 3591.0 and 3504.5 kg ha^{-1} under the planting densities of 82500 and 112500 plants ha^{-1}, respectively. Suitable planting density increased the seed cotton yield mainly by regulating the boll number per unit area and boll weight. In conclusion, in order to obtain high seed cotton yield, quality and economic benefits, the optimal planting densities for Wanmian-191 and Wankemian-1 would be 79000 and 115000 plants ha^{-1}, respectively, in Anhui cotton region, China. Although the results of this study may need to be verified by further multiple-year experiments, the suitable planting density of the two varieties provided useful reference and guidance for the fellow researchers and cotton growers.

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THE ROLE OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AND PHOSPHORUS FERTILIZATION IN IMPROVING PHENOLOGY AND PHYSIOLOGY OF BEAN (*PHASEOLUS VULGARIS* L.)

FILIZ, O. – TAKIL, E.* – KAYAN, N.

Department of Field Crops, Faculty of Agriculture, Eskisehir Osmangazi University, 26480 Eskisehir, Turkey
(e-mail: olcay.filizz@gmail.com; nkayan@ogu.edu.tr; phone: +90-222-324-2991; fax: +90-222-324-2990)

**Corresponding author*
e-mail: etakil@ogu.edu.tr

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Abstract. The field experiment was conducted during 2017 and 2018 at the experimental area of the Faculty of Agriculture, Eskisehir Osmangazi University, Eskisehir, Turkey. It was designed as factorial arrangement in the complete randomized block design with three replications. Three phosphorus doses (0, 30 and 60 kg ha^{-1} P_2O_5) were investigated with different biofertilizers (Control, Bontera (*Bacillus amyloliquefociens, Bacillus pumilus, Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Trichoderma harzianum, Trichoderma kanigi*), Bactoboost (*Bacillus subtilis, Bacillus magaterium, Loctococcus* spp.), Koklendirici (*Bacillus subtilis, Bacillus magaterium, Loctococcus* spp.) Lifebac NP (*Bacillus subtilis, Bacillus magaterium*), NSAH (15% organic matter, 6% organic carbon, 13% humic + fulvic acid), Rhizobia (*Rhizobium leguminosorum*). Emergence time, flowering time, maturity time, chlorophyll content, nodule number, nodule fresh weight, nodule dry weight, leaf area index, normalized difference vegetation index, and grain yield were investigated during the research. All of the investigated characteristics were higher in the first year than in the second year due to high temperature in the second year. Temperature stress negatively affected phenological and physiological characters during the research. Increasing phosphorus doses raised maturity time, chlorophyll content, leaf area index, NDVI and grain yield. In the research biofertilizer positively affected all of the investigated characters except for nodulation potential.
Keywords: *NDVI, leaf area index, chlorophyll content, biofertilizer*

Introduction

Phosphorus is the second most important macronutrient after nitrogen that is required by the plants (Sarwar et al., 2016). Chemical fertilizers are needed to get good crop yields but their abuse and overuse can be harmful for the environment and their cost cannot make economic and profitable agricultural products (Bobade et al., 1992). Simultaneous inoculation with *Rhizobium* and other plant growth-promoting bacteria has shown potential to enhance plant growth, nodulation and nitrogen fixation of several legumes. (Remans et al., 2007). N_2-fixing and P-solubilizing bacteria may be important for plant nutrition by increasing N and P uptake of the plants, and playing a significant role as plant growth-promoting rhizobacteria (PGPR) in the biofertilization of crops (Yazdani et al., 2009). PGPR is thought to stimulate plant growth through any of the following mechanisms: (1) by altering the hormone balance in the host plant; (2) by increasing mineral nutrient solubilization; and (3) antagonism towards plant pathogens. In addition to the improvement of plant growth, PGPR is directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals such as phosphorus and

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production of siderophores that chelate iron and make it available to the plant root (Glick, 1995). Rhizobacterial-based technologies have been investigated for their use as alternatives to synthetic fertilizers for sustainable crop production (Patel and Minocheherhomji, 2018). Economic and environmental benefits can include increased income from high yields, reduced fertilizer costs and reduced emission of the greenhouse gas, N_2O as well as reduced leaching of NO_3^-, N to ground water (Yazdani et al., 2009). Wang et al. (2016) reported that PGPR can improve seed germination. Fatnassi et al. (2015) found that PGPR increased nodule numbers and nodule dry weight by 50% for Vicia faba. Elkoca et al. (2010) reported that single, dual and triple inoculation with *Bacillus subtilis, B. megaterium* and *Rhizobium leguminosarum* bv. *phaseoli* increased chlorophyll contents, nodule dry weight, and nutrient uptake of *Phaseolus vulgaris* L. Inoculation with *Bacillus* spp. and *Rhizobium* or *Bradyrhizobium* spp. developed the nodulation and plant growth of common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* (L.) Merr*)* (Srinivasan et al., 1997; Camacho et al., 2001; Bai et al., 2003).

Plant growth-promoting rhizobacteria (PGPR) actively participated in the transformation of phosphate in the soil and made phosphorus available to the plant. (Bechtaoui et al., 2020) The use of biofertilizers along with chemical fertilizers may serve as an effective approach for enhancing the crop nutrient requirements, thereby leading to sustainable crop production. (Israr et al., 2016). Plant growth-promoting rhizobacteria (PGPR) exert a beneficial effect on plant growth in several ways, e.g. phosphate-solubilizing ability can increase the availability of phosphorus (P) in the soil from the residual soil P, produce growth-promoting substances and improve N-fixation, and thereby increase the overall growth and physiology of the crop (Singh et al., 2018).

We aimed to elucidate the effects of PGPR and different phosphorus doses on phenological and physiological characters of bean under field experiments.

Materials and methods

The field experiment was conducted during 2017 and 2018 at the experimental area of the Faculty of Agriculture, Eskisehir Osmangazi University, Eskisehir, Turkey (39°48’ N; 30°31’ E, 798 m above sea level). Climatic data for long-term and experimental years are shown in *Figure 1*.

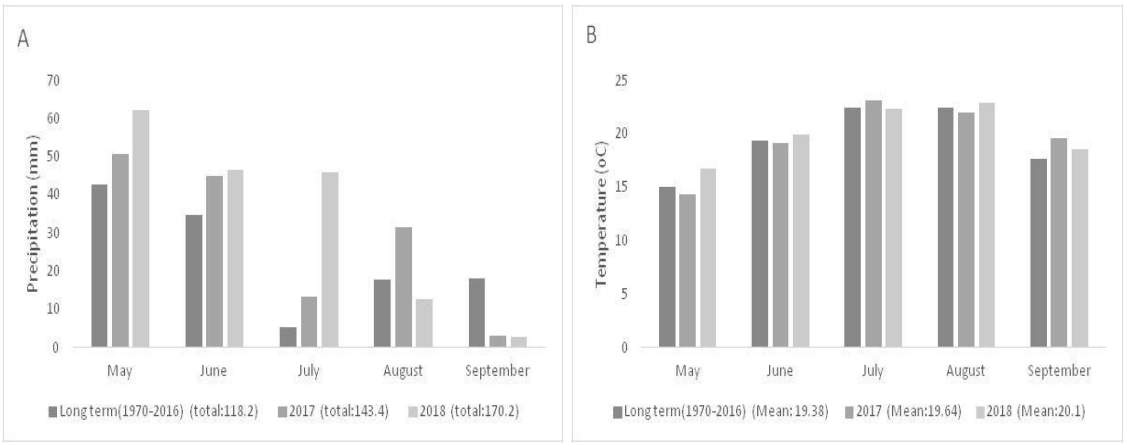

Figure 1. *Climatic data of the research area*

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Long-term annual total precipitation is 104.1 mm and it was 143.4 and 170.2 mm in the experimental years, respectively. The annual average temperature was 19.64 °C in 2017 and 20.1 °C in 2018. Physical and chemical properties of the soil in the experimental areas are presented *Table 1*.

Table 1. *Soil physical and chemical properties of the experimental area*

Depth (cm)	pH	Lime (%)	Organic matter (%)	P_2O_5 kg ha^{-1}	K_2O (kg ha^{-1})	N (%)	Ca (mg/kg)	Mg (mg/kg)	Cu (ppm)	Mn (ppm)	Fe (ppm)	Zn (ppm)
0-30	7.83	5.40	0.79	40.55	1810	0.03	4197	876.30	0.95	3.16	1.56	0.66
0-30	7.71	7.56	1.65	170.75	2450	0.08	2061	482.8	0.82	2.94	2.84	0.32

The experiment was designed as factorial arrangement in the complete randomized block design with three replications. Three phosphorus doses (0, 30 and 60 kg ha^{-1} P_2O_5) were investigated with different biofertilizers (Control, Bontera (Bacillus amyloliquefociens, Bacillus pumilus, Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Trichoderma harzianum, Trichoderma kanigi), Bactoboost (Bacillus subtilis, Bacillus magaterium, Loctococcus spp.), Koklendirici (Bacillus subtilis, Bacillus magaterium, Loctococcus spp.) Lifebac NP (Bacillus subtilis, Bacillus magaterium), NSAH (15% organic matter, 6% organic carbon, 13% humic + fulvic acid), Rhizobia (Rhizobium leguminosorum). Bean varieties Topcu were used as research materials. Each plot was 7.2 m^2 (4 m x 1.8 m) and bean was sown with 45 cm row spacing and seeding rate was 26 seeds m^{-2}. The sowing time was 04 May and 04 May in 2017 and 2018, respectively. Triple super phosphate containing 43-45% P_2O_5 was used as phosphorus fertilizer. Ammonium sulfate fertilizer (21%) was applied to all of the plots at 25 kg ha^{-1} N at sowing time, emergence time was when 50% of plots were emerging, flowering time was when 50% of plots were in flowering; maturation time was observed when 90% of the plots were mature. Chlorophyll content (spad) was evaluated on 5 randomly selected plants in each plot at the time of flowering with the Minolta Spad 502 Plus chlorophyll meter. The nodule number, nodule fresh and dry weight (g) were determined in 5 plants taken from all parcels to determine the nodulation potential in June when flowering started. Leaf area index was measured at the beginning of the podding stage with a portable field meter Delta-T SunScan in the middle of the two rows. Normalized difference vegetation index was measured in the middle of each plot by a hand-held optical sensor with Greenskeer^{TM} at the beginning of the podding stage. Each plot was harvested, blended and grain yield (kg ha^{-1}) was estimated.

The variance analysis was based on General Linear Model using the Statview package (SAS Institute). Means were compared by Least Significant Differences (LSD) test.

Results and discussion

The effects of years and bacteria were significant for all of the investigated characters but differences between phosphorus doses were insignificant for some investigated properties such as flowering time, nodule number and nodule dry weight (*Tables 2* and *3*).

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Table 2. *Effects of different phosphorus doses and bacteria on some traits of bean*

	Emergence time (day)	Flowering time (day)	Maturity time (day)	Chlorophyll content (spad)	Nodule number
2017	15.96 A	39.67 A	116.25 A	44.29 A	24.03 A
2018	15.32 B	38.16 B	106.60 B	37.74 B	15.28 B
Mean	15.64	38.91	111.42	41.01	19.65
0 kg ha^{-1} P_2O_5	15.69 A	38.96	110.77 B	38.00 C	19.51
30 kg ha^{-1} P_2O_5	15.79 A	38.91	111.71 A	40.44 B	17.72
60 kg ha^{-1} P_2O_5	15.44 B	38.88	111.78 A	44.61 A	21.74
Mean	15.64	38.91	111.42	41.01	19.65
Control	15.18 C	38.79 B	112.53 AB	38.68 D	32.64 A
Bontera	15.27 C	38.23 C	109.78 E	36.33 E	14.80 C
Bactoboost	15.76 B	39.04 B	110.72 D	40.03 CD	24.67 AB
Koklendirici	15.80 AB	38.95 B	111.64 C	42.69 B	17.37 BC
Lifebac NP	15.67 B	38.83 B	110.31 D	43.26 B	18.07 BC
NSAH	15.73 B	38.93 B	112.74 A	40.62 C	17.24 BC
Rhizobia	16.08 A	39.63 A	112.23 B	45.50 A	12.81 C
Mean	15.64	38.91	111.42	41.01	19.65
General mean	15.64	38.91	111.42	41.01	19.65
Year	**	**	**	**	**
Phosphorus doses	**	ns	**	**	ns
Bacteria	**	**	**	**	**
Year x phosph.	ns	ns	**	*	ns
Year x bacteria	**	**	**	**	ns
Phosp. x bacteria	**	**	**	**	ns
Year x phosp. x bac.	**	**	**	**	ns

ns: non-significant, *: $p \leq 0.05$, **: $p \leq 0.01$

While emergence time and flowering time were higher in 60 kg ha^{-1} P_2O_5 plots in 2017 rhizobia and bacteria showed lower values in the same plots in 30 kg ha^{-1} in the same year (*Fig. 2A, B*). While chlorophyll content and leaf area index were higher in 60 kg ha^{-1} P_2O_5 plots in 2017 for Lifebac NP, the same bacteria showed lower values in the same plots in 2018 (*Figs. 3B, 4A*). While NDVI was higher in 60 kg ha^{-1} P_2O_5 plots in 2017 for control, in the second year control plots showed lower values in the same doses (*Fig. 4B*). Nodule fresh weight and nodule dry weight showed superior performance in control plots in 2018 but same plots showed lower values in 2017 (*Fig. 5 A, B*). While maturity time and grain yield showed superior performance in control plots in 2017, same plots showed lower values in 2018 (*Figs. 3A, 6*). Therefore, year x rhizobia x nitrogen fertilization interaction was significant.

All of the investigated characters were higher in the first year than in the second year (*Tables 2* and *3*). The temperature was higher in the second year than in the first year in our research. In the second year, higher temperatures in May when experiments were established caused earlier emergence. In addition, higher June temperatures in the flowering stage caused earlier flowering in the second year (*Fig. 1*). Chlorophyll content was 44.29 spads in 2017 but it was 37.74 spads in 2018 (*Table 2*). High temperature caused lower photosynthetic activity and therefore chlorophyll content

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decreased (Xu et al., 1995). High temperatures in the second year may be caused by low chlorophyll content. Nodule number, nodule fresh weight and nodule dry weight were lower in 2018 (*Tables 2* and *3*). High temperatures negatively affected nodule formation and nitrogenase activity (Yavas and Unay, 2018). Temperature stress before flowering causes nodules degeneration (Gaur et al., 2015). The higher temperatures in the second year had negative effects on the nodule number, nodule fresh weight and nodule dry weight. Leaf area index was 3.18 in 2017 but it was 2.33 in 2018 (*Table 3*). Oner and Sezer (2007) reported that the leaf area index increased with increasing light intensity and low temperatures. Low temperatures in the first year may be caused by higher leaf area index. NDVI is defined as the ratio of radiation reflected from healthy vegetation to radiation reflected from all other sources. There is a positive relationship between NDVI and leaf area index and grain yield (Tahir et al., 2020). Grain yield was lower due to total high temperature especially grain-filled period in the second experimental year (*Table 3*). High temperatures reduced the total leaf area and net assimilation amount and therefore plant growth also reduced (Rodríguez et al., 2005; Ashraf and Hafeez, 2004).

Table 3. *Effects of different phosphorus doses and bacteria on some traits of bean*

	Nodule fresh weight (g)	**Nodule dry weight (g)**	**Leaf area index**	**Normalized difference vegetation index**	**Grain yield (kg ha^{-1})**
2017	0.29 A	0.18 A	3.18 A	0.69 A	2110 A
2018	0.24 B	0.15 B	2.33 B	0.59 B	1490 B
Mean	0.26	0.16	2.75	0.64	1800
0 kg ha^{-1} P_2O_5	0.24 B	0.17	2.47 C	0.62 C	1650 C
30 kg ha^{-1} P_2O_5	0.29 A	0.16	2.82 B	0.63 B	1810 B
60 kg ha^{-1} P_2O_5	0.26 B	0.15	2.98 A	0.67 A	1950 A
Mean	0.26	0.16	2.75	0.64	1800
Control	0.36 A	0.22 A	2.60 C	0.62 D	1930 B
Bontera	0.28 B	0.18 B	2.49 C	0.62 D	1490 D
Bactoboost	0.26 B	0.15 C	2.71 BC	0.64 C	1580 C
Koklendirici	0.21 C	0.13 C	2.86 AB	0.65 B	1930 B
Lifebac NP	0.21 C	0.14 C	3.03 A	0.63 C	1630 C
NSAH	0.26 B	0.15 BC	2.71 BC	0.65 B	2100 A
Rhizobia	0.27 B	0.16 BC	2.91 AB	0.67 A	1930 B
Mean	0.26	0.16	2.75	0.64	1800
General mean	0.26	0.16	2.75	0.64	1800
Year	**	**	**	**	**
Phosphorus doses	**	ns	**	**	**
Bacteria	**	**	**	**	**
Year x phosphorus	**	**	**	**	**
Year x bacteria	**	**	**	**	**
Phosp. x bacteria	**	**	**	**	**
Year x phosp. x bac.	**	**	**	**	**

ns: non-significant, *: $p \leq 0.05$, **: $p \leq 0.01$

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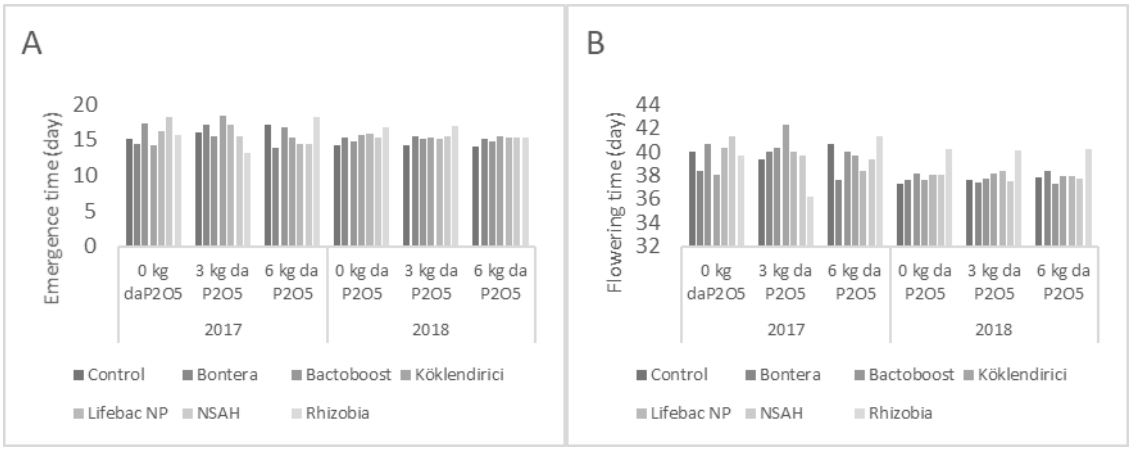

Figure 2. *The interaction between year, phosphorus doses and bacteria on emergence time (A) and flowering time (B) of bean [LSD 1%: 0.657 (A);1%: 1.146 (B)]*

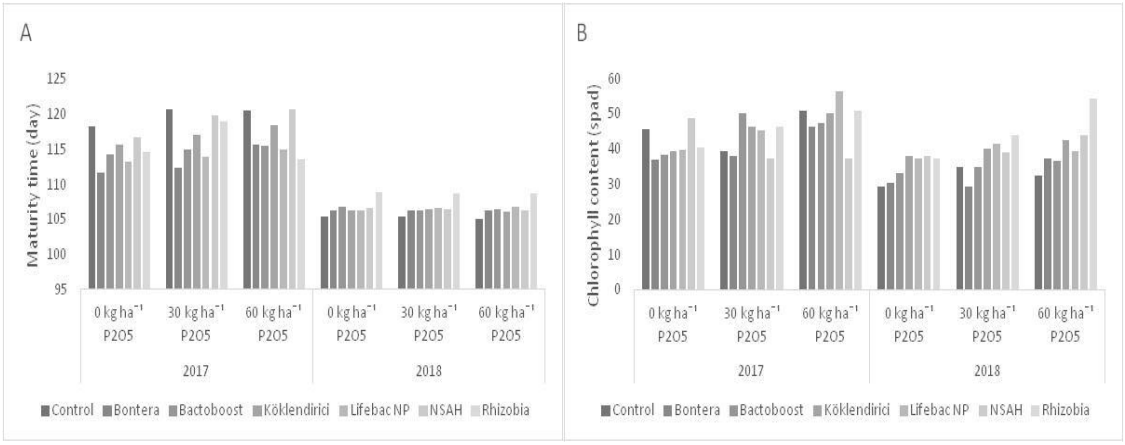

Figure 3. *The interaction between year, phosphorus doses and bacteria on maturity time (A) and chlorophyll content (B) of bean [LSD 1%: 1.040 (A);1%: 3.597 (B)]*

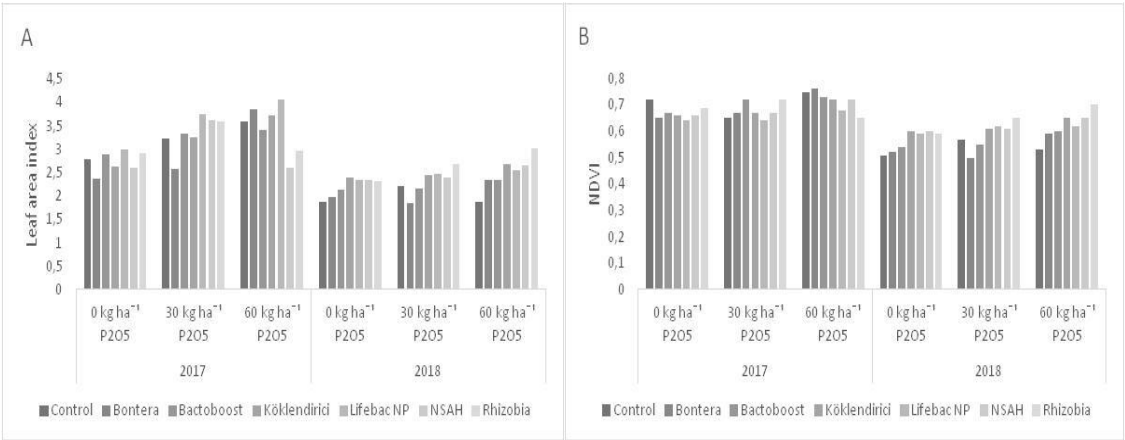

Figure 4. *The interaction between year, phosphorus doses and bacteria on leaf area index (A) and NDVI (B) of bean [LSD 1%: 0.476 (A);1%: 0.002 (B)]*

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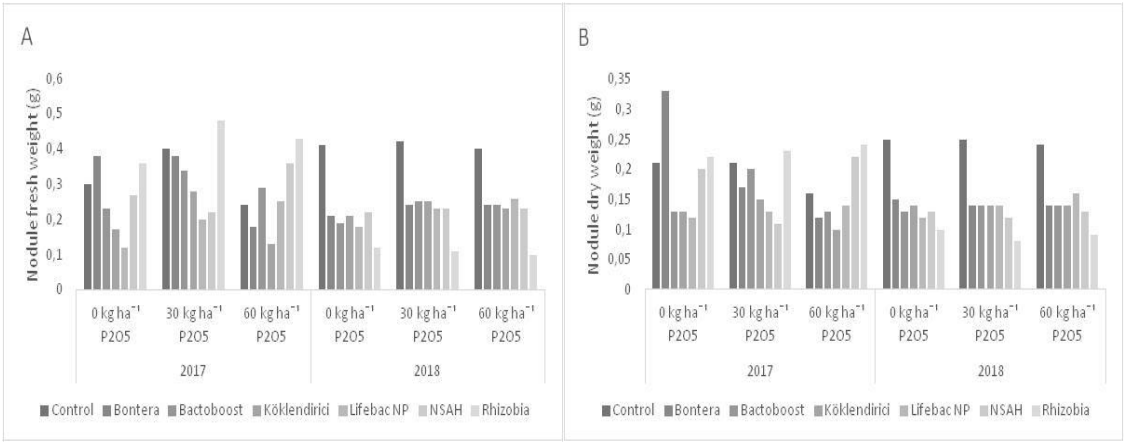

Figure 5. *The interaction between year, phosphorus doses and bacteria on nodule fresh weight (A) and nodule dry weight (B) of bean [LSD 1%: 0.068 (A);1%: 0.068 (B)]*

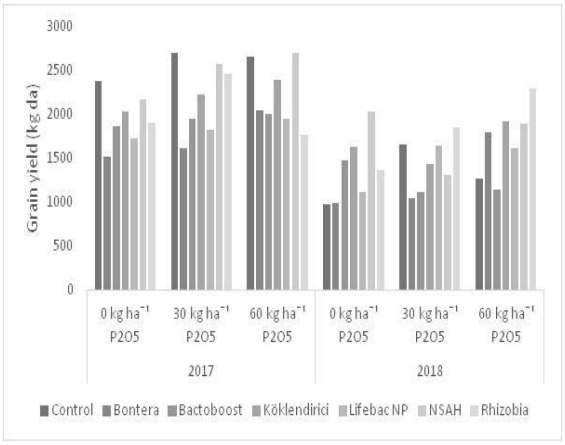

Figure 6. *The interaction between year, phosphorus doses and bacteria on grain yield of bean [LSD 1%: 14.15]*

Increasing phosphorus doses decreased the emergence time but increased maturity time (*Table 2*). 0 kg ha^{-1} and 30 kg ha^{-1} phosphorus doses show similarity regarding emergence time. 30 kg ha^{-1} and 60 kg ha^{-1} phosphorus doses show no difference for maturity time. The highest chlorophyll content was obtained when 60 kg ha^{-1} P_2O_5 was applied (*Table 2*). Chlorophyll content increased with increasing phosphorus doses. Mtua (2015) reported that phosphorus doses increased chlorophyll content for bean. Phosphorus fertilization increased nodule number but it was not significant statistically. 0 kg ha^{-1} and 60 kg ha^{-1} phosphorus doses show similarities for nodule fresh weight. The highest nodule fresh weight was obtained from 30 kg ha^{-1} P_2O_5. Yilmaz (2010) indicated that phosphorus fertilization increased nodule number. The highest leaf area index was obtained from 60 kg ha^{-1} P_2O_5 (*Table 3*). Turuko and Mohammed (2014) indicated that leaf area index increased with increasing phosphorus doses for bean. Many researchers reported that phosphorous fertilization increased grain yield in bean (Baydemir, 2013; Turuko and Mohammed, 2014; Mtua, 2015).

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The latest exit time was determined in the rhizobi parcels and the earliest exit time was determined in the control parcels, but this was followed by the bontera. Control plots and bontera are in the same statistical group. The earliest flowering time was in Bontera but the latest was in rhizobia. Flowering time is similar for control plots, Bactoboost, Koklendirici, Lifebac NP and NSAH content. The latest maturity time was determined in NSAH plots but this was followed by the rhizobia and the earliest maturity time was determined in bontera plots. Control plots are in the same group with NSAH and Rhizobia plots, Life NP and Bactoboost plots are statistically in the same group for maturity time. Bontera biofertilizer provided earlier emergence, flowering and maturity in our research. While the highest chlorophyll content is in rhizobia plots, it was the lowest in Bontera plots but this was followed by the control plots. Control plots and Bactoboost applications, Koklendirici and Lifebac NP applications, and Bactoboost and NSAH applications are statistically in the same group. Baset Mia et al. (2010) and Ahamd et al. (2014) reported that biofertilizer increased chlorophyll content. The highest nodule number, nodule fresh weight and nodule dry weight were determined for the control plots (*Tables 2* and *3*). Nodule number is similar to the control and Bactoboost plots. Also Koklendirici, Lifebac Np and NSAH are in the same statistical group. Biofertilizer had no effect on nodulation potential. The highest nodule wet weight was observed in control plots. Bontera, Bactoboost, NSAH and Rhizobia showed similar values. Nodule dry weight was observed in the highest control plots. These are followed by Bontera, NSAH and rhizobia plots. The highest leaf area index was determined in Lifebac NP plots and the lowest was determined in Bontera plots but this was followed by the control plots. Metwali et al. (2015) indicated that PGPR increased leaf area index. The highest Normalized Difference Vegetation Index (NDVI) was determined for rhizobia plots and lower value was observed in control plots (*Table 3*). Biofertilizer positively affected NDVI. While grain yield was 1930 kg ha in control plots, it was 2100 kg ha in NSAH plots (*Table 3*). NSAH increased grain yield but other biofertilizers showed no positive effect on grain yield in our research. After NSAH plots, the highest yields were obtained from the plots using rhizobia and Koklendirici in the same statistical group. They were followed by Lifebac NP and Bactoboost plots in the same statistical group. Many researchers reported that grain yield was increased with PGPR application (Zahir et al., 2007; Ardekani et al., 2008; Akhtar et al., 2013; Naseri et al., 2013; Fatetorbay et al., 2014; Talat, 2019).

Conclusions

Considering the climatic data of the years when the experiment was established although the amount of precipitation was higher in the second year compared to the first year, lower values were observed in all the characteristics examined. The reason for this was irrigation in the experimental areas. The second year the temperature was higher. All of the investigated characters were higher in the first year than in the second year due to high temperature in the second year. Temperature stress negatively affected phenological and physiological characters in our research. Increasing phosphorus doses increased maturity time, chlorophyll content, leaf area index, NDVI and grain yield. When the phenological and physiological characteristics were examined, the most appropriate phosphorus doses were found to be 60 kg ha^{-1}. Effective biofertilizers are promising tools to maintain agricultural resources to improve soil fertility and plant growth. Biofertilizers positively affected all of the investigated characters except for

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nodulation potential in our research. While good results were obtained from Rhizobia bacteria in terms of the investigated characters, the highest grain yield was determined in NSAH plots. The effect of PGPR depends on the type and number of bacteria, plant-bacteria combination, plant genotype, harvest date, soil type, soil organic matter and environmental conditions. Some unpredictable conditions in field experiments, sometimes prevents correct results. Therefore, field experiments should be increased.

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ASSESSING LANDSCAPE FRAGMENTATION EFFECTS ON ECOSYSTEM SERVICES IN A SEMI-ARID MOUNTAINOUS ENVIRONMENT: A CASE STUDY ON ABHA WATERSHED, SAUDI ARABIA

BINDAJAM, A. A.[1] – MALLICK, J.[2*] – MAHATO, S.[3] – TALUKDAR, S.[3] – ALQUADHI, S.[2]

[1]Department of Architecture and Planning, College of Engineering, King Khalid University, Abha, Kingdom of Saudi Arabia
(e-mail: abindajam@kku.edu.sa (Bindajam, A. A.))

[2]Department of Civil Engineering, College of Engineering, King Khalid University, Abha, Kingdom of Saudi Arabia
(e-mail: jmallick@kku.edu.sa (Mallick, J.); sdalqadi@kku.edu.sa (AlQuadhi, S.))

[3]Department of Geography, University of Gour Banga, West Bengal, India
(e-mail: mahatosusanta2011@gmail.com (Mahato, S.); swapantalukdar65@gmail.com (Talukdar, S.))

[*]Corresponding author
e-mail: jmallick@kku.edu.sa; phone: +966172428439; fax: +966172418152
address: Department of Civil Engineering, College of Engineering, King Khalid University, P.O. Box: 394 Abha 61411, Kingdom of Saudi Arabia

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Abstract. The effect of land use land cover (LULC) change is well documented, but the impact of landscape fragmentation on Ecosystem Service Value (ESV) has not been quantitatively explored yet in the study area. The present study designed to evaluate the landscape fragmentation effect of Abha watershed, Saudi Arabia, a new method was proposed by integrating the ESV with the analytical hierarchy process (AHP) and landscape fragmentation model. Results of LULC dynamics showed that urban area has increased significantly by 1648.8 hectares (ha) to 6379.2 ha from 1990 to 2018. While the scrubland has covered a half (15173.6 ha) of the total study area (37001.08 ha) in 1990, but it was significantly reduced to 8907.93 ha in 2018. The calculated ESV of dense vegetation was 0.2 million dollars/year, according to the results of the integrated ESV model, but it was 2 million dollars/year using the initial coefficient in 2018. In 2018, the estimated ESV for water bodies using AHP's integrated ESV model was 7 times lower than the estimated ESV with an initial coefficient That is, a declining trend was discovered after integrating landscape fragmentation for ESV estimation in this study area.
Keywords: *LULC dynamics, analytical hierarchy process, ESV change, fragmentation analysis, spatial mapping*

Introduction

The natural habitat has been hampered and structurally and functionally changed as human interference has increased. This has caused the fragmentation of the landscape, which negatively affects the biodiversity and ecosystem (Sanon et al., 2020; Lhoest, 2020). The landscape fragmentation can be defined as the breaking of a large natural land cover unit into several smaller patches limited by a matrix. The mechanism of fragmentation can take two forms, LULC changes due to natural causes (Flowers et al., 2020; Gerner, 2020; Venier et al., 2020) and anthropic activities (Riechers et al., 2020; Siqueira-Gay et al., 2020). The key anthropogenic processes include urban development,

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infrastructure development, the intrusion in various cultural environments and so on (Onilude and Vaz, 2020; Kamwi and Mbidzo, 2020; Arroyo-Rodríguez et al., 2020). Many researches have been conducted on the dynamics of LULC over time in a different environment (Motlagh, et al., 2020; Li et al., 2020). The LULC dynamics are highest in the flood plain regions (Thonfeld et al., 2020; Adnan et al., 2020) because of the easy conversion process compare to the mountainous and island regions. Earlier studies show that LULC changes can occur due to severe damage caused by natural and anthropogenic disturbances (Ntihinyurwa and de Vries, 2020; Snep and Clergeau, 2020). The dynamics of LULC cause the landscape to disintegrate, which is described as fragmentation. The fragmented landscape can be turned into other LULC categories quickly. The fragmentation of the landscape will then adversely affect the environment in various ways, such as the deduction of ecological continuity, productivity, functional strength, connectivity, ecological richness etc. (Sanderson, 2020; Jupiter, 2020). Aside from these, landscape fragmentation can impede ecosystem services by interfering with the movement of various matters, species, humans, and energy (Sanderson, 2020). Natural habitats have been increasingly transformed all over the world as a result of human intervention, which has a detrimental impact on natural resources and biodiversity (Patru-Stupariu et al., 2020; Perennes et al., 2020). As a result, it is critical to recognise landscape fragmentation areas that impact ecosystem services. The impact of fragmentation drives biodiversity depletion and shifts in ecological structure (Chase et al., 2020; Herrera et al., 2020; Suárez-Castro et al., 2020). The effect of fragmentation can be different, as it depends on the degree of landscape fragmentation (Rybicki et al., 2020; Yezzi et al., 2020). The fragmentation of the landscape prevented the daily movement of plant and animal species between breeding, feeding habitat, and migration (Rycken et al., 2020). Furthermore, fewer species are assisted by smaller habitat patches, which can only accommodate a smaller number of populations, placing them at risk of extinction (Plaza, 2020). The edge, on the other hand, has a greater impact than ever on the sustainability of native species (Kiene et al., 2020). Lin et al. (2020) investigated the structural change in connectivity for ecological land units using graph theory and the distance of a suitable landscape threshold. Many researchers used an ecological manifestation assessment predictor method to produce a realistic analysis of pattern changes, as well as changes in the vulnerability of ecology and ecosystem resources of respective landscape units (Abad-Segura et al., 2020). The resulting conditions of landscape fragmentation include an increase in patch frequency, edge length, edge-to-area ratio, patch size reduction, and so on. Previous studies reported very clearly that because of the landscape fragmentation, the fragmented units have been exposed to be converted or captured by the anthropogenic activities (Vergara et al., 2020). The diversity of ecology, the quality of habitat, and the security of ecology can be seen to a greater extent in large core areas, while near to fragmented units, such as patch and edge, these have been drastically diminished (Pritchard et al., 2019). For example, if a forest landscape bifurcated by road or railways, which causes the ecological shortage across the buffer distance of railway line or road based on the degree of vehicle frequency, uncertainty and effect of pollution (Kulkarni et al., 2014). Even where forest land is fragmented by highways, the accessibility of networks for certain animals, such as elephants, is hindered. As a result, the accidental demise of many critical species is common (Braulik et al., 2014; Cudowski and Pietryczuk, 2020), exacerbating the conflict between animal and man (Cretois et al., 2019; Mota-Rojas et al., 2020). These combined phenomena have the potential to reduce ecosystem services. Only a few studies have conceptualised the concept of

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fragmentation's effects on ESV (Buckwell et al., 2020). To make it clear about the exposure of natural landscape due to fragmentation to the human inference, the schematic diagram (*Figure 1*) can be substantiated. For the first case (a stretch of landscape continuously), 100 units are considered as the total patch area, while 40 units are edge area. The estimated ratio is 1:2.5 for this. But after the landscape fragmentation, the land has fragmented into four and sixteen patches, the ratio of edge and area would be 1:1.25 and 1:0.625, respectively, which depicts the declining of ESV due to human interference.

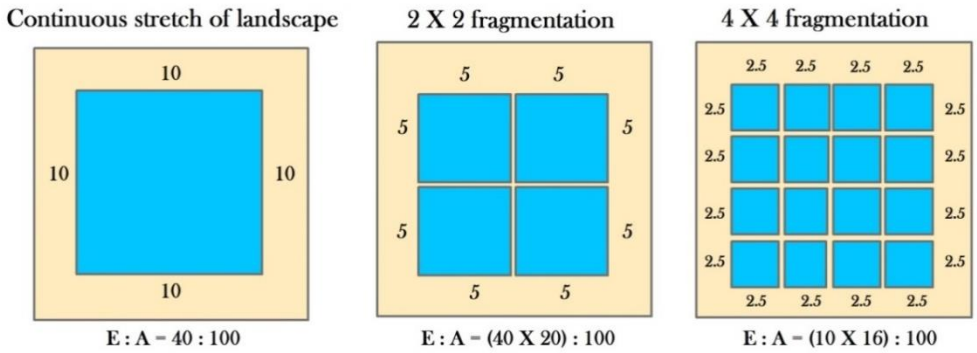

Figure 1. *Schematic diagram showing possible edge effect on fragmented landscape (E= Edge, A= Area)*

Along with other ecosystem services, the landscape fragmentation can hamper the biodiversity in terms of reducing the richness and diversity of the tree species significantly in any regions (Zoderer et al., 2019; Canedoli et al., 2020; Carlucci et al., 2020). Thus, with the declining of biodiversity due to landscape fragmentation has been commonly observed. Also in marine and riverine ecosystem, the fragmentation has largely affected significantly, which includes the aquatic biodiversity and abundance of fish (Kaus et al., 2017; Heino et al., 2020; Bertolini et al., 2020; Sahoo and Swain, 2020; Wang et al., 2020) On the other hand, few services have experienced the restriction of flows of matter or organisms due to fragmentation. Across the natural landscape, the shape, size, arrangement of space and the isolation of patches are affected by fragmentation, which influences the flow of water, organisms, soil and energy negatively or positively (Douglas et al., 2018; Suchara et al., 2019; Rotem et al., 2020). The fragmentation can have negative or positive impacts on the flow of services based on the process and the structure of the landscape (Andriamparany et al., 2020). For example, breakdown of forests due to logging, road construction, the spread of urban land and agriculture can alter the growth and development of many species of plant, which affects negatively the regulation of carbon sequestration and water quality (Mayer et al., 2020). The capacity of the species for travelling across the landscape has decreased due to the effect of the fragmentation for diurnal movement (Benoit et al., 2020; Suraci et al., 2020). For this reason, the risk of species extinction has increased with the fragmented land (Zungu et al., 2019; Zengeya et al., 2019). Apart from it, the ESV of different LULC classes have also decreased due to the landscape fragmentation. Many researchers also integrated the biodiversity with the decreasing ESV for analyzing the effect of landscape fragmentation (Van Bussel et al., 2020). Even, enlarged tree species and diverse flowers can supply various ES with significant amounts (Corcket et al., 2020). Consequently, the ecosystem services have been reduced with the declining of landscape size due to fragmentation effect (Valdés et

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al., 2020). The carbon sequestration and biomass production get affected by the disintegration of the forest or forest fragmentation (Baskent, 2019). This is also applicable in case of water bodies. Big size of the continuous wetlands can supply a larger amount of ecosystem services than a fragmented one, therefore, the wetland with a large number of fragmentations can lead to wetland loss (Gómez-Baggethun et al., 2019). The loss of diversity of animal and plant including water supplies and storage reduction, protection and floodplain loss, groundwater recharge, increasing sedimentation, and soil erosion can happen greatly than many times before due to the degradation as well as fragmentation of wetlands (Zhao et al., 2018; Boardman et al., 2019; Huang et al., 2019; Teixido et al., 2020). Despite the immense values, anthropogenic activities have been affecting the freshwater wetlands and other natural resources, which caused the landscape fragmentation and degradation of such ecosystems (Talbot et al., 2018; Guevara-Ochoa et al., 2020; Han et al., 2020). The conversion of natural resources may be beneficial for the short term in case of the economy, but for the long term, these will be harmful to the environment as well as humans.

To estimate the ESV of different land use land cover classes, several well-known and widely used methods are used, such as the cost-based method, energy analysis model, Integrated Valuation of Environmental Services and Trade-offs (InVEST) model, value/benefit transfer model (VTM), contingent valuation, and so on (Costanza et al., 2014; Yang et al., 2018; Sun et al., 2018; Shi et al., 2020; Chen et al., 2020; Talukdar et al., 2020). Although these approaches have many challenges in estimating ESV on real-world ground. This, too, necessitate extremely sophisticated field data. It is exceedingly difficult to achieve a sophisticated response from locals in countries such as Saudi Arabia, where very few studies on ESV estimation have been performed. As a consequence, people lack adequate understanding of the ESV, which can lead to a great deal of uncertainty and mistake. As a result, we had to focus on Costanza et al. (2014)'s global coefficient value to estimate the ESV of different LULC categories.

Many scholars have researched the effects of land use land cover dynamics on ESV estimation (Shiferaw et al., 2019; Clerici et al., 2019) method. The researchers just looked at the ESV in terms of LULC dynamics. However, no studies have taken into account integrating the dynamics problem with the ESV estimate. As a result, in the current research, we integrated the LULC dynamics in terms of fragmentation for correcting the ESV estimation. We also applied weights (through AHP) to the fragmented units based on the field survey and expert opinion to improve the precision of the ESV estimation. As a result, the aim of this analysis was to calculate the ESV in relation to LULC dynamics over a 30-year period. Another big goal was to investigate the impact of fragmentation on ESV.

Materials and Methods

Study area

Abha, a semi-arid mountainous watershed of Saudi Arabia, is considered as the study area. It is located in Aseer province of Saudi Arabia. It covers an area of 370 km^2. The geographical location is extended between 18°10'12.39"N and 42°21'41.58"E to 18°23'33.05"N and 42°39'36.09"E. A part of Abha's highland is linked with the Arabian shield in the western part of the kingdom (*Figure 2*). The study area observes heavy rainfall for short period, while the surrounding rural areas have witnessed flash flood in winter. The elevation of this region varies between 1954 meters to 2989 meters above

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mean sea level with undulating topography. The watershed is elongated and dominated by many small wadies, which drain their water into this watershed. The slope of this region ranges between 0^{O} to 52.32^{O}. The landscape of the study area is heterogeneous because of the complexity in terrain. The slope, geological weakness, rain etc. have accelerated the erosional problem in the study area, which affects agricultural productivity, forestland, sedimentation etc. The semi-arid climatic condition is the main climatic feature of this region. The study area observes an average rainfall of 214 mm per year (Since three decades) 1990 - 2018. The northwestern part of this watershed with 3000 m altitude has high richness in flora. Because of the climatic and topographical variation in this region, a diverse plant community has been found (Abbas et al., 2020). In the western part of the study area, a huge amount of acacia trees has been found. Therefore, it can be stated that the study area is rich in natural resources. The socio-economic activities have been formed based on natural resources. For this reason, immediate attention should be paid towards the development and conservation of the natural environment. This is the reason; we have selected Abha watershed as the study area.

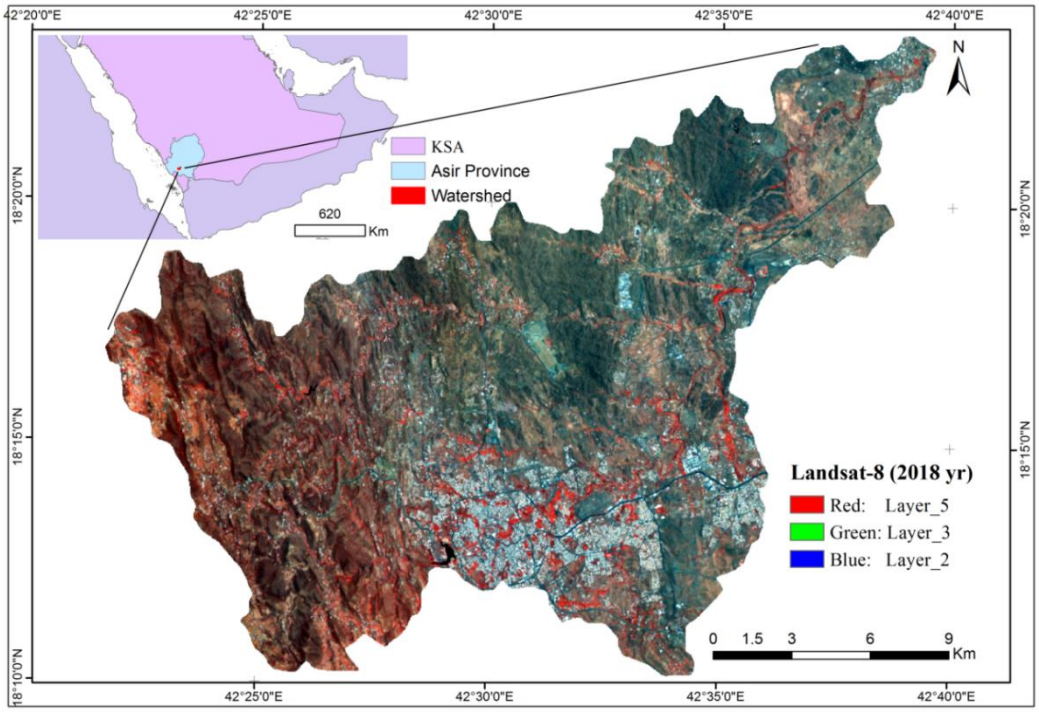

Figure 2. *Location of Abha Watershed*

Methodology

Classification of land use land cover and validation

The LULC change is an identified aspect for analyzing the alteration of global environment and effect on the ecosystem services (Costanza et al., 1997; Clerici et al., 2019; Jiang et al., 2020). In the present study, Landsat 4-5^{TM} and Landsat 8 OLI were utilized for preparing the LULC maps for the year 1990, 2000, and 2018. The required satellite images (Details see *Table 1*) were downloaded from the United State Department of Geological Survey (USGS) (https://earthexplorer.usgs.gov).

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Table 1. *Details of Satellite image*

Satellite data	Path/row	Date and Local Time	Spatial resolution	Number of bands
Landsat TM	167/47	1990-06-02/9.51 am	30	6 (excluding thermal band)
Landsat TM	167/47	2000-05-28/10.07am	30	6 (excluding thermal band)
Landsat 8	167/47	2018-06-15/10.30am	30	9 (excluding thermal band)

The geometrically and radiometrically correction was made on these satellite images using ERDAS software (version 2014). The maximum likelihood classifier (MLC), a supervised image classification technique, was used for LULC classification in ArcGIS 10.5 software (Mazhar and Fadia, 2019). On the theory of probability, the MLC is considered as dependent. In the time of training the data, the statistics of training data for all classes in the area of the band is Gaussian distributed (Alam et al., 2020). We collected 50-70 spectral signatures for each LULC classes, which were used for training the classifier. Based on the collected spectral signatures, eight LULC classes were identified, such as Urban, waterbodies, dense vegetation, sparse vegetation, agricultural cropland, Scrubland, bare soil and exposed rock.

The accuracy evaluation of the LULC map is crucial for the user's confidence. The accuracy measurement quantifies the degree of similarity between the LULC map obtained from satellite images and ground reality (Sánchez-Espinosa et al., 2019). The ground truth samples in this analysis were taken at random from Google Earth real-time data. Statistical methods were used to equate the prepared LULC to the collected ground truth samples. The Kappa coefficient was used to calculate the accuracy of LULC maps. In this analysis, 200 sample sites were chosen at random from Google Earth Pro real-time data, and the considered sites were checked precisely. The Kappa statistics were computed using Stehman's (1996) proposed method. The Kappa coefficient ranges from 0 to 1, with 0 representing the least accuracy in the case of field reality and classified images and > 0.85 representing very high accuracy (Monserud and Leemans, 1992).

Method for analyzing the LULC dynamics

The change detection technique was used in this research to analyse the dynamics of LULC maps for the years 1990, 2000, and 2018. (Kalinicheva et al., 2020; Fahad et al., 2020; Mishra et al., 2020). Change detection techniques are divided into two categories: pre-classification and post-classification approaches (Haque and Basak, 2017). The post-classification technique was used in this research to determine LULC changes from 1990 to 2018.

Methods for computing the ESV

Simulated market approach (Caparrós et al., 2020), benefits transfer approach (Msofe et al., 2020; Custodio et al., 2020), the surrogate (proxy) market approach (Phoomirat et al., 2020) are widely applied approaches for estimating the ESV. The benefit transfer approach is considered as one of the standard method, used for estimating service values (Poudel et al., 2020). Costanza et al. (1997) used a simple benefit transfer approaches for estimating global ESV and provided global coefficients for different types of biomes, which have been widely used to calculate the ESV. Researchers have used LULC classes

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as a proxy of biomes. Then, the area of different LULC classes was computed and integrated with the respective coefficients using equation 1. In this way, the ESV from different LULC classes was estimated.

$$ESV = A \times VC \quad \text{(Eq.1)}$$

where 'ESV' denotes the value of ecosystem services for various LULCs, 'A' denotes the region of each LULC type, and 'VC' denotes the coefficient value of each LULC type. *Table 2* shows the coefficient value of six biomes or LULC categories. The maps for ESV mapping were generated by assigning the computed ESV values to the relevant LULC classes.

Table 2. *Land use land cover and ecosystem service values as per Costanza et al. (1997)*

Land use types	Equivalent biome	ESV coefficient (USD/ha/yr)
Urban	Urban	6661
Water bodies	Lakes and river	12512
Dense vegetation	Forest	3800
Sparse vegetation	Grassland	4166
Agricultural cropland	Cropland	5567
Scrubland	Grassland	4166
Bare soil	Barren land	0
Exposed rock	Rock	0

Modelling landscape fragmentation

Landscape fragmentation is defined as the division of a natural landscape or a broad unit of the landscape into multiple landscapes or units. Landscape fragmentation has an effect on both environmental changes and biodiversity. Until now, very few methods for modelling landscape fragmentation at a spatial scale have been created. The landscape fragmentation tool in ArcGIS 10.5 software was used to model the landscape fragmentation in this analysis. At the spatial scale, the tool distinguishes six types of landscape fragmentation, such as patch, edge, perforated, small core (<250 acres), medium core (250-500 acres), and large core (>500 acres). *Table 3* provided the overview of the above fragmentation categories. In the present study, we prepared fragmentation for LULC maps of 1990, 2000, and 2020.

Table 3. *Reference landscape unit definition*

Landscape	Definition
Patch	Relatively discontinuous areas (spatial domain) or periods (temporal domain) or environmental condition which is relatively homogeneous is represented by patches.
Edge	An edge represents an area where the rapid changes of observed value are found or where the change rate is very high.
Perforated	The edge habitat generated by a small area of non-forest habitat which is enclosed by core habitat is the perforated section.
Small core	The internal area of any landscape which covered <250 acres refers to an as small core.
Medium core	The internal area of any landscape which covered 205 – 500 acres.
Large core	The core area is defined as the internal area of patches after the elimination of edge buffer which is specified by a user

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Computation of ESV by proposing fragmentation integrated method

Previous studies have shown that a number of key ecosystem services have declined due to Landscape fragments, such as carbon sequestration, soil formation, seed dispersal and pollination; and nutrient cycling (Leal Filho et al., 2020; Loewen, 2020; Wang and Dai, 2020). In order to formulate ecosystem service coefficient Costanza et al. (1997) did not consider the impact of landscape fragmentation. Although the ESV produced from a broad and compact landscape unit cannot be identically produced with the ESV produced from a landscape fragmentation unit. From the compact and fragmented landscape, different ESVs should be created. However, no such different coefficient for estimating the ESV has been discovered. If we apply the same coefficient to all broken units of the landscape, the qualitative deterioration of the landscape would not be expressed in calculating the ESV. As a result, to capture the effect of fragmentation on ESV estimation, we used the AHP methodology (Saaty, 2004; Mehdipour et al., 2019), which took the comparison pair matrix into account for six hierarchic units of landscape fragmentation. These fragmented units were weighted using AHP based on expert opinion and local people's perception. Consistency ratio findings showed <3% that was found satisfactory and should be continued for further study. Using equations 2–7 was calculated for the ESV of the patch, edge, perforated, small core, medium core and large core. The patch's ESV was calculated by multiplying the assigned patch weight of one landscape unit by the unit's respective areal coverage and CV (*Eq.2*). Similarly, ESV was measured for various hierarchic landscape units, but when assigning weights to edge, perforated, small core, medium core, and large core, the total weight of the corresponding lower hierarchic landscape unit was taken into account (*Eqs.2 to 7*).

To compute the effect of landscape fragmentation on ESV, first LULC specific ESV was estimated using the coefficient mentioned in *Equation 1* similar to patch and edge etc. Finally, the total ESV from the fragmented units was estimated using *Equation 8*. The ecosystem service values of LULC in total were calculated by *Eq.9*. Then, the difference between these two ESV has been considered as the effect of fragmentation on ESV (*Eq.10*).

$$ESV_p = W_p \times CV_i \quad \text{(Eq.2)}$$

$$ESV_e = ((W_p + W_e) \times CV_i) \quad \text{(Eq.3)}$$

$$ESV_{pr} = ((W_p + W_e + W_{pr}) \times CV_i) \quad \text{(Eq.4)}$$

$$ESV_{sc} = ((W_p + W_e + W_{pr} + W_{sc}) \times CV_i) \quad \text{(Eq.5)}$$

$$ESV_{mc} = ((W_p + W_e + W_{pr} + W_{sc} + W_{mc}) \times CV_i) \quad \text{(Eq.6)}$$

$$ESV_{lc} = ((W_p + W_e + W_{pr} + W_{sc} + W_{mc} + W_{lc}) \times CV_i) \quad \text{(Eq.7)}$$

$$ESV_{flti} = \left(ESV_p \times A_p\right) + \left(ESV_e \times A_e\right) + \left(ESV_{pr} \times A_{pr}\right) + \left(ESV_{sc} \times A_{sc}\right) + \left(ESV_{mc} \times A_{mc}\right) + \left(ESV_{lc} \times A_{lc}\right) \quad \text{(Eq.8)}$$

$$ESV_{ti} = (CV_i \times A_i) \quad \text{(Eq.9)}$$

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$$ESV_{fe} = (ESV_{ti} - ESV_{flit}) \quad \text{(Eq.10)}$$

where, the ecosystem service values of patch, edge, perforated, small core, medium core and large core are represented by ESV_p, ESV_e, ESV_{pr}, ESV_{sc}, ESV_{mc}, ESV_{lc}, respectively. CV_i indicates the Coefficient value of ith LULC types. The weight values based on AHP are indicated by W_p, W_e, W_{pr}, W_{sc}, W_{mc}, W_{lc}, respectively. ESV_{flti}, ESV_{ti}, ESV_{fe} refer to the total value of ecosystem service of fragmented landscape, ecosystem service values of LULC in total and effect of fragmentation on ecosystem service respectively. A_i is the areal coverage of i^{th} wetland type and the A_P, A_e, A_{pr}, A_{sc}, A_{mc}, A_{lc} are the areal coverage of patch, edge, perforated, small core, medium core and large core, respectively.

Results

LULC mapping and validation

For the years 1990, 2000, and 2018, six LULC classes were identified, such as urban area, water bodies, dense forest, sparse forest, agricultural cropland, and scrubland (*Figure 3*). *Table 3* displays the area of six LULC classes that were computed. Around 1990 and 2018, the urban area grew from 1648.8 hectares to 6379.2 hectares. Vegetation covers the western portion of the study area. Dense forest coverage rose from 219.15 hectares in 1990 to 780.57 hectares in 2018 (*Table 4*). The sparse vegetation, on the other hand, covered 10250 ha in 1990, but has shrunk to 9963.72 ha in 2018 (*Table 4*). In 1990, the cropland area was 1644.75 ha, but by 2018 it had shrunk to 604.8 ha. Scrubland occupied half of the study area in 1990, but it was reduced to 24.07 percent in 2018. The study area has relatively few water bodies, and even those that do exist have been converted into other land uses over time. The region of water sources in 1990 was 17.28 ha, but it has now shrunk to 15.48 ha. Over the study period, the metropolitan region experienced the greatest transformation, going from 4.46 percent to 17.24 percent.

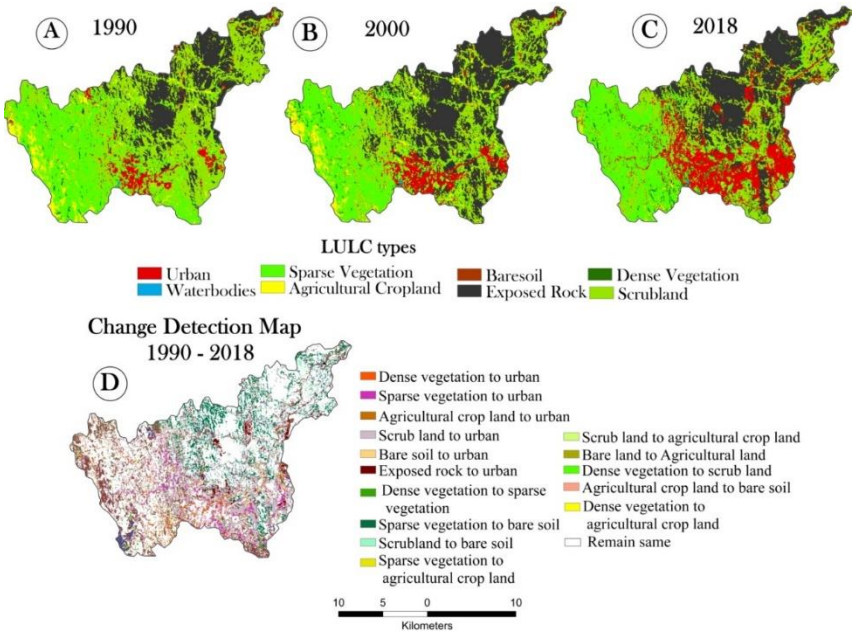

Figure 3. *Year wise change of LULC (1990 - 2018) (A,B,C) and change detection map (1990-2018)(D) of Abha watershed*

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Table 4. *Areal coverage of different LULC type from 1990 to 2018*

Land use types	1990		2000		2018	
	Area (ha.)	Area in %	Area (ha.)	Area in %	Area (ha.)	Area in %
Urban	1648.8	4.46	2211.93	5.98	6379.2	17.24
Water bodies	17.28	0.05	18.36	0.05	15.48	0.04
Dense vegetation	219.15	0.59	435.69	1.18	780.57	2.11
Sparse vegetation	10250.8	27.70	8058.33	21.78	9963.72	26.93
Agri. Cropland	1644.75	4.45	1666.71	4.50	604.8	1.63
Scrubland	15173.6	41.01	11928.1	32.24	8907.93	24.07
Bare soil	100.35	0.27	291.96	0.79	237.87	0.64
Exposed rock	7946.37	21.48	12390	33.49	10111.5	27.33
Total	37001.08	100	37001.08	100	37001.08	100

The LULC maps were validated using the Kappa coefficient. We calculated the kappa coefficient values for 1990 and 2018 using 250 Google Earth reference points and 60 ground control points. The kappa coefficient for all of the LULC maps is greater than 0.84, suggesting very strong conformity between classified maps and ground reality (K=>0.84).

Analysis of LULC dynamics

The urban area has improved dramatically from 5.98 percent to 17.24 percent, according to the findings of the change detection. Sparse vegetation, on the other hand, declined from 27.70 percent to 26.93 percent over the study period (*Figure 3D*). Scrubland also substantially reduced from 41.01% to 27.07%. Agricultural land, in addition to other natural resources, also declined spatially from 4.50 to 1.63 percent. The field of research includes smaller parts of waterbodies, but during the study time it was transformed. The rate has dropped from 0.05% to 0.04% (*Figure 3D*). The results showed that vegetation cover in terms of dense vegetation, sparse vegetation, and sparse land deteriorated over time as a result of urbanisation or human activity (*Figure 3D*).

Ecosystem service values (ESV) and its change

Total ESV maps for 1990, 2000, and 2018 were generated at a spatial scale using the coefficient of Costanza et al. (2014) (*Figure 4A-C*). The results revealed that the ESV of water bodies, sparse forest, agricultural land, and scrubland decreased, while the ESV of the urban area increased significantly. We calculated ESV for all LULC classes in 1990, 2000, and 2018. Then, during the periods from 1990-2000 and 2000-2018, we measured changes to the ESV for all LULC classes as defined in *Table 5*. Results showed that there was a declining trend of -3, 0.01, 0.8, - 0.1 and -43 million USD/y, respectively, in the period 1990–2000 for urban area, water bodies, dense vegetation, agricultural cropland and scrubland (*Table 5*). While there has been an increasing trend in the ESV in sparse vegetation of $9 million per year. However, since bare soil and exposed rocks did not have any ESV, these LULC classes did not see any changes. The ESV of water bodies, agricultural cropland, and scrubland increased by 0.03, 5, and 12 million USD/y, respectively, in the second phase (2000 to 2018) (*Table 3*). The ESV of the urban area, dense vegetation, and sparse vegetation, on the other hand, have demonstrated negative trends of -27 million USD/y, -1 million USD/y, and -7 million USD/y, respectively. ESV from urban areas was found to have risen in both phases.

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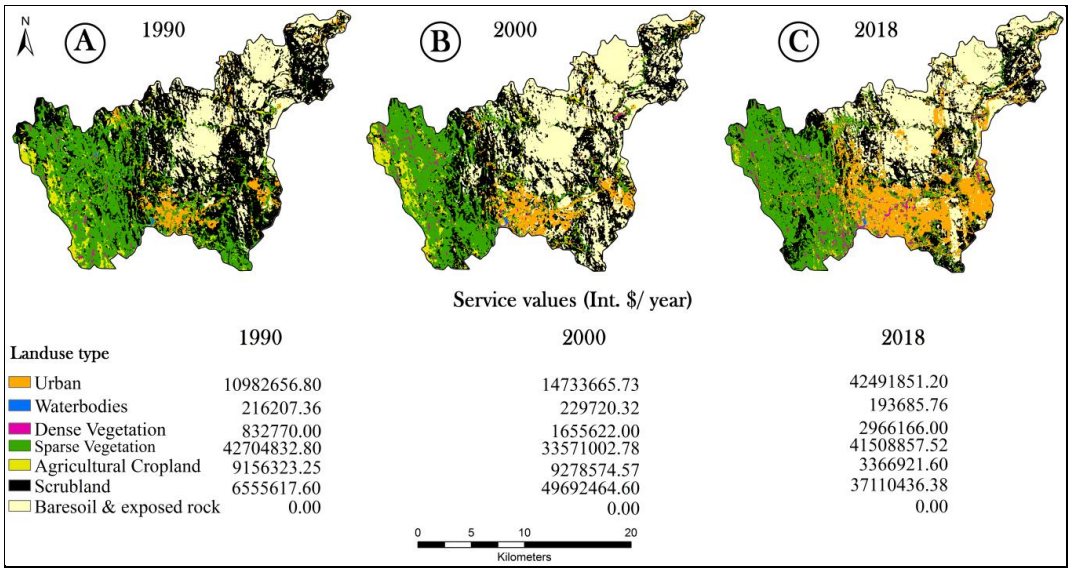

Figure 4. *Estimated total ESV since 1990-2018 by using the CV of Costanza (2014)*

Table 5. *Total ESV Change from 1990 to 2018 obtained from the CV of Costanza et al.*

Land use types	**ESV Change (1990 – 2000) ESV (USD /yr)**	**ESV Change (2000 – 2018) ESV (USD /yr)**
Urban	-3751008.93	-27758185.47
Water body	-13512.96	36034.56
Dense vegetation	-822852	-1310544
Sparse vegetation	9133830.02	-7937854.74
Agricultural cropland	-122251.32	5911652.97
Scrubland	-43371142.84	12582028.22
Bare soil	0	0
Exposed rock	0	0

Analysis of the effect of landscape fragmentation on ESV

Figure 5 shows the fragmentation state of the landscape of the years considered and its associated ESVs. *Table 6* presented the measured ESV of various fragmented categories such as patch, edge, perforated, small, medium, and large core of six LULC categories. During the study period, the area under edge was higher in the urban area, followed by patch, small core (<250 acres), and perforated (*Figure 5A,B,C*), indicating that the new urban area has been expanded around the main city and into natural resources, such as vegetation cover. During the study period, the region underneath water bodies was fragmented. The Edge of water bodies had the highest amount of area. In 1991 the area was 9.81 hectares and in 2000 and 2018 it steadily fell to 11.7 ha and 10.98 ha (*Table 6*). Dense vegetation had the highest area, followed by an edge between 1990 and 2018 (*Figure 5A,B,C*), indicating a considerable fragmentation of the dense forest over the years. During periods of study, the large core and medium core of sparse vegetation found that the sparse vegetation remained exactly the same throughout the study period. It was not substantially fragmented. In the case of agricultural cropland, the edge and

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patch had the greatest area as compared to another fragmented group in 1990 (patch 570.33 ha, edge 694.26 ha), 2000 (patch 599.13 ha, edge 656.37 ha), and 2018 (patch 545.31 ha, edge 50.31 ha) (*Table 6*). In the case of scrubland, the patch and edge occupied the most area and demonstrated a growing pattern over time (patch area: 1095.75 ha in 1990, 1256.13 ha in 2000, 1743.3 ha in 2018).

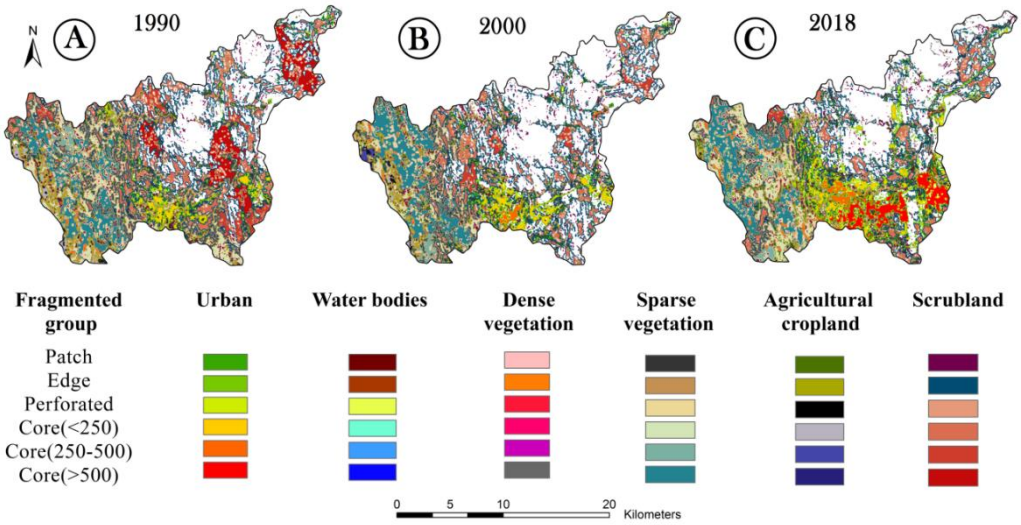

Figure 5. *Different fragmented landscape categories year wise*

The ESV of six LULC classes was calculated using the fragmentation integrated approach in 1990, 2000, and 2018 (*Table 7*). By taking fragmentation into account, the ESV of urban areas decreased during the study period (*Table 7*). In 1990, 2000, and 2018, the total ESV of urban areas was 9 million USD/year, 11 million USD/year, and 28 million USD/year, respectively (*Table 7*). As a result, the study area observed an increasing trend in projected ESV for urban areas. Since the spatial extension of water bodies was negligible, the ESV would be insignificant as well. Despite having a small spatial coverage, the ESV of water bodies showed a declining trend. This is a cause of concern about environmental changes. Over the study period, the ESV of dense vegetation changed by 0.7 million USD/year, 1 million USD/year, and 2 million USD/year, while the ESV of sparse vegetation changed by 27 million USD/year, 18 million USD/year, and 3 million USD/year (*Table 7*). For the years 1990, 2000, and 2018, the ESV adjustment after fragmentation for agricultural cropland was 0.7 million USD/year, 7 million USD/year, and 3 million USD/year, respectively (*Table 7*).

Scrubland's ESV adjustment was 12 million USD/year in 1990, 40 million USD/year in 2000, and 31 million USD/year in 2018 (*Table 7*). According to the results of the study, the ESV of all LULC classes has decreased after integrating the fragmentation effect (*Table 7*). In 1990, the largest change in ESV was seen in sparse vegetation (27 million USD/year), followed by urban areas (9 million USD/year) and agricultural cropland (7 million USD/year). Scrubland (40 million USD/year) and urban areas (31 million USD/year) witnessed the largest ESV transition between 2000 and 2018 (*Table 7*).

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Table 6. *Area of landscape fragmentation and estimated ESV in USD/year*

1990												
Fragmented group	**Urban**		**Water bodies**		**Dense vegetation**		**Sparse vegetation**		**Agricultural cropland**		**Scrubland**	
	Area in ha.	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**
Patch	427.59	122471.6	5.94	3195.815	162.36	26529.62	782.91	140248.9	570.33	136526.2	1095.75	196290.5
Edge	774.45	557130	9.81	13256.21	52.29	21459.82	4054.59	1824274	694.26	417414.1	6617.61	2977448
Perforated	110.43	154470.6	0	0	0	0	1845.36	1614432	60.84	71126.22	2154.96	1885288
Core(<250 acres)	336.33	831149.1	1.53	7102.187	4.5	6344.1	1159.83	1792617	319.32	659509.8	2601.27	4020486
Core (<250-500acres)	0	0	0	0	0	0	193.86	500724.9	0	0	788.49	2036607
Core (>500acres)	0	0	0	0	0	0	2214.27	9224649	0	0	1915.47	7979848
Total	1648.8		17.28		219.15		10250.82		1644.75		15173.55	
2000												
Fragmented group	**Urban**		**Water bodies**		**Dense vegetation**		**Sparse vegetation**		**Agricultural cropland**		**Scrubland**	
	Area in ha.	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**
Patch	485.19	138969.6	3.51	1888.436	350.55	57279.87	996.12	178442.9	599.13	143420.3	1256.13	225020.6
Edge	964.44	693806.6	11.7	15810.16	76.86	31543.34	2532.33	1139366	656.37	394633.3	6126.12	2756313
Perforated	165.24	231139.4	0	0	0	0	1175.94	1028783	94.95	111003.2	1567.8	1371606
Core(<250 acres)	445.68	1101378	3.15	14622.15	8.28	11673.14	531.18	820984.4	208.26	430131.2	2385.09	3686362
Core (<250-500acres)	151.38	625172.2	0	0	0	0	184.68	477013.7	108	372766.3	592.92	1531465
Core (>500acres)	0	0	0	0	0	0	2638.08	10990241	0	0	0	0
Total	2211.93		18.36		435.69		8058.33		1666.71		11928.06	
2018												
Fragmented group	**Urban**		**Water bodies**		**Dense vegetation**		**Sparse vegetation**		**Agricultural cropland**		**Scrubland**	
	Area in ha.	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**	**Area in ha.**	**ESV**
Patch	1239.03	354886.7	1.98	1065.272	512.82	83794.79	1305.09	233791.2	545.31	130536.9	1743.3	312291.3
Edge	2476.53	1781586	10.98	14837.23	250.38	102756	2476.08	1114058	50.31	30248.18	4421.7	1989447
Perforated	590.4	825857.4	0	0	1.44	1149.12	2336.49	2044102	5.49	6418.194	871.83	762729.2
Core(<250 acres)	612.45	1513505	2.52	11697.72	15.93	22458.11	978.75	1512742	3.69	7621.167	1744.74	2696646
Core (<250-500acres)	268.65	1109476	0	0	0	0	264.24	682510.8	0	0	126.36	326377.8
Core (>500acres)	1192.14	7940845	0	0	0	0	2603.07	10844390	0	0	0	0
Total	6379.2		15.48		780.57		9963.72		604.8		8907.93	

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Table 7. *Computed ESV gap after fragmentation in different LULC units*

Land use types	1990	2000	2018
	ESV change (USD /year)	ESV change (USD /year)	ESV change (USD /year)
Urban	9317435.5	11943199.93	28965695.1
Water body	192653.148	197399.574	166085.538
Dense vegetation	778436.46	1555125.65	2756007.98
Sparse vegetation	27607887	18936171.78	16431594
Agricultural crop land	7871746.93	7826620.27	3192097.159
Scrubland	12774645.74	40121698	31022945.08
Bare soil	0	0	0
Exposed rock	0	0	0

Discussion

It is evident from the research that the study region has experienced massive LULC dynamics over the last 30 years. Land use change has been noted in all LULC classes. Other land use types, such as sparse vegetation, scrubland, and dense vegetation, have been reclaimed by the built-up area as a result of the rapid and incremental urbanisation process. As a result of this type of urbanisation trend, environmental changes have worsened, affecting the landscape as well as biodiversity (Dong and Xu, 2019). Agricultural land, on the other hand, showed a declining trend over time. These results are primarily in contrast to those of other researchers (Han and Song, 2019). The reason behind the decline in agriculture is that the study area is a hilly region, so a limited amount of flat or slope-based areas for conventional agriculture are available. Since the year 2000, urbanisation has resulted in the conversion of certain agricultural lands. Rapid and unscientific urbanisation of mountainous regions is a cause for concern, since the mountain environment is regarded as one of the most natural locations on the world (Han and Song, 2019). While the conversion of vegetative land to other commercial land has been noted in other mountainous regions as well (Corton et al., 2020; Shi et al., 2020). Previous research has found that one of the causes of the declination of the vegetative area and other natural resources is the growth of the settlement area. The study area therefore noted an enormous shift in land use that resulted in a fragmentation of the landscape, particularly vegetation cover and other natural resources. This landscape fragmentation would have an effect on the ESV, which in turn has an impact on the environment as well as the livelihood status of the local people.

Based on the interpretation of ESV changes, the study area found a declining trend in natural resources such as vegetation cover and water bodies over time. The ESV in urban areas has been rising. This has occurred as natural land covers, such as dense vegetation, sparse vegetation, agricultural lands, and water bodies, have been transformed into anthropogenic land uses, such as settlement areas and barren land. Not only in this region, but also in other countries, such as India, China, Bangladesh, etc., this condition has been noticed. Because of the high demand, unscientific resource discovery and mismanagement lead ESV to decline over time. The total ESV of 2.43 billion USD in northeast China was damaged in just 35 years because the grasslands were produced for minimum benefit (Wang et al., 2015). Similarly, ESV from natural land cover has

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decreased in the present study area. With the advent of commercial operations barren land and exposed rock have declined over time (Mallick et al., 2014).

The present study, on the other hand, stated that the ESV was estimated using the global coefficient given by Costanza et al. (2014) and newly developed fragmentation corrected weights. The coefficient ESV and the fragmentation corrected ESV were found to be quite altered. The integration of fragmentation and weightage by AHP has improved the accuracy of ESV estimation.

Conclusion

This analysis calculated the cumulative ESV of various land use and land cover units over time, as well as the impact of fragmentation on ESV. The LULC change was discovered. The LULC transformation affects the ESV of this region. Research has however been done on the mountainous areas, but changes to ESV and LULC would also influence the plain climate. In addition, this analysis also modelled the fragmentation effect on ESV. The declining trend for ESV was observed to be diminished by the increased fragmentation of natural land use classes. The effect of fragmentation is measured by calculating the distance between ESVs without and with fragmentation. In dealing with this problem, the calculation of ESV gain or loss and its spatial distribution is novel in this work.

The large core areas were considered to be in ecologically good shape in order to provide the ESV. The theoretical weight was assigned for determining the effect of fragmentation based on this definition and expert opinion. It did better when calculating the ESV. Although the current research has certain drawbacks, such as the use of coarse resolution satellite images, the use of conventional MLC classifiers, and a smaller field survey. One challenge for this research is obtaining cloud-free images, without which we cannot accurately evaluate the area, and this is one of the study's main limitations. Many times, these images provided inaccurate results due to spectral mixing in the zone of hill shadow. Obtaining a specific result for the truth of the field was another obstacle to overcome for this work. However, in the future, ESV estimation can be enhanced by addressing the issues listed above, such as high-resolution satellite images, detailed field surveys, and the use of machine learning algorithms, deep learning algorithms for LULC classification, and sophisticated econometric models.

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Conflicts of Interests. The authors declare no conflict of interests.

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WATER PRODUCTIVITY, EXPLOITATION AND FOOTPRINT: OBSOLETE CONCEPTS OR REPRESENTATIVE TOOLS IN UNDERSTANDING EUROPEAN ENVIRONMENTAL POLICY?

ANDREI, J. V.[1*] – BĂLĂCESCU, A.[2] – CHIVU, L.[3] – GOGONEA, R. M.[3] – PATRASCU, A.[1] – ZAHARIA, M.[1]

[1]*Petroleum - Gas University of Ploiesti, Faculty of Economic Sciences*
B-dul Bucuresti, No.39, 100680 Ploiesti, Prahova, Romania

[2]*Faculty of Economics, Constantin Brâncuși University of Targu Jiu, Gorj County, Romania*

[3]*National Institute for Economic Research - Romanian Academy*
Calea 13 Septembrie 13, Casa Academiei Romane, 050711 Bucharest, Romania

[4]*Faculty of Cybernetics, Statistics and Economic Informatics, The Bucharest University of Economic Studies, No.6, Piata Romana, 010374 Bucharest, Romania*

**Corresponding author*
e-mail: andrei_jeanvasile@yahoo.com; phone: +40-72-761-5540

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Abstract. In contemporary economies, water represents a key resource in ensuring the sustainable economic development, being equally a factor of production in the economic branches, and also a relevant indicator in shaping the evolution of human activity. Water resources are vital for humanity, ecosystems and the economy, and its inefficient use is a serious problem. The main aim of the present research was to analyse and evaluate the impact of water use, from the perspective of a sustainable economic development, and to determine to what extent it can become a relevant indicator in this respect. For this purpose, three indicators were considered: water productivity, water exploitation index and water footprint. Based on the findings highlighted in the research, it can be concluded that the considered indicators can constitute representative instruments in understanding the European environmental policy.
Keywords: *aquatic systems, ecosystem, environmental efficiency, hydrographic shortage, water management, water deficit, pollution, sustainability*

Introduction

In the context of contemporary economic evolutions marked by increasingly pronounced manifestations of some negative climatic phenomena the reconsideration of the water resource management and its adaptation to the new global exigencies became mandatory. It also emphasized the necessity for an integrative approach in regard to these resources. Water use analysis is a mandatory instrument of sustainable development. It may serve as a sensitive compass ready to show the degree of economic growth, and as an instrument orienting decision makers to those economic policies that do not ignore the limited nature of this resource, all while recognising the need to reconcile accessibility to water.

The worsening of the water deficit, wastefulness, reckless pollution of water sources, the ever-increasing length of droughty periods, the poor management for water, the irresponsible catchment of flowing water are all arguments in favour of a new approach to water management as a way to ensure the balanced development of economic activities, and a sustainable future of human communities. The misuse of water

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resources may have serious and irreversible effects on economies and societies: it may jeopardise very existence of industries relying on intensive use of water, or it may cause, by depletion, the abandonment of entire geographic areas.

In this context, the water footprint may be viewed as one of the specific analytical indicators of social and economic development, and, no less, as a challenge for experts, because this indicator must be able to reveal, as true to fact as possible, the complex aspects of the quality of human life, accepting the prerequisite that water is both an economic resource (Winpenny, 2005), and a vital source of life (Oki and Kanae, 2006). This may be equally a challenge to experts, because the indicator must be able to reflect the complex aspects of human life in a realistic manner, knowing that water is not only an economic resource, but also the vital source of human life, and that we are already witnessing the globalisation of the water issue. This, in turn, reflects on the environmental challenges affecting societies and economies, and therefore requires a multidisciplinary and trans-societal approach (Hogeboom, 2020).

The debate on water use as a possible indicator which reflects the sustainability of the economic development returns in the literature with a new perspective, following the transformations of the paradigms of reference for the contemporary society. The interdependence between the sustainable management of water and economic development is obvious and holds the attention of the dedicated literature. It is generally recognised that irresponsible consumption and wasteful use of water are two factors that add to the already existing pressure on the uncontrollable exploitation of ground and surface water. The reckless use of water generates a wide range of risks and vulnerabilities in contemporary economies, from the irreversible degradation of the environment, or the appearance of an unsustainable cycle of economic and social activities, or even social unrest, to jeopardising food safety and security, or endangering aquatic systems and landscapes.

The literature related to this subject (EESC, 2013; Sima and Gheorghe, 2015; D'Ambrosio et al., 2020) is unanimous in asserting the vital role of water in supporting life, human activities, nature, and economy. Although permanently regenerated in its natural circuit, water is however a limited resource that cannot be replaced or substituted for.

With all these aspects in mind, understanding water and its specific indicators as possible instruments for the determination of the level of economic development is part of the attempts of identifying indicators relevant for measuring sustainable development, in close connection with the global changes in the paradigm of contemporary economy. As Wada et al. (2016) pointed out, water consumption by humans in the 20th century grew six times as a consequence of the Globe's fourfold population increase, and the rising of its standard of living. This increased the pressure on the water resources, through even more waste. From this perspective, the concept of water footprint (WF) may gain recognition as an instrument orienting towards more sustainable water use patterns and consumption habits (Aldaya et al., 2010).

Starting from the realities of the contemporary economy, the concepts of water footprints and the efficiency of water use can join the concept of sustainable economic growth, taking into consideration that water resources are indispensable to the evolution and the development of the human society.

The water footprint can represent a much more comprehensive and complex concept (Jean et al., 2018; Gogonea, 2019), of multidimensional nature which also implies different interpretations and meanings, compared to the classical indicators for

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measuring sustainable development. The necessity of changing the referential imposed by the current economic demands requires the approach of the human system from the perspective of its relations with the ecosystem, and the water footprint can realize this sort of connection.

The responsible management and use of water resources have become one of the most provocative challenges the contemporary society is facing, in the context of the necessity to adapt to the new transformations and requirements of sustainable economic development. Starting from the fact that the environment represents a fundamental element of sustainable development, which should not be understood only as a source of production factors or a support element for the human activity, but also as a result of the complexity of the evolution of the contemporary society, environment has become a topical issue which regards the identification of those relevant factors imprinting the evolution of the current global economic transformations. From this perspective, the problematic of the efficient use of water, and also the identification of certain reliable indicators which would allow the mapping of this resource is of actuality and widely debated in the specialized studies, highlighting numerous correlations, limits and interdependencies between the multiple aspects of sustainable development. Thus, numerous researches have analysed the efficiency of water use as a significant component of the environment, at territorial level (Dietzel et al., 2016; Hernández et al., 2015; Andrei et al., 2020). However, there is also a certain limit, respectively the efficiency of water use cannot be measured directly on a global scale, which requires the deepening of the knowledge and management of the water processes (Xiao et al., 2013), simultaneously with the development of the remote sensing technology.

The responsible management of water resources, but also the efficiency of its use are fundamental and of major importance due to the fact that water represents a vital resource for humans and ecosystems, but with a tendency of becoming an economic asset under the conditions of an increasingly insufficient access to safe, clean water at an affordable price for the population which has become more and more numerous. As observed by Vörösmarty et al. (2015), the local water consumption has become a global problem, requiring global approaches.

The transfer and relocation of water implies not only the realization of certain substantial and long-term investments but can also irremediably affect the environment and the natural habitats. Meanwhile, water is indispensable in carrying out the activities for all economic sectors, but the most consuming economic branches, as proved by a number of specialized studies, are agriculture and manufacturing, a fact that determined us to consider them for this research. In this context, regional and global analyses have become more and more relevant for a domain in which water is understood not only from the perspective of a rational economic use, but also as a vital source, indispensable for human evolution. Considered to be a significant component of the environment, water as an economic resource can be analysed and understood through certain important indicators such as: water productivity (WP) and water exploitation index (WEI).

As Lipinska (2016, p. 114) notes the high intensity of the water resources exploitation in the case of numerous European states, exposes these countries to incidence of phenomena such as ‘severe water scarcity’ or ‘water scarcity’. In line with this approach, Arnel et al. (2011) argues that European countries do not always use water efficiently, in order to contribute in reducing the access and the availability of

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water resources, or by contrary their behaviour provoke even dramatically impact on climate changes.

The water deficit, doubled by the inconsistency of the management measures taken to protect this strategic resource emphasizes the necessity for a more thorough research in the field, and the application of new, environment-friendly technologies, in the domains with intense water consumption. The problematic generated by the water deficit has accentuated, as proved by the much more dynamic frequency of the drought periods and the extension of the geographic areas of manifestation of hydrographic shortage. From this perspective, the necessity of convergence, with a particular stress on the EU space, for the construction of a European model of sustainable development has brought to the fore the tools specific for footprints. Very often, the water footprint goes hand in hand with a carbon footprint (Kubova et al., 2018; Poom et al., 2017; Schlegel et al., 2016) or with the field footprint (Chen et al., 2018; de Ruiter, 2017).

The responsible management of water resources, the appropriate management of waste, the reduction of the greenhouse effect and of energy consumption, the sustainable exploitation of sources of energy, the protection of the environment and natural landscapes, are all important goals to be pursued for the purpose of ensuring the sustainable development of human society. This paper is intended as a background analytical research of the correspondence between the three indicators and the exigencies of a sustainable development in the two economic sectors chosen for this purpose: manufacturing and agriculture.

With the aid of the three indicators considered, water may act as a relevant sensor of the level of sustainable development and of the interaction between human existence and environment. As such, water can be used in practice as a scientifically reliable unit of measure for those aspects of real economy that are fundamental for the wellbeing of human condition. Therefore, water footprint, water productivity, and water exploitation are concepts that should not be regarded or understood as models or indicators per se, but rather as a complement to the ample debate pursuing to clarify the mechanisms that underlie sustainable development in our times, and to render efficiency to environmental policies. It is from this perspective that the title question arises: are these obsolete concepts or representative tools in understanding environmental policy?

Until now, the importance and usefulness of the water footprint as an indicator for the analysis of sustainable development has been insufficiently highlighted in the context of the indicators developed and diversified for the purpose. The literature is still endeavouring to find suitable indicators to measure the water consumption needed to manufacture a product or provide a service, but also capable to assess the environmental impact of various consumption and production patterns.

The study carried out and presented in the current article aimed at two complementary, interconnected objectives, respectively one prior to the analysis itself and another one, the main one, which took into consideration the analysis itself which meant the identification of the main realities and tendencies. The first objective is to emphasize the image regarding, on the one hand, the relation between water exploitation indices and its productivity, and on the other hand, the relations between internal water footprints used for the production of agricultural products and those used for the production of industrial ones. The second objective, the main objective of the study, was to identify and evaluate several possible relations between the internal water footprint used for the generation of a product and the efficiency of its utilization. To lay emphasis on the elements specified above, the article was structured in six sections:

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introduction, a systematic review of the specialized literature, the methodology used to validate the objectives proposed in the research, a section presenting the results obtained and the related discussions, and a section of conclusions and references. Also, an additional section of research limits and further directions of investigation was included in the article.

Literature review

The water footprint concept constitutes not only an opportunity to understand the water consumption from the perspective of sustainability and the choice of practices of responsible water use as sustained by Aldaya et al. (2010), but also constitutes an instrument of integrative knowledge of the aspects related to the water use in its close connection with the social challenges, the economic environment or related to ensuring sustainable economic development, as described by Chapagain and Tickner (2012).

The water footprint belongs to a much wider and diverse family of analytical instruments. It is the family of footprints, which complements and adds to a range of indicators that help us measure and understand the concepts of sustainability and wellbeing. A drawback but also an opportunity for research along this line is the very diversity of the methodologies used to give a scientific basis to such footprints, and their limited capacity to substantiate environmental policies and to integrate into the existing specific indicators. At the same time, as Chapagain and Tickner (2012) have also demonstrated, the water footprint with its various components can have a significant contribution to enriching and diversifying the knowledge about the closely-knitted connections between the use of water, sustainable economic development, and the contemporary social and environmental requirements.

The methodology based on the use of footprints has become a widespread instrument in the specialized studies, and is utilized to understand the different aspects regarding the sustainable development. The water footprint does nothing more than to complete this diverse scientific landscape, and contribute to the refining of the entire concept of sustainable development.

The concept of the water footprint was initially introduced in Hoekstra's study, in 2003, which proposed a specific indicator which would directly or indirectly measure the volume of freshwater consumed in order to produce goods or services consumed by an individual, a country, a community, a campaign or another organization. This indicator has been refined and supplemented by other methodologies specific to the domain such as Water Remaining (AWARE) method (Ansorge and Beránková, 2017) or water resources exploitation (WRE) (He et al., 2019). As shown in the literature (Harding, 2019), terms such as water footprint, water accounting, water use intensity, are used to describe the quantity of water used or incorporated to obtain a product, process or service, or as the case may be, the efficiency of water use.

Studies, such as the ones conducted by Lovarelli et al. (2016) and Jeswani and Azapagic (2011), analysing the existing approaches in the literature regarding the water footprint emphasize the strong points and the conceptual limitations, concluding that there are immense variations in the results obtained in these studies, as a cause of either the methodologies utilized, the data availability or other random causes. Returning to the water footprint and the efficiency of water use, the specialized literature has offered a wide range of studies, methodologies and areas to which this can be applied.

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Researches regarding the water footprint aimed especially at agriculture, as proven by Ewaid et al. (2019), Barbosa et al. (2017), Lovarelli et al. (2016) and Zhao and Chen (2014), taking into consideration the quantity of water used in this domain, the obtainment of animal products and their processing to which the animal husbandry processes can be added (Mourad et al., 2019; de Miguel et al., 2015; Lee et al., 2015).

Thus, Scheepers and Jordaan (2016) analysed the intensity of blue and green water use in the case of alfalfa used in the feeding of lactating cows, Owusu-Sekyere et al. (2016) quantified the amount of water used for the milk production and processing in South Africa, Zonderland-Thomassen and Ledgard (2012) used and compared in their study two methodologies for estimating the water footprint in the case of milk production in two regions of New Zeeland, Zoumides et al. (2014) economically evaluated the efficiency of water use for the production of several agricultural crops in Cyprus, Dourte et al. (2014) using a web-based tool estimated the water footprint of different agricultural products in different regions of the USA, and Chiu et al. (2015) calculated the water footprint in the case of second-generation bioethanol. In studies such as the one conducted by Borsato et al. (2018) Schafer and Blanke (2012) and Stoessel et al. (2012), both the water footprint and the carbon footprint generated during the production of numerous fresh agricultural products are calculated. Roibas et al. (2015) investigated the sustainability of banana culture systems in several organic plantations, from the perspective of the water use availability. Suttayakul et al. (2016), using the WFA methodology, quantifies in his research the volume of freshwater consumed and the degradation in the case of palm tree oil plantations. Page et al. (2012) concludes that the use and especially the efficiency of freshwater use is closely determined by the production system in use. Serio et al. (2018) applied the Grey Water Footprint (GWF) for the determination of groundwater nitrate contamination level of soil, generated by agriculture in the Southern Apulia Region, Italy.

Several studies such as the ones elaborated by Ababaei and Etedali (2017) used the water footprint to estimate the production of wheat, barley and corn in Iran and as noted by Garofalo et al. (2019), in the case of Germany and Italy, two important countries in the winter wheat production, the emphasized models underlined a decrease of the future water footprint precisely due to the efficiency of water use and the improvement of the plants in water capitalization and the stocking of this resource in the soil.

In what regards the industry, Gerbens-Leenes et al. (2018) evaluated the water footprint in the case of two types of steel: alloyed and unalloyed, using the data from global databases available. Ma et al. (2018) and Gu et al. (2015), also evaluated the water footprint generated by the steel production in China, and Burchart-Korol and Kruczek (2015) evaluated the water deficit in the case of steel production in Poland. Similarly, Grey Water Footprint can be used to measure the level of contamination of water resources with pollutants that have a much higher noxious effect on health, such as: Mercury (Hg), Vanadium (V) and Ammonium (NH^{4+}), as Miglietta et al. (2017) argues.

However, there are studies such as Jamshidi (2019) which argue the limitations of the concept, that the water footprint measures only the quantities used in agriculture, manufacturing or households, without incorporating, for example, activities such as aquaculture (Vanham, 2016), which is strictly based on the use of water as a support. Taking into consideration the diversity of the elaborated studies focusing on water as a complement, our research comes to add to the range of specialized studies devoted to the evaluation of sustainable development from the perspective of the intensity of the

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use of this resource, trying to contribute to the expansion of the inventory of specific knowledge in the field.

As Hogeboom (2020) also suggests, there is an increasing number of studies in the literature, which use the concept of water footprint, in a variety of forms and applications that have been developed due to the complexity, gravity, and topicality of the critical issues arising from the need to manage and use water in a responsible manner. For example, according to the opinion of Gómez-Llanos et al. (2020), the water footprint has gained recognition as a multidimensional indicator for the direct measurement of the water consumption with the aid of the two parameters, blue and green water, but also as a measure of the pollution level, based on the grey water concept. WF tools, alongside with indicators like water productivity and water exploitation, may be regarded as representative tools in understanding environmental policy, as part of a general framework in which effort is being made to better understand and broaden the system of indicators needed to measure the environmental dimension of sustainable development.

Research methodology

The methodology applied in this manuscript for arguing the main objectives described in this research follows the concepts already described in previous studies as Andrei et al. (2018) and Andrei et al. (2020) with some particularities presented in this section. Also, the possible linkage and mutuality effects of water productivity, exploitation and footprint on understanding the tendencies and exigencies of the European environmental policy are analyzed using the comparative analysis of the absolute values of the considered indicators, recorded for each of the EU countries and the cluster methodology.

The first indicator used is water productivity (WP) indicates the quantity of economic production per cubic metre of freshwater extracted (Arnell et al., 2011) (in EUR per m^3) emphasizing the efficiency of its use. In its evaluation, the water from any source of fresh water, permanently or temporarily, mining water, flowing water, as well as the water from precipitations is considered. For a more conclusive picture of the availability and the efficiency of the use of water resources, the study included the water exploitation index (WEI). WEI measures the total annual freshwater extraction in a country as a percentage of the long-term annual average (30 years) of available water from renewable freshwater resources (Sheikhipour et al., 2018; Visentin and Guilhoto, 2019; Eurostat, 2020a) and water from any freshwater source, permanently or temporarily, mining water, flowing water, as well as water from precipitations.

Internal water footprint of consumption of agricultural and industrial products (Hoekstra and Chapagain, 2006) includes water from internal sources and which is consumed for the production of industrial and agricultural products. This includes water from underground or surface resources (blue water), the amount of freshwater needed to assimilate pollutants in order to meet the quality standards specific to water (gray water), as well as rainwater (green water) in the case of agricultural production.

The data set employed in designed and emphasizing the main objectives asset and described in the current research were retrieved from Eurostat (2020 a, b) in case of water productivity and exploitation indicators. Some statistics related to water footprints are collected from a deviated case study of Mekonnen and Hoekstra (2011). The

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indicators to assess the water sustainability as representative tools in understanding European environmental policy were: water productivity, exploitation and footprints as they are described in *Table 1*.

The research is performed on the EU level for the countries where the data were available to estimate if the analyzed water indicators are obsolete concepts or representative tools in understanding European environmental policy. Then the results are discussed and compared in a larger framework in order to identify the determinants between water exploitation indices and its productivity, on one hand, and to evaluate several possible relations between the internal water footprint used for the generation of a product and the efficiency of its utilization the on the other hand.

The current study works with various water indicators and measurements analyzed in case of the EU countries. It was also taken into consideration the fact that data and information related to water footprint estimation are scarce in some EU countries, therefore, the analyses and discussions were limited and focused on to these aspects. Taking into consideration that out of the 27 EU member states, for four states (Austria, Finland, Ireland and Italy) there is no available data on water productivity, they were not included in the analysis of the possible relations between the internal water footprint consumed for the production of agricultural and industrial products and the efficiency (the productivity) of its utilization. As data series used also included the UK, as well as the relationships existing at that time between the UK and the EU, we included UK in our analysis as well.

Taking these aspects into account, six indicators were used in the study. The abbreviations, their significance and their units of measurement are presented in *Table 1*.

Table 1. *Significance and units of measurement for the utilized indicators. (Source: authors based on Eurostat, 2020 a, b; Mekonnen and Hoekstra, 2011)*

Indicator	Significance	Unit
WP	Water productivity	Euro per m^3
WEI	Water exploitation index	Percentage
GWFAP	Green Water footprint of consumption of agricultural products	m^3/yr/cap
BWFAP	Blue Water footprint of consumption of agricultural products	m^3/yr/cap
GyWFAP	Gray Water footprint of consumption of agricultural products	m^3/yr/cap
BWFIP	Blue Water footprint of consumption of industrial products	m^3/yr/cap
GyWFIP	Gray Water footprint of consumption of Industrial products	m^3/yr/cap

An overview regarding the relations between the water exploitation indices and its productivity, as well as the relations between the internal water footprints used to obtain agricultural products and those used to produce industrial products, was emphasized by applying a quantitative descriptive method. This method is based both on the comparative analysis of the absolute values of the six indicators, recorded in each of the states included in the study, as well as through the relations between them.

To achieve the second objective of this study, the identification and the evaluation of several correlative relations between the internal water footprint used for the production of products and the efficiency of its use, the cluster methodology was used. For this purpose, starting from the vectors corresponding to the six indicators analysed above, the matrix Z was constructed.

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$$Z = \left\| z_{ij} \right\|_{\substack{i=\overline{1,6} \\ j=\overline{1,24}}} . \quad \text{(Eq.1)}$$

Proximity matrix was obtained using Euclidian distance:

$$W = \left\| w_{jl} \right\|_{j=\overline{1,24},\, l=\overline{1,24}}, \quad w_{jl} = \sqrt{\sum_{j=1}^{24} \left(z_{il} - z_{ij} \right)^2}, \; i = \overline{1,6}, \; l = \overline{1,p}, \; j \neq i, \; k \neq i, \; w_{ii} = 0 \quad \text{(Eq.1.1)}$$

In *Equation 1,* d_{ij} represents the square average of the sum of the square differences between each of the six types of water footprint of national consumption per capita registered in the countries i and j.

Ward's method was generated to determine the distance between clusters (Gogonea, 2019):

$$\Delta(A,B) = \sum_{i \in A \cup B} \left\| x_i - m_{A \cup B} \right\|^2 - \sum_{i \in A} \left\| x_i - m_A \right\|^2 - \sum_{i \in B} \left\| x_i - m_{\mathbf{B}} \right\|^2 - \frac{n_{A \cap B}}{n_{A \cup B}} \left\| m_A - m_B \right\|^2 \quad \text{(Eq.2)}$$

In *Equation 2,* A and B are two clusters, m_i is the centroid, n_i is the number of elements from cluster *i*. and x_i an item. Levene's Test and Robust Tests of Equality of Means were used to choose the method for testing the significance of the six variables at the clusters.

Let be a group in r clusters. The null hypothesis of Levene's Test is:

$$H_{0\_1} : \sigma_1^2 = \sigma_2^2 = \sigma_3^2 = \ldots = \sigma_r^2 \quad \text{(Eq.3)}$$

The acceptance condition of the null hypothesis H_{0_1} is:

$$Sig.F > \alpha \quad equivalent \quad to \quad F_{stat} < F_{\alpha, r-1, n-r} \quad \text{(Eq.4)}$$

In case of accepting the null hypothesis (*Eq. 3*) the methodology ANOVA can be applied to test the statistical significances of the appurtenance of the variables to the clusters. Otherwise, we analyse the results of Robust Tests of Equality of Means, whose null hypothesis is:

$$H_{0\_2} : m_1 = m_2 = m_3 = \ldots = m_r \quad \text{(Eq.5)}$$

The acceptance condition of the null hypothesis H_{0_2} is the same (*Eq. 4*). In case of accepting the null hypothesis H_{0_2}, it results that the averages of the variables at cluster level do not differ significantly and the appurtenance of the variables to the clusters is significant. Consequently, the appurtenance of the variables to the clusters is significant only if for all the six variables the hypothesis H_{0_2} is rejected.

For the validation of statistical hypothesis, the Confidence level of 95% ($\alpha = 0.05$) was used. In exceptional cases, the Confidence level of 90% ($\alpha = 0.10$) was also admitted. The lack of a standardized approach to the special changes regarding the water availability in dynamic environments allows the analysis of the capacity to

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evaluate the ecological impact of the changing of water availability, prioritizing the sustainable management strategies of water capitalization, of the efficiency of its use.

Results and discussion

The distribution of the forms of relief, as well as the climatic conditions in the European states make the sources and the water reserves of each country differ significantly. Starting from the assumption that the abundance or lack of this resource can lead to different attitudes about the efficiency of its use from one state to another, a first analysis aimed to identify, on the one hand, both the water reserves (water obtained from lakes, artificial basins, rivers and underground) used by each of the 23 countries included in the research, and on the other hand the efficiency of the water quantities used.

In this case the two indicators (WEI and WP) show significant differences between the countries analysed and presented in *Figure 1*. A first observation is that there are countries with significant water reserves, which record very low productivity levels: Bulgaria 7.9 euro/m^3, Estonia 11.6 euro/m^3, Greece 16.4 euro/m^3. At the same time, there are countries with small water reserves, which use them in very large proportions as in case of Malta, 49.7% and Cyprus, 72.3% and have much higher productivity values (Malta 229.3 euro/m^3).

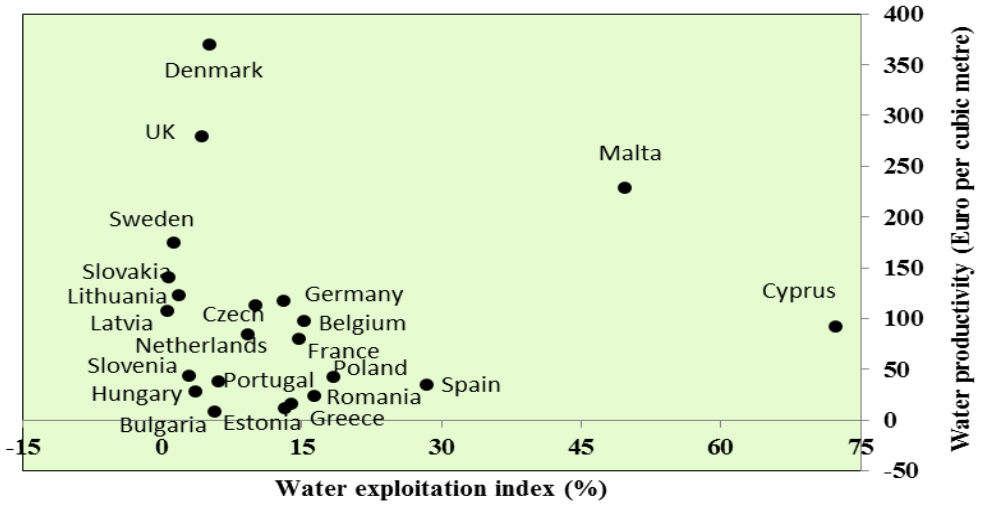

Figure 1. *Water exploitation index vs. water productivity. (Source: authors' own design)*

This is also emphasized by Lipinska (2016) who also points out that Malta and Cyprus are island nations with severe water scarcity but some countries like Sweden, Slovakia, Latvia and Croatia are countries in the bottom 1% of the WEI and they are not experiencing water-stress challenges. In this regard, it should be underlined that, a value of the water exploitation index of over 20% implies that the water resource is under stress, but over 40% indicates stress and even severe stress regarding its most efficient use (Raskin et al., 1997).

A second observation highlights the fact that there is no determination between the percentage of water exploitation and its productivity, which leads to the conclusion that the efficiency of water use is not given by the restrictive nature of this resource in some

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European countries but by the quality and productivity of the technologies used in the production processes of manufacturing and agriculture. This conclusion is strongly supported by the position of Denmark, the European leader in the sustainable water use (370 euro/m3), which uses only 5% of the water supply in the production processes. Denmark is followed by the UK with a productivity of 280 Euros/m^3 and Sweden with 175 Euros/m^3, although they consume only 4.2%, respectively 1.2% of their water reserves.

Regarding the ratio between the indicators Blue water footprint of consumption of agricultural products and Blue water footprint of consumption of industrial products (*Fig. 2*), it can be noted that, countries from southern Europe (Cyprus, Greece, Spain, Portugal) are detaching clearly compared to the other EU member countries, as the values recorded for the two variables in these countries are extremely different from those recorded by the other EU countries. Spain is the most arid country of the European Union and the one that devotes most of the water resources to irrigation, which has led to the development of the agri-food system (Clar et al., 2017) involving pressures on water.

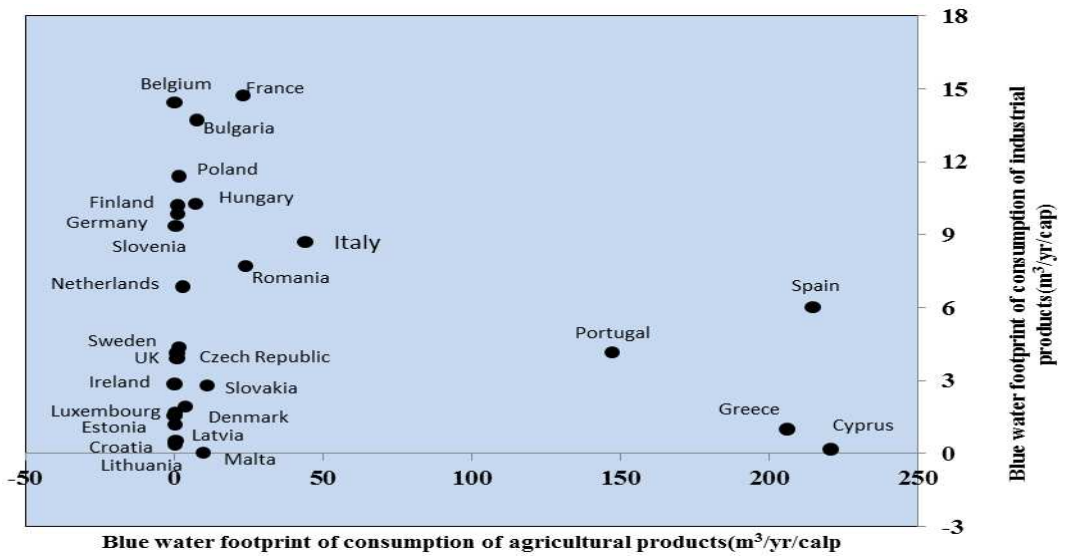

Figure 2. *Agricultural products vs. industrial products blue water footprints. (Source: authors' own design)*

The abstraction of water for industrial use has decreased in the last 2 two decades, partly due to the general decrease of the heavy industry as an intensive water consumer, but also due to the increase of the efficiency of water use as a result of the implementation of advanced cooling technologies that require less water. In the top of the EU countries, the highest water consumption values for agricultural and industrial products from the blue water footprint are recorded in Belgium and France, followed by Bulgaria.

Belgium is the country where industrial production uses the largest share of the total water footprint in the country. The water footprint of industries in Belgium measured between 1996 and 2005; used 41% of the total water footprint in the country, and the agricultural production used 53% (Hoekstra and Mekonnen, 2012).

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The blue water footprint of production in France is dominated by consumption for agricultural products, especially maize. Other crops with a significant share in the blue water footprint are fodder crops, potato, soy, rice and apples. The priority basins, as regards the blue water footprint of the French production, are the Loire, Garonne, Seine and Rhone basins.

A marked deficit of Blue water footprint of consumption of agricultural products and Blue water footprint of consumption of industrial products can be found in countries such as Malta, Lithuania, and Croatia. In fact, Malta has the highest level of external water dependence (92% dependence), its freshwater supply coming from other countries.

The spatial distribution of Gray Water footprint of consumption of agricultural and industrial products in UE is shown in *Figure 3*. This highlights substantial differences in the Gray Water footprint of consumption of agricultural and industrial product in Europe. Thus, Bulgaria is the largest consumer of gray water, largely due to agricultural products, while Germany is one of the main consumers of gray water in economic sectors such as: agriculture, food, textile and electricity. In turn, Poland is the largest supplier of gray water included in the exports of agricultural and chemical products to Germany and other European countries (Serrano et al., 2016).

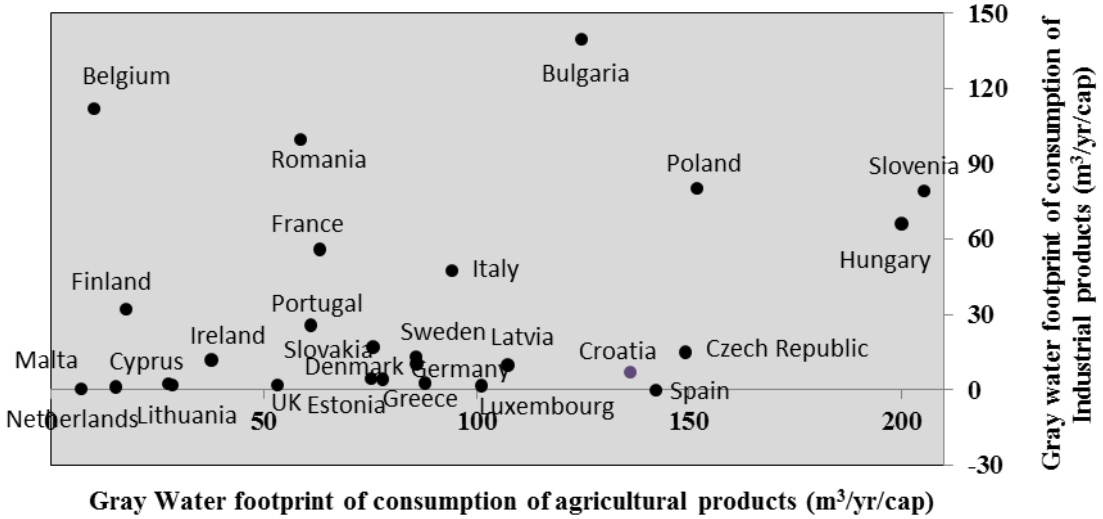

Figure 3. *Agricultural products vs. industrial products gray water footprints. (Source: authors' own design)*

The second part of the study aimed to identify the grouping mode of the 23 states in clusters, taking into account five indicators: WEI, GWFAP, BWFAP, GyWFAP, BWFIP, and GyWFIP. The dendrogram associated with the hierarchical clustering analysis developed as in Marinoiu (2016) and Andrei et al. (2018) is shown in *Figure 4*. The dendrogram shows the evolution of the states analysed by clusters, based on the similarity between them revealed by the indicators applied to the analysis.

According to *Figure 4,* the first level of grouping comes with six groups, each of them formed of two states (Slovakia, Sweden; Estonia, Lithuania; Denmark, UK; Germany, The Netherlands; Greece, Portugal; Poland, Slovenia), and a cluster formed of three states (Croatia, Latvia and The Czech Republic), while the other states are left outside any cluster. The second level of grouping already forms Cluster C1 (Denmark,

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Malta and UK), Cluster C2 (Croatia, The Czech Republic, Estonia, Latvia, Lithuania, Slovakia and Sweden), Cluster C4 (Cyprus, Greece, Portugal and Spain), and two other clusters, one formed of Belgium and France, and the other formed of Romania and Bulgaria. Finally, at the next level of grouping, the clusters formed of Belgium-France and Germany-The Netherlands group into Cluster C3, and the clusters Romania-Bulgaria and Poland-Slovenia, together with Hungary, form Cluster C5.

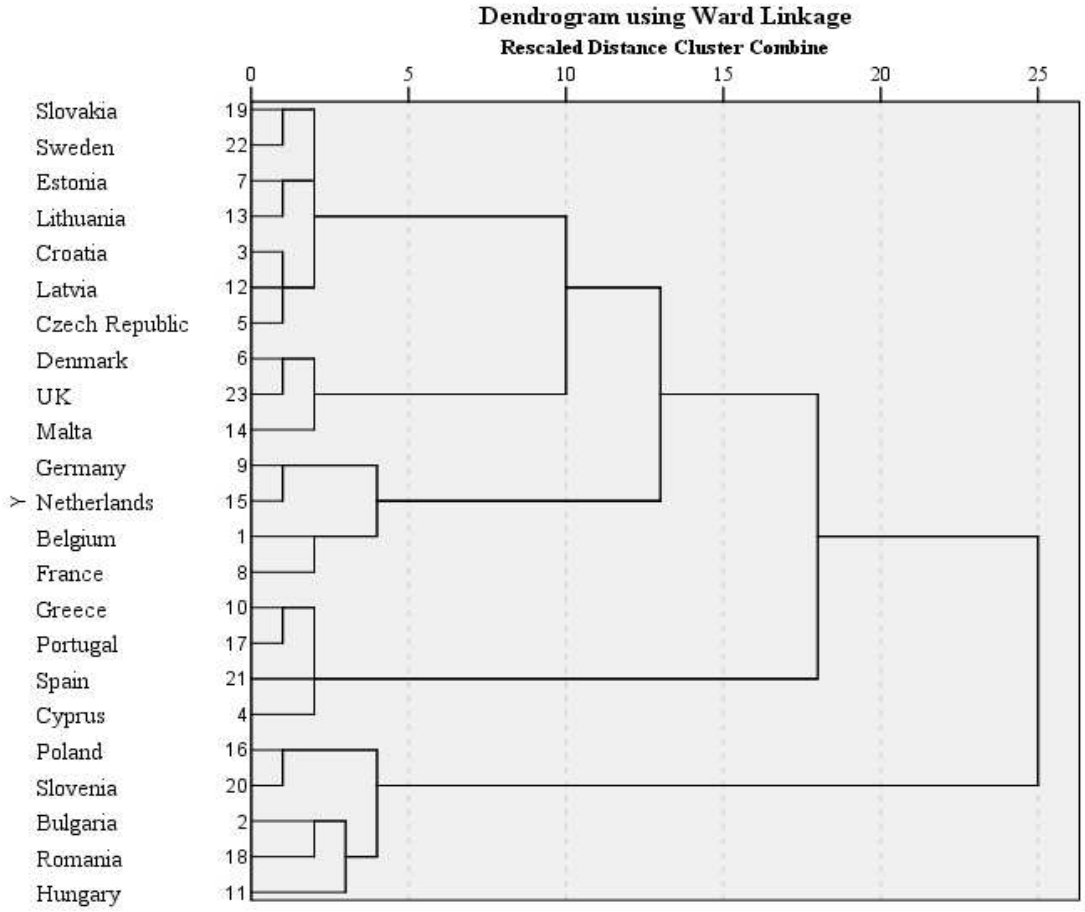

Figure 4. *The cluster generation dendrogram using Ward Linkage Method. (Source: authors' own design)*

Following the tests and analyses carried out using the hierarchical cluster methodology and taking into account the relevance of the results, a group of the states analysed in five clusters was chosen. In order to identify the method of testing the statistical significance of the membership of the analysed variables in the clusters, the homoscedasticity of the dispersions of the corresponding data series was verified. The results of Levene's test (*Table 2*) highlights the fact that for two of the six indicators under analysis, respectively BWFAP and GyWFIP, at a significance level $\alpha = 0.05$, with a value of Sig.F < α (BWFAP - 0.006 and GyWFIP - 0.02), the null hypothesis H_0 must be rejected and, consequently, the ANOVA methodology cannot be applied.

Under these conditions, the statistical significance testing of the membership of the variables in the clusters was performed using two tests, namely Welch and Brown-Forsythe. Following the application of the Welch test, the results show that for four of

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the analysed variables (BWFAP, BWFIP, GyWFIP, WP) Sig.F values are lower than $\alpha = 0.05$, therefore, the null hypothesis H0 is rejected and, consequently, H_0 is rejected, their environments differ significantly from one cluster to another.

Table 2. *Results of Levene's tests to verify the homoscedaticity of the dispersions of the analysed data series. (Source: authors' own computations)*

Indicator	Levene statistic	Degrees of freedom		Sig.
		df1	df2	
GWFAP	2.189	4	18	.111
BWFAP	5.207	4	18	.006
GyWFAP	.411	4	18	.799
BWFIP	2.250	4	18	.104
GyWFIP	6.407	4	18	.002
WP	1.948	4	18	.146

In *Table 3* are presented the results of the robust tests of equality of means to verify the statistical significance of the membership of the variables in the clusters

Table 3. *Results robust tests of equality of means to verify the statistical significance of the membership of the variables in the clusters. (Source: authors' own computations)*

Indicator	Test	Statistic[a]	Degrees of freedom		Sig.F
			df1	df2	
GWFAP	Welch	4.520	4	7.119	.039
	Brown-Forsythe	4.946	4	11.968	.014
BWFAP	Welch	25.796	4	6.816	.000
	Brown-Forsythe	106.924	4	4.317	.000
GyWFAP	Welch	3.024	4	7.570	.090
	Brown-Forsythe	3.938	4	14.730	.023
BWFIP	Welch	13.705	4	6.998	.002
	Brown-Forsythe	15.145	4	10.708	.000
GyWFIP	Welch	11.815	4	7.781	.002
	Brown-Forsythe	9.487	4	5.261	.013
WP	Welch	15.302	4	7.118	.001
	Brown-Forsythe	19.684	4	5.407	.002

[a]Asymptotically F distributed

Considering that for the GyWFAP variable, at the chosen significance threshold, the Welch test leads to the acceptance of the null hypothesis and its rejection for confidence level 90% ($\alpha = 0.10$), and the Brown-Forsythe test indicates the rejection of the null hypothesis, we considered that this variable can also be taken into account when analysing the characteristics of the generated clusters.

Considering the values of the six variables considered in characterizing the sustainable water use by referring to the existence and water use resources in the economic sectors of agriculture and manufacturing in the 23 EU member countries, the

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structure and geographical distribution of the clusters is shown in *Figure 5*. The characteristics of the clusters are mainly highlighted by their classification in relation to the determined average values. The statistical characteristics associated to each cluster in terms of the analysed indicators is presented in *Table 4*.

Cluster	Countries included
C1	Denmark, Malta, UK
C2	Croatia, Czech Republic, Estonia, Latvia, Lithuania, Slovakia, Sweden
C3	Belgium, France, Germany, Netherlands
C4	Cyprus, Greece, Portugal, Spain
C5	Bulgaria, Hungary, Poland, Romania, Slovenia

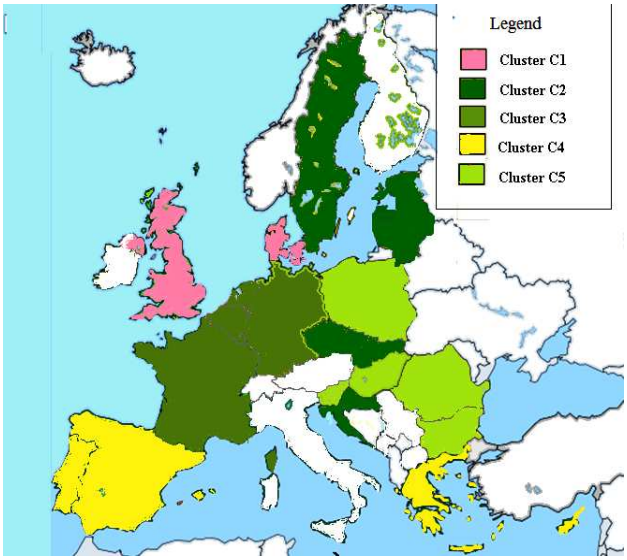

Figure 5. *Cluster structure and geographic cluster distribution. (Source: authors' own design)*

Table 4. *Characteristics of the groups of countries according to the cluster average values of water footprint of consumption of agricultural and industrial products and water productivity. (Source: authors` own computations)*

Cluster	Number of countries	Water footprint of agricultural and industrial products (m^3/yr/cap)					Water productivity (Euro per m^3)
		GWFAP	BWFAP	GyWFAP	BWFIP	GyWFIP	WP
C1	3	276.77	4.80	45.20	2.00	2.40	293.10
C2	7	821.21	2.11	94.31	2.13	9.73	106.11
C3	4	281.20	6.75	43.55	11.48	44.90	94.98
C4	4	739.13	197.08	79.70	2.85	7.70	45.30
C5	5	1072.22	8.18	148.06	10.50	92.98	29.22

As can be seen from the data in *Table 4,* the average values of the six indicators (GWFAP, BWFAP, GyWFAP, BWFIP, GyWFIP, and WP) differ significantly, which means that there are major discrepancies between the 5 clusters formed.

While in cluster 1, countries characterized by sustainable water use were identified, cluster 5 includes countries with significant water resources, but with a poor use of them. Denmark is part of Cluster 1, the European leader in water rationalization, whose main tools used to control water consumption are water prices and its metering. With an average GWFAP value far above all the other 4 clusters, which is almost 4 times higher than that of cluster 1, cluster 5 has the lowest mean WP value. Also, cluster 5 has the highest average values for the other 2 indicators, respectively GyWFAP and GyWFIP. These countries must adopt and apply more drastic measures to solve the problem of

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pollution, to reduce the value of the gray water footprint of the consumption of agricultural and industrial products (Gogonea, 2019).

The positioning of the countries included in cluster 2 in areas with high rainfall, and on the other hand the abundance of lakes and rivers (Sweden, Slovakia, and Croatia) resulted in lower water consumption for agriculture, recording the lowest average BWFAP value among all clusters.

Cluster 3, consisting of countries with high operating indices (Belgium, France, Germany, Netherlands), recorded the highest average value of BWFIP, reflecting the predominant water use for consumption of industrial products.

Cluster 4 includes 4 countries in southern Europe (Cyprus, Greece, Portugal, Spain), large water-consuming countries, recording the highest average value of BWFAP. Also, the clustering of the states included in the analysis highlights the significant differences between them and in terms of existing long-term links between total internal water footprint (TWF) and water productivity (WP), and also between total internal water footprint for industrial products (TotalIND) and total internal water footprint for agricultural products (TotalAGR).

Thus, from the point of view of existing long-term relations between WP and TWP (*Fig. 6*), the large gap must be noted between Denmark, Malta, UK (countries included in cluster 1) and the other 20 countries in terms of water productivity. Also, Belgium, France, Germany and The Netherlands, although they are far from Denmark, Malta, and UK in terms of water productivity, hold a special position compared to the countries included in clusters C2, C4 and C5, due to the fact that the average value of TWF (393.38 m^3/yr/cap) is less than half of the average values of TWF recorded in them.

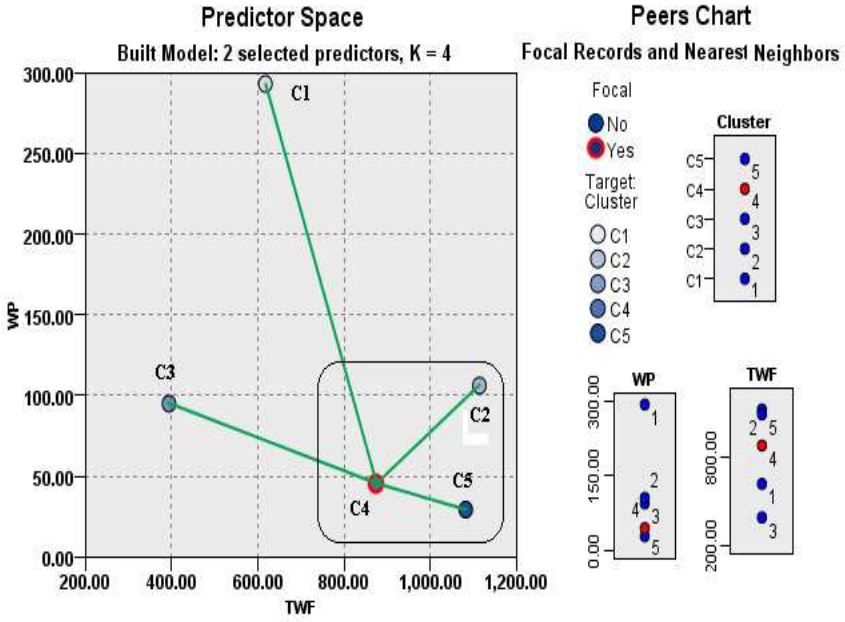

Figure 6. *The highlighting of the distances between clusters according to WP (euro m^3) and TWF (m^3/yr/cap). (Source: authors' own design)*

Another significant analysis of the clusters also includes a graphical representation (*Fig. 7*) through which the distances between the clusters according to WP (euro/m^3), TotalIND (m^3/yr/cap) and TotalAGR (m^3/yr/cap) are highlighted.

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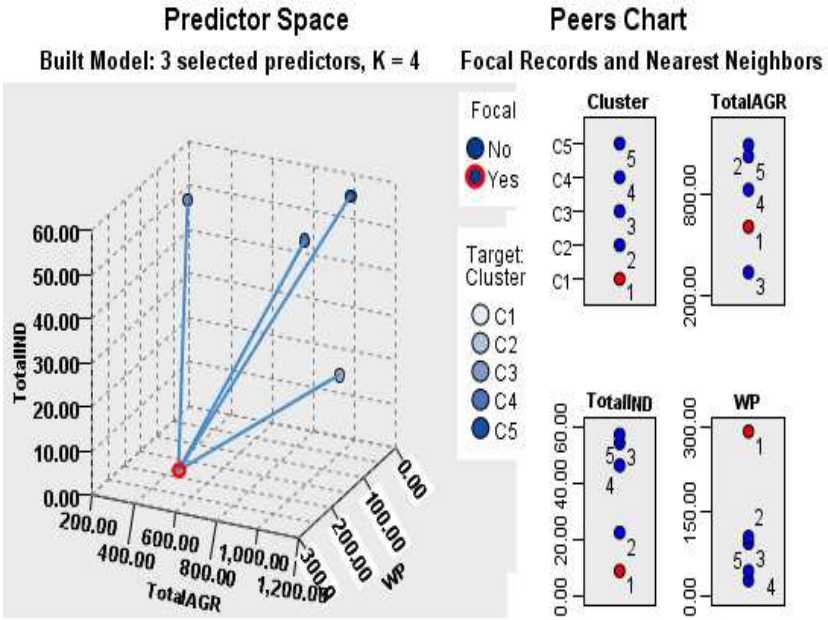

Figure 7. *The highlighting of the distances between clusters according to WP (euro/m^3), TotalIND (m^3/yr/cap) and TotalAGR (m^3/yr/cap). (Source: authors' own design)*

In terms of the dependencies between water productivity, total water footprint for industrial products (TotalIND) and total internal water footprint for agricultural products (TotalAGR), in this case the countries included in the C1 cluster (Denmark, Malta, UK) are significantly different from the others countries included in the analysis. The difference is evident not only in terms of WP values, but also in the fact that the lowest average value of TotalIND (9.16 m^3/yr/cap) is recorded here, 2.5 times lower than in the C2 cluster and over 5 times smaller than in clusters C3, C4 and C5.

From the point of view of TotalAGR, the closest to the C1 cluster is the C4 cluster (Cyprus, Greece, Portugal, Spain) with an average value of 827.13 m^3/yr/cap (with 218.96 m^3/yr/head more than in Cluster C1). On the other hand, Cluster C3 also occupies a separate place, being the only one where the TotalAGR has a lower value than Cluster C1 (338.87 m^3/yr/cap, compared to 608.17 m^3/yr/cap).

However, if we consider the TotalIND values, the closest one to Cluster C1 is Cluster C2 (Croatia, Czech Republic, Estonia, Latvia, Lithuania, Slovakia, Sweden) with an average value of TotalIND of 22.74 m^3/yr/cap (with 13.57 m^3/yr/cap more than in Cluster C1). At the same time, Clusters C3, C4 and C5 form a fairly tight group with values of TotalIND ranging from 46.58 m^3/yr/cap (cluster C4) to 57.5 m^3/yr/cap (cluster C5).

Summarizing the performed analysis, the usefulness of continuous monitoring and study of the efficiency of the use of water resources can serve to identify efficiency as clearly as possible at territorial level, this being considered a significant indicator for the description of water use in agricultural and industrial production (Gang et al., 2016; Huang et al., 2017).

Conclusions

Considering that the two economic branches use the three categories of water (Green, Blue, Gray), the analysis in this research is realised taking into account the

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interdependencies of all types of water. Thus, five other study variables (Green, Blue, Gray water footprint of consumption of agricultural products and Blue, Gray water footprint of consumption of industrial products) were introduced into the research.

In Europe, for all the 23 countries included in the study, for the agricultural sector, on average per year, approximately one third of the available water is used. The irrigation of agricultural crops differs from one country to another, mainly due to climatic conditions, but also to the technological systems in place. Different characteristics are also noticeable in what regards the manufacturing technologies used by each European country, which has determined the detection of different consumption patterns, with quite significant differences from one country to another.

Northern and Western European countries are characterized by abundant rainfalls that form the basis of their water resources, paralleled by a high degree of water rationalization, while in Central and Southern Europe we either have countries with rich water reserves, but poor use of them (Bulgaria, Hungary, Poland, Romania, Slovenia), or countries with arid or semi-arid conditions requiring the use of irrigation in a large proportion (Cyprus, Greece, Spain). Thus, many countries in southern Europe, with limited resources, have to cope with a high water pressure due to the strongly developed agri-food system, while other countries have considerably reduced water consumption in manufacturing through advanced technologies. In both manufacturing and agriculture, the biggest water consumers are: Belgium, France and Bulgaria, while Malta is the country with the highest volume of imported water.

The results obtained from the application of the analysis (another analysed object) emphasize the same aspects of the territorial concentration of water types in countries. The six clusters formed complement the analysis of the relations between the indices of water exploitation and its productivity, as well as the relations between the internal water footprints used for the production of agricultural and industrial products, with the identification and the evaluation of some possible relations between the internal water footprint used for the production of products and the efficiency of its use. The highest average value of water productivity in the European Union was recorded by Cluster 1 (Denmark, Malta and UK), while southern countries remain in cluster 5, characterized by low performances in terms of sustainable water use. In this context, in the future, a priority for the countries, in relation to the problems they are facing, would be their orientation towards the cultivation of less water-consuming plants, and technologies which will sharply reduce the water consumption in the industry.

The EU countries must join efforts in the direction of water saving, its efficient use, thus following in the direction of sustainable development. As a consequence of the application of the quantitative descriptive method between the water reserves of each state and the efficiency of the water quantities used, it was observed that, in general, the efficiency of the water use does not depend to a large extent on the restrictive nature of the resource.

The results of this research allow us to conclude that water-related problems may hamper severely the economic development of a region or country, depending on the availability of this resource, or on the degree of accessibility to it. Other studies as Ibáñez et al. (2017) emphasise that water footprint could be used to identify the main environmental impacts on the high risks areas. In the same time water resources could become an important and restrictive factor to social and economic growth in numerous countries as Li et al. (2021) highlights.

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Indicators like water productivity, water exploitation and water footprint may reflect accurately the level of development of the two economic sectors considered in our study, agriculture and manufacturing, and, as such, the three indicators can guide towards developing and enforcing appropriate measures to save water and, thereby, reduce the level of gray water in the manufacturing and farming production chains. The assessment of resilient development and of the efficiency of an environmental policy with the help of the water footprint and of other, related, indicators, opens a broad field of interdisciplinary research against the background of ever wider analyses purporting to identify new indicators for the sustainable development of contemporary economies. The application of the water productivity, exploitation and footprint indicators develop new approaches and highlight the global dimension on the water management strategies and offers in an equal measure a better understanding of the countries` dependence on water resources and their usage. This study argues a proactive need for a more rational-allocative and intelligent water usage in competitive economies. In this context, in literature is a massive need for future studies in assessing the environmental performance by evaluating water indicators. Developing a specific framework in assessing water performance and consumptions patterns would be one of the major challenges in the near future. In this context, this paper may serve as a basic instrument in building the structure required to understand concepts and principles like impartiality and efficiency in the use of water, sustainability of water exploitation, and the rational management of water as an effect of using specific indicators, such as those generated by the water footprint system.

Research limits and further directions of investigation

Understanding the mechanisms that foster sustainable development, and the extensive use of the indicators that come to our aid in doing so require a minute analysis of the existing literature covering this subject, and, no less, the functional relations of the available statistic data used to substantiate the propounded methodology and research model. In view of the above, our research attempts to answer the question to what extent the three indicators examined herein - water productivity, water exploitation and water footprint - are obsolete concepts or representative tools in understanding environmental policy?

Our research follows the current trends in the literature aiming the water economics. Notwithstanding, our study has its own limitations deriving from the very working model and variables chosen for the purpose. Among the main limitations are the number of variables used and the relatively small reference span. Also, the fact that the cluster model is based only on the values of a single year. These limits however may become the starting point of new lines of investigation for future studies. One other limitation arises from the understanding and implementation of the concepts employed, and from the fact that the results obtained cannot be statistically extrapolated, despite the highly explanatory nature of the model and methodology applied.

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INTRASPECIFIC VARIATION IN THE INTERNAL TRANSCRIBED SPACER (ITS) REGION OF GREEN PEACH APHID *MYZUS PERSICAE* [(SULZER) (HEMIPTERA: APHIDIDAE)] UNDER ELEVATED ATMOSPHERIC CO_2 PRESSURE

KARACAOĞLU, M.

Department of Plant Protection, Faculty of Agriculture, Malatya Turgut Ozal University, Battalgazi/Malatya, Turkey
e-mail: mehmet.karacaoglu@ozal.edu.tr

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Abstract. The continuously increasing concentrations of atmospheric CO_2 is predicted to affect biological processes at many levels of organisms. Yet, no study exists in the literature attempting to describe that the elevated atmospheric CO_2 (eCO_2) concentration may cause an evolutionary response on nucleotide sequences of ribosomal DNA of *Myzus persicae* [(Sulzer) (Hemiptera: Aphididae)]. Here, we provide a preliminary study to understand how the insect ribosomal DNA sequences are influenced under the elevated CO_2 levels after several generations. Four *M. persicae* populations were established for 35 days under ambient CO_2 (a CO_2) (400 ppm), e CO_2 (600 ppm), e CO_2 (800 ppm) and e CO_2 (1000 ppm) at 29ºC in moisture-controlled greenhouse chambers. Intraspecific variation of *M. periscae* was assessed by the sequencing and analyzing the internal transcribed spacer (ITS) region of *Myzus persicae* (Sulzer) under elevated atmospheric CO_2 pressure. Based on our results, the phylogenetic analysis of ITS sequences differentiated the individuals grown at 800 ppm CO_2 level. The alignment of ITS sequences of all specimens revealed several single-nucleotide substitutions on the nucleotide sequence of *M. persicae* samples grown at 800 ppm CO_2 level. Overall results show that the elevated atmospheric CO_2 levels could be a powerful evolutionary force than expected on *M. persicae* reared on eggplants.
Keywords: *evolution, atmospheric CO_2, alignment, intraspecific variation, nucleotide comparison*

Introduction

Species of the Aphididae (Hemiptera) family species are found almost everywhere in the world, but these pests are more common in temperate regions than in the tropics. It has been reported that there are approximately 5000 species belonging to the Aphididae family in the world and 1600 species in Europe (Nieto Nafria et al., 2013; Blackman and Eastop, 2020). Considering aphids in Turkey, the number of species identified is reported to be 558 (Akyürek et al., 2019; Özdemir, 2020). Aphids are among the most significant pests in agricultural areas around the world. *Myzus persicae* Sulz. (Hemiptera: Aphididae) causes serious damage both in cover crop and the open field (Blackman and Eastop, 2006; Van Emden and Harrington, 2017). Aphids are generally considered polyphagous pests. Among them *M. persicae* feed on phloem tissue of plants (Pollard, 1973). As a result of feeding, the growth of the plant may stunt, produce plant galls, cause deformation of leaves, buds, and flowers and transmit plant virus diseases. *M. persicae* produces large amounts of a sugary liquid waste called "honeydew", and as a result of this secreted substance, saprophyte fungi can grow on honeydew that accumulate on leaves resulting in a decrease in the plant's photosynthesis (Lodos, 1982). This pest completes its life cycle in a short time when there are suitable climate conditions and continues its life throughout the season. Temperature and humidity play an important role in its development.

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M. persicae alone is reported to carry more than 150 plant virus diseases from different crops, including vegetables belonging to the Solanaceae family (Sharma et al., 2008).

Atmospheric CO_2 has increased from about 280 ppm to 400 ppm since the Industrial Revolution in the mid-1700s (Bonan and Doney, 2018). In the inter-country climate panel held in 2014, it was reported that the atmospheric CO_2 rate will increase between approximately 750 and 1300 ppm by the year 2100 (IPCC, 2014). The increase in the CO_2 ratio in the atmosphere in the next century will cause a decrease in the nutrient content in the host of many insect species, thus, it is expected that both larval development time and mortality rate will increase (Carlos and Trumble, 1998). Whether these changes will affect insects' feeding behavior, biology, host preferences, adaptation to climatic conditions and genetics is not clearly known. In the context of climate change, the impact of increased CO_2 on insect species has become a major issue over the last three decades. Elevated CO_2 levels may modify the insect behavior for feeding, but precise effects on insect genetics are poorly known. There have been many studies on the effects of enhanced CO_2 on behavior of insect species (Stiling et al., 2002; Chen et al., 2007; Sudderth and Sudderth, 2014) however, there has been no investigation of the effects of elevated CO_2 levels on intraspecific variation. The purpose of the study was to obtain some initial data regarding the possible effects of ambient and elevated CO_2 levels on intraspecific variation on one example of a species (*Myzus persicae*) using the internal transcribed spacers of the ribosomal DNA (ITS rDNA) region.

Materials and Methods

Source of green peach aphid and eggplants

The colonies of green peach aphids trapped in the eggplant fields. Once aphids were identified, they were maintained in the temperature controlled growing chamber on the same species (*Solanum melongena* L. cv. Pala-49) at 14 h day length alternating at temperature of regimes (29/19±1ºC) under constant relative humidity (60±10% RH with 14:10 h L:D photoperiod at 8-10 klux light intensity photoperiod conditions). The aphids were sourced from the colony cultured on these plants. The eggplants (*Solanum melongena* L. cv. Pala-49) were then transferred to a temperature, humidity and CO_2-controlled greenhouse at the Malatya Turgut Ozal University to provide plants for the experiments. A separate concentration of carbon dioxide level was applied in each compartment of the greenhouse including ambient (400 ppm) and elevated 600 ppm, 800 ppm, and 1000 ppm CO_2 levels, consisting of four compartments.

Extraction of genomic DNA from the green peach aphid

Genomic DNA of single aphid was simply and effectively extracted at room temperature using DNeasy® Blood & Tissue Kit (Qiagen, Germany). Seventeen specimens of *M. persicae* were collected from eggplants grown at ambient (400 ppm) and elevated (600, 800, 1000 ppm) CO_2 levels. The four samples of *M. persicae* from ambient CO_2 level (400 ppm) were served as controls (*Table 1*). In order to eliminate the external microbial contaminants, the aphid samples were rinsed in 70% ethanol for 5 min and then rinsed four times with sterilized water to eliminate the external microbial contaminants. The whole body of an adult aphid was used to purify total genomic DNA (*Table 1*). All DNA preparations were stored at -20°C until use.

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Table 1. *Characteristics of aphid samples used in this study grown under ambient and elevated CO_2 levels*

Number of rooms	CO_2 level	Number of individuals produced	Number of samples sequenced	Number of generations	Temperature of each room	After the third generation duration of rearing (day)
1st room	400 ppm	150	4	4	29/19*	35
2nd room	600 ppm	180	5	4	29/19	35
3rd room	800 ppm	130	4	4	29/19	35
4th room	1000 ppm	140	4	4	29/19	35

*At 14 h day length at temperature of 29 /19ºC (L:D) photoperiod at 8-10 klux light intensity

Elevated CO_2 conditions and experimental design

Five adult aphids maintained in the growing chamber were removed from their host plant and placed on young eggplant leaves grown at ambient (400 ppm) and elevated 600 ppm, 800 ppm and 1000 ppm levels of CO_2 in the green house. The trials were carried out at four-room temperature, humidity and CO_2 controlled greenhouse facility located at Malatya Turgut Ozal University. Each had alternating (29/19±1ºC) temperature regimes under constant relative humidity and photoperiod conditions (60±10% RH with 14:10 h L:D photoperiod) at 8-10 klux light intensity. *M. persicae* stock culture was established on the eggplant plant in each CO_2 level room. Four young eggplant plants were placed in each room and five *M. persicae* individuals were transferred on each plant. The aphid samples were taken from the individuals grown in each room, after 4 generations. Individuals taken from each climate room were stored in sample bags at -80ºC, and total DNA isolation was made from these samples. Genomic DNA isolation was performed from a single individual.

PCR amplification of 16S rRNA and sequencing

An approximately 730 bp DNA fragment of ITS region of nuclear ribosomal RNA (nrRNA) gene containing ITS1, 5.8S and ITS2 was amplified by polymerase chain reaction (PCR) with the primers (ITS4:5'-TCCTCCGCTTATTGATATGC-3'and ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990) (*Figure 1*). The PCR mixture consisted of 5 µL of 10×reaction buffer (200 mMTris–HCl pH: 8.4, 500 mMKCl), 2 µL of genomic DNA, 1 µL of dNTPs (10 mM each), 3 µL of $MgCl_2$ (25 mM), 1 µL of each primer (100 pmol), 0.4 µL of DNA polymerase, and 36.6 µL of DNase free sterile water. PCR amplification was performed using the following thermocycling program: a 2 min initial denaturation at 94°C, followed by 36 cycles of 94°C for 1 min, annealing at 55°C for 1 min and an extension of 72°C for 2 min, and a final extension of 72°C for 10 min. The PCR amplified DNA fragments were separated on 2% agarose gel containing fluorescent dye and recovered by agarose gel extraction kit (Bioline, Germany). A total of 17 nuclear ribosomal DNA fragments of *M. persicae* samples, originated from the same population and reared at four levels of CO_2, were sequenced after completing their 4th generations in growing chamber. All the sequences studied in this study are deposited in GenBank database under the accession numbers given in *Table 2*.

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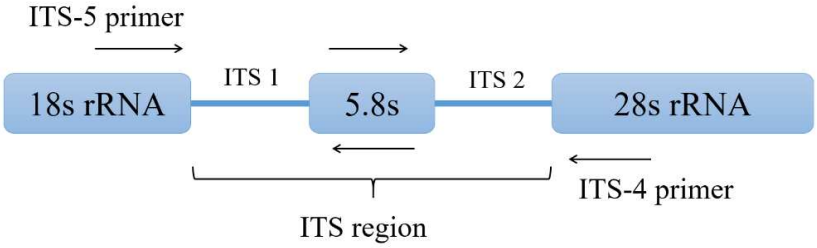

Figure 1. *Diagram of nuclear ribosomal RNA (nrRNA) gene containing ITS1, 5.8S, and ITS2 regions with primer binding sites*

Table 2. *List of ITS sequences of Myzus persicae samples used in this study, length and GenBank accession numbers*

Species	CO_2 level (ppm)	Name of the sample	Length (bp)	Accession no
Myzus persicae	400	Sample 1	698	MW581037
M. persicae	400	Sample 2	699	MW581033
M. persicae	400	Sample 3	699	MW581028
M. persicae	400	Sample 4	699	MW581038
M. persicae	600	Sample 1	699	MW581034
M. persicae	600	Sample 2	699	MW581035
M. persicae	600	Sample 3	698	MW581032
M. persicae	600	Sample 4	698	MW581029
M. persicae	600	Sample 5	699	MW581031
M. persicae	800	Sample 1	699	MW581036
M. persicae	800	Sample 2	699	MW581030
M. persicae	800	Sample 3	698	MW581039
M. persicae	800	Sample 4	699	MW581073
M. persicae	1000	Sample 1	699	MW581070
M. persicae	1000	Sample 2	698	MW581071
M. persicae	1000	Sample 3	699	MW581072
M. persicae	1000	Sample 4	699	MW581069

Bioinformatic analysis

The ITS sequences of aphid samples were initially edited manually than aligned using CLC Main Workbench Version 6.2 (CLC bio, Denmark) software. To determine the intraspecific variation among the seventeen *M. persicae* samples grown in four different levels of CO_2, we constructed a phylogenetic tree using ITS sequences obtained in this study. Intraspecific pairwise alignments of all loci considered (ITS1, 5.8S, ITS2) in this research were generated using CLC Main Workbench Version 6.2 (CLC bio, Denmark) software for all *M. persicae* samples. The phylogenetic tree was built under the neighbor joining algorithm. The relationships were assessed using 1000 bootstrap replicates.

Secondary structure analyses

In order to predict the most stable secondary structure of the nuclear ribosomal DNA, containing ITS1, 5.8S and ITS2 sequences, the established full sequences were folded

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and visualized using the mfold structure prediction package of CLC RNA Workbench Version 6.2 (CLC bio, Denmark) software by energy minimizing. Each consensus sequence, belonging to a a particular CO_2 level, was folded separately to build the full structure.

In silico virtual RFLP analysis

Delimitation of the start and end points of each sequence was carefully trimmed manually in order to arrange the nucleotide bases at the start and end of the sequences were identical for all individuals. Computer-simulated (*in silico*) RFLP analysis of the ITS sequences of all amplified specimens was performed using pDRAW32 (AcaClone Software). The following 17 restriction enzymes which are commonly used in bench digests and identification *in silico* RFLP analysis for phytoplasmas (Lee et al., 1998; Oksal et al., 2017; Usta et al., 2018) were adapted and screened: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. Among these enzymes, HaeIII, HpaII and TaqI did not cut any of the sequences submitted to digestion, but were retained in the data set for comparison. Following the *in silico* restriction digestion, a virtual 1.0% agarose gel image plotted automatically to the computer screen to capture the RFLP pattern of 16Sr DNA sequences using the program pDRAW32 (AcaClone Software).

Results

An approximately 700 bp single amplified PCR product was obtained in all *M. persicae* samples for the complete rDNA ITS region. The ITS sequence ranged from 698 bp to 699 bp in all accessions. Only three base difference was detected within the 800 ppm CO_2 level of growing condition of *M. persicae*. The divergence in the ITS genes among the individuals grown at 800 ppm tested was low (*c*. 0.4% informative sites) (*Figure 2*). However, the variation among the individuals within the individual grown at ambient OC2 (400 ppm and elevated CO_2 levels (600 and 1000 ppm), appears to be very low, with no informative positions. GenBank accession numbers of the ITS sequences and the origin of samples are given in *Table 2*.

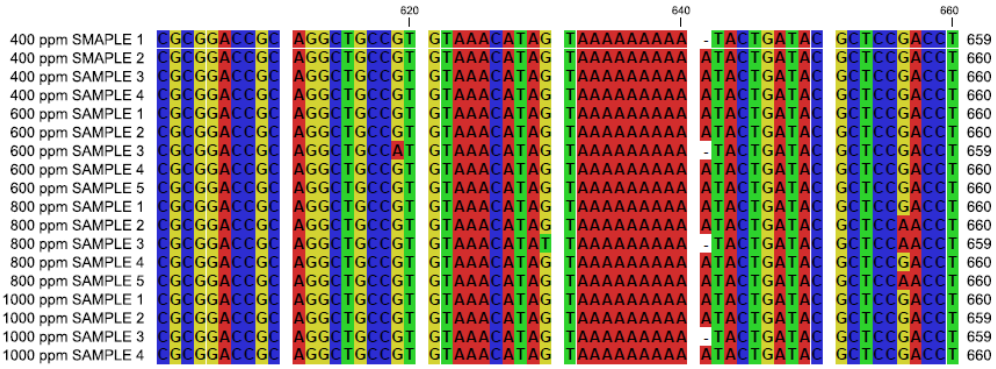

Figure 2. *Sequence alignment of ITS region of M. persicae individuals grown at ambiante and elevated CO_2 levels. Mutational sites of ITS region of M. persicae grown at 800 ppm CO_2 level are boxed*

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The intraspecific variation among the individuals of *M. periscae* resolved by ITS sequence comparisons. Our results show that the intraspecific variability on ITS sequence of *M. persicae* was noticeable at 800 ppm level. In comparison to ambient CO_2, 600 ppm and 1000 ppm CO_2 levels, except one specimen, the ITS sequences of a *M. persica* specimens grown at 800 ppm CO_2 level was phylogenetically diverse. The green peach aphids grown at 800 ppm are clustered in the same branch (Group I) which clearly distinguished from the other individuals grown under ambient CO_2 and 600 and 1000 ppm CO_2 levels (*Figure 3*).

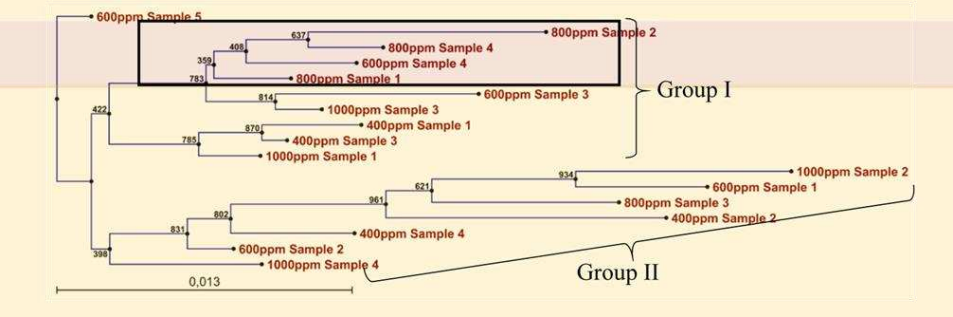

Figure 3. *Phylogram generated from ITS nucleotide sequence data of 17 Myzus persicae specimens grown at elevated CO_2 (600, 800 and 1000ppm) levels and ambient CO_2 level using the Neighbor Joining algorithm. The value of 1000 was used for bootstrap analysis and corresponding values are shown on individual branches. The green peach aphids grown at 800 ppm CO_2 level are boxed*

In *Figure 3*, the neighbor joining phylogram displays the genetic relationships between the aphid samples grown at different CO_2 levels. The Group I composed of 2 subgroups. One of the subgroups consists of 3 green peach aphids all from the same chamber of greenhouse having 800 ppm CO_2 level, indicating their genetic similarity. This cluster received 78% support in the bootstrap analysis. The Group I consist also individuals from ambient and other elevated CO_2 levels, as do Group II, reflecting intraspecific species similarities. However, an aphid sample from the same origin grown at 600 ppm CO_2 level (sample 5) did not grouped together by other aphid samples but instead formed a separate individual branch rooting the tree, indicating its divers feature (*Figure 3*). In Group II, the entire ITS sequence was typically fully conserved within the species, and the variation observed was negligible. In general, no distinct treatment effects were observed on ITS sequences in elevated CO_2 levels among the aphid samples of Group II. Overall results show that the green peach aphid reared on eggplants at different levels of increased atmospheric CO_2 showed a low level of intraspecific variation for Group II members.

Along with nucleotide sequences, the most stabile secondary structure of these sequences was also obtained to compare the topology of sequence paring. What is most significant is the strong distinction in the secondary structure pattern of consensus sequence of individuals grown at 800 ppm CO_2 level (*Figure 4*). The most stable secondary structure alone provided a clear and informative secondary structure topology.

The *in silico* RFLP analyses revealed that the ITS region produced similar banding patterns for all seventeen specimens of *M. persicae*. None of the restriction enzyme used in this study were able to generate different banding patterns (*Figure 5*). The banding patterns obtained from restriction digestion was not sufficient to differentiate the tested specimens with no informative positions.

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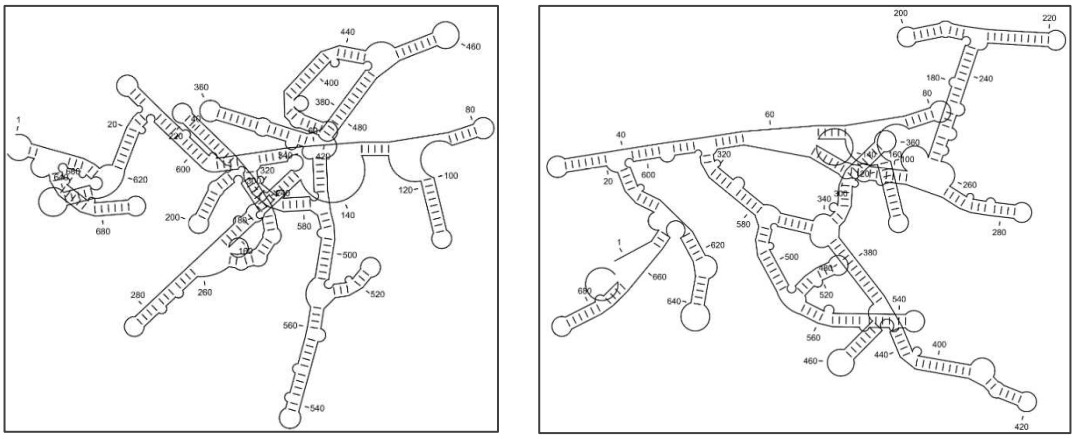

Figure 4. *The most stabile secondary structure based on 730 bp of consensus ITS sequence of green peach aphids grown at ambient CO_2 (A) and elevated (600, 800 (B) and 1000 ppm) CO_2 levels*

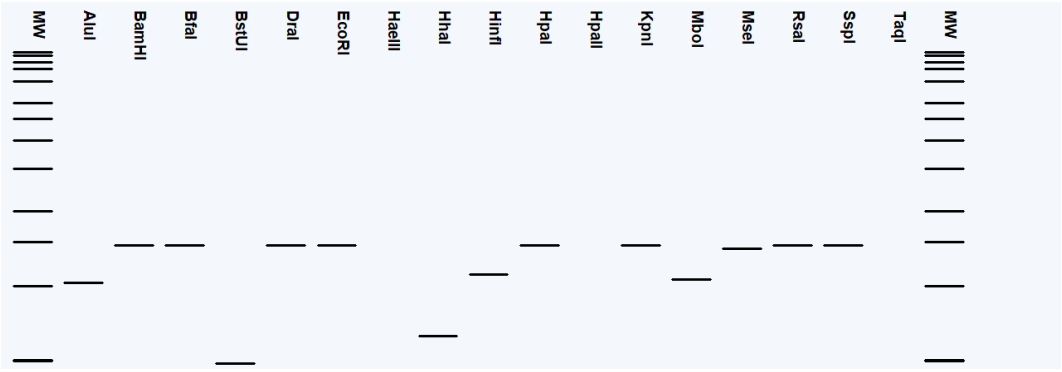

Figure 5. *In silico virtual restriction endonuclease digestion profile of ITS sequence of M. persicae grown at 400 ppm CO_2 level. In the simulated digestions for the recognition sites 17 restriction enzymes were used. The restriction patterns of the all samples were identical. MW: 1 kb DNA ladder*

Discussion

The present study was undertaken to investigate the level of intraspecific variation within the rRNA gene sequences of *M. persicae* based on sequence alignment, secondary structure prediction and *in silico* PCR-RFLP under the CO_2 pressure. It is frequently reported that the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nr DNA) has been widely used for identification and phylogenetic analysis of many microorganism, plant and insect families (Lee et al., 1998; Mir et al., 2009; Keskin et al., 2017; Oksal et al., 2017; Usta et al., 2018).

For comparative purposes, we focused on the prediction of the most stabile secondary structure of ITS sequences and *in silico* virtual RFLP analysis to our consensus sequences of aphids grown at 400, 600, 800 and 1000 ppm CO_2 levels. Point mutations (insertions, deletions or nucleotide substitutions) in ITS sequence of individuals grown at 800 ppm result in a change in secondary structure configuration and fragment size. However, this change did not affect the *in silico* virtual RFLP profiles of aphids tested in this study. The all ITS sequences used in our study showed identical virtual RFLP patterns indicating

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that all these samples cannot be distinguished by this method. The present data suggest that the *in silico* virtual RFLP patterns is not sufficient to understand the intraspecific variation within the species and cannot be perceived by a consideration of virtual RFLP pattern alone. For instance, in this study, the *in silico* approach implementing virtual digestion by 17 key restriction enzymes, did not enabled easy visualization of polymorphisms in ITS sequences of *M. persicae*. However, it may have done for phytoplasma detection and identification and would ultimately facilitate the discovery of new phytoplasma lineages (Lee et al., 1998; Ramdeen and Rampersad, 2012). In species identification and differentiation, recent entomological applications of RFLP studies have focused on mtCOI gene to solve known mitochondrial DNA polymorphism in Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera (Germain et al., 2013; Arimoto and Iwaizumi, 2014; Ovalle et al., 2014; Vesterlund et al., 2014). Comparison of virtual RFLP analysis versus the prediction of secondary structure analysis revealed that the interpretive feature of the later one seems to be higher in understanding of the intraspecific sequence divergence of a given species.

Mutational variation within ITS sequences of *M. persica* specimens grown at 800 ppm CO_2 level probably due to the rate of sexual and asexual reproduction, the number of generations annually, the rate of mutation, and the environmental conditions. Coviella and Trumble (1998) emphasize that the extended period of elevated CO_2 level seems to be a strong evolutionary force in many insect species, who has relatively short generation times and potential for rapid genetic turnover.

The ITS region of nuclear ribosomal is separated into ITS 1 and ITS 2. The ITS 1 is present between 18S and 5.8S rRNA whereas ITS 2 is present between 5.8 and 26s rRNA. 5.8S rRNA is a highly-conserved region (Baldwin et al., 1995). Generally, the *M. persicae* individual grown in ambient and elevated CO_2 levels separated into two groups by phylogenetic analysis of ITS sequences. Except one specimen of 800 ppm CO_2, the aphid samples grown at 800 ppm CO_2 level were clustered in the same group. This is more likely to be related to an elevated CO_2 factor, since some characters are strongly affected by different selection pressures (Mir et al., 2010) and, at some level, expected due to the variable nature of the ITS1 and ITS2 sequences (Nilsson et al., 2009). Here, we used elevated CO_2 concentration as selection pressure, however, the data presented above leave little room for interpretation on the actual influence of elevated CO_2 on ITS sequences, thus the reason that mutational changes increased elevated CO_2 level in this study remain unknown. The present results should be tested with more specimens experimentally. Pillmann et al. (1997) reports that for the measurement of the amount of intraspecific variation within a particular population may not require sampling of many individuals. In the point of view of the conserved nucleotide sequences, long term studies will make critical contribution to our understanding of elevated CO_2 effects on sequence divergence within a species.

Conclusion

Although there are many studies investigating the possible implications of the increasing CO_2 level in the Earth's atmosphere on the genetic characteristics of living organisms, the evolutionary affects remain uncertain. We conducted a preliminary study to analyze the possible changes on the green peach aphid ribosomal DNA sequences grown under ambient CO_2 (a CO_2) (400 ppm), e CO_2 (600 ppm), e CO_2 (800 ppm) and CO_2 (1000 ppm) CO_2 levels. Among the green peach aphid individuals who gave several

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generations after adapting to different CO_2 levels (400 ppm, 600 ppm, 800 ppm and 1000 ppm), striking and exciting differences were found in the ITS region of those who developed at 800 ppm carbon dioxide level. Under the light of these results, it has been seen that elevated atmospheric CO_2 levels may have an evolutionary force on *M. persicae*. However, additional studies are needed to test this hypothesis with more aphid samples and high number of offspring. Continued scientific studies are required to determine the relationship between elevated CO_2 levels and the evolutionary link.

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COMBINED EFFECTS OF WARMING AND SHADING ON GROWTH AND PHOTOSYNTHETIC PERFORMANCE OF SUBMERGED MACROPHYTES FROM SONGKHLA LAGOON, THAILAND

CHOTIKARN, P.[1,2,3] – KAEWCHANA, P.[2,3] – ROEKNGANDEE, P.[2,3] – PRAMNEECHOTE, P.[3] – PRATHEP, A.[4] – SINUTOK, S.[2,3*]

[1]*Marine and Coastal Resources Institute, Faculty of Environmental Management, Prince of Songkla University, Hat Yai, Thailand*

[2]*Faculty of Environmental Management, Prince of Songkla University, Hat Yai, Thailand*

[3]*Coastal Oceanography and Climate Change Research Center, Prince of Songkla University, Hat Yai, Thailand*

[4]*Seaweed and Seagrass Research Unit, Excellence Center for Biodiversity of Peninsular Thailand, Division of Biological Science, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand*

[*]*Corresponding author*
e-mail: ssutinee@gmail.com

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Abstract. Macrophytes play an important role in maintaining high physical and biological diversity in freshwater ecosystems. However, during the past several centuries since the industrial revolution, human activities and climate change have caused significant changes in the structure and function of aquatic environments, for example via increased temperature or high sedimentation that reduces light penetration. This study investigated the combined effects of elevated temperature and low light on growth and photosynthetic performance of submerged macrophytes *Ceratophyllum demersum* and *Elodea canadensis* and identified temperature and light thresholds for these species. Photosynthetic performance, chlorophyll *a* and *b* concentrations, organic and carbon contents, and growth rates were estimated in these two dominant macrophytes sampled from the middle of Songkhla lagoon, subjected to 9 treatments (3 light intensities and 3 temperatures) for 9 weeks. The results show that photosynthetic performance according to the indicators EQY, α and $rETR_{max}$ was inhibited by low light in both species. *C. demersum* was more tolerant to temperature and light stresses. This study provides an understanding of physiological tolerance and response to light and temperature stresses, and improves the understanding of how aquatic macrophytes respond to future climatic and anthropogenic changes, supporting the development of sustainable lagoon management plans.
Keywords: *climate change, organic carbon, PAM fluorometry, chlorophyll, shading*

Introduction

During the past several centuries since the industrial revolution, human activities such as industrialization, urbanization and intensive agriculture activities have caused strong changes in the structure and function of nearby aquatic environments (Smith et al., 1999). The growth of human population has increased the pressure on both aquatic and terrestrial ecosystems causing land transformation and altering hydrological processes and geochemical cycles of carbon (C), nitrogen (N) and phosphorus (P) (Vitousek et al., 1997; Smith et al., 1999). Land transformation and altering hydrological process led to soil erosion and sedimentation in the waterways and in lakes (Pradit et al., 2010). High

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turbidity from sediment load affects vertical light penetration, which leads to low-light conditions and disrupts ecosystem functions (Mi et al., 2019). Furthermore, the anthropogenic release of greenhouse gases into the atmosphere, predominantly carbon dioxide (CO_2), has led to increased global temperatures through the trapping of heat in the Greenhouse Effect. As a consequence, surface water temperature has increased and is predicted to increase in the future. Climate change is expected to have profound impacts to terrestrial and aquatic ecosystems and their resident organisms (Johnson et al., 2007).

Macrophytes play an important role in maintaining high physical and biological diversity and act as ecosystem engineers (Li et al., 2018) that provide nutrient cycling capacity, and also act as habitat structure and refugia for aquatic organisms (Wigand et al., 2000; Qiu et al., 2001; Cronk and Fennessy, 2016). Macrophytes are potentially used as powerful natural tools for water quality improvement in lakes and reservoirs due to their capacity in nutrient uptake and in preventing phytoplankton blooms (Liu et al., 2000; Lone et al., 2014). The growth and photosynthesis of submerged macrophytes can be affected by temperature (Chalanika De Silva and Asaeda, 2017), light (Chen et al., 2016), and high organic loads in sediments (Barko and Smart, 1983) due to several coupled biological, physical and chemical modifications of the benthic system (Sand-Jensen et al., 2005; Raun et al., 2010). Diurnal light changes affect photosynthetic activities, and Jiang et al. (2018) found that the Maximum Quantum Yield of photosystem II (MQY) of six common submerged macrophytes decreased at midday under ambient light, but there was no significant change under shade, and MQY was negatively correlated with photon radiance, except for *Ceratophyllum demersum*, which probably could support high light levels.

Light is a major environmental factor influencing photosynthetic organisms (Hanelt, 1992). Light penetration through a water column is influenced by several factors including depth, suspended particles, and dissolved compounds. A high load of suspended particles can reduce light transmission to photosynthetically active leaf surfaces (Reitsema et al., 2018) and alter gas and nutrient exchanges in submerged macrophytes (Korschgen et al., 1997). Chen et al. (2016) found that *Potamogeton maackianus* and *Vallisneria natans* increased their initial slope of RLC (α) and decreased their minimum saturating irradiance (E_k) and maximum relative electron transport rate (ETR_m) under low light stress, while higher Relative Growth Rate of *P. maackianus* than *V. natans* was seen with a stronger light intensity but it was decreased in a low light intensity. Shading caused a decrease in net photosynthesis in *Chara aspera* and *Chara canescens* within 24 hours, but their photosynthetic performances recovered within a short period, suggesting that these charophytes are able to adapt to low light conditions (Kovtun-Kante et al., 2014). In contrast, increasing irradiance in oligotrophic lake can lead to an increase in photosynthesis, oxygen production and growth rate of macrophytes (Eller et al., 2015). An increase in shoot density and biomass of *Vallisneria americana* was observed on increasing light intensity from 100 to 600 μmol photons m^{-2} s^{-1} (Barko et al., 1984). However, exposure to a high level of light at a shallow depth can lead to a loss of photosynthetic activity through light-dependent down-regulation of photosynthesis, or a rise in photoinhibition and photodamage, which further inhibits growth (Jin et al., 2020).

Many studies have shown that an increase in global temperature influences the health and survivorship of aquatic organisms (Eissa and Zaki, 2011). Each species responds differently in terms of growth, photosynthesis, reproduction and survivorship to global warming (Gonzalez, 2010; Eissa and Zaki, 2011). Temperature influences the

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physiological processes, including photosynthesis and biomass growth rate of macrophytes (Atta-Boateng et al., 2019). Thermal stress can reduce shoot elongation and increase hydrogen peroxide (H_2O_2) level, damaging photosynthetic pigments and cell membrane structures (Chalanika De Silva and Asaeda, 2018), and may result in shifts in distribution and abundance (Fernández et al., 2020; Miller et al., 2020). Barko and Smart (1981) found that aboveground biomass of submerged macrophytes increased with temperature. However, a recent study showed a decrease in biomass under increasing temperature alone, while an increase in temperature with elevated CO_2 increased photosynthetic performance and growth of *V. natans* (Cao and Ruan, 2015).

Songkhla Lagoon is a tropical estuarine lagoon system located on the eastern side of the southern Thai peninsula, and consists of four interconnected water bodies: Thale Noi, upper lagoon, middle lagoon, and lower lagoon (Pongpiachan et al., 2019). Songkhla Lagoon not only supports biodiversity, but also a large number of people whose livelihoods depend on that biodiversity via fishery, aquaculture and tourism (Community Development Department, 2009). Songkhla Lagoon is currently experiencing serious water pollution and sedimentation due to human activities (Pradit et al., 2010; Somboonsuke et al., 2018). This could lead to losing their valuable ecosystem services and functions such as carbon sequestration. The submerged macrophytes found in Songkhla Lagoon include *Ceratophyllum demersum*, *Cladophora* sp., *Najas malesiana*, *Najas marina*, *Najas graminea*, *Hydrilla verticillata*, and *Potamogeton malaianus* (Thongkao et al., 2001). Climate change is likely to affect each organism in each system differently due to interactions with other factors such as nutrient loading and light. Moreover, studies on effects of light and temperature on growth and photosynthesis of submerged macrophytes in a tropical estuarine lagoon system (like the Songkhla Lagoon) are still lacking. Hence, it is essential to consider the effects of climate change together with low light to successfully manage the eutrophic lagoon (Howarth et al., 2000; Yang et al., 2008).

This study aims to investigate the combined effects of elevated temperature and low light on growth and photosynthesis of submerged macrophytes *C. demersum* and *E. canadensis* and sought to identify temperature and light thresholds for these species. An understanding of physiological tolerance and response to light and temperature stresses is critical for identifying the main driver for growth photosynthesis and carbon capture potential of submerged macrophytes, and for predicting their performances under these stresses. Furthermore, this study could contribute to understanding how aquatic macrophytes will respond to future climatic and anthropogenic changes in temperature and light, and support the development of sustainable lagoon management plans.

Materials and Methods

Sample collection and experimental design

Samples of *Ceratophyllum demersum* and *Elodea canadensis* (*Fig. 1*) were randomly collected from the middle of Songkhla Lagoon (7˚ 28’ 09.0’’ N, 100˚ 23’ 45.0” E) (*Fig. 2*) in May 2018 and were maintained in an aquarium (50 L) at 30˚C, 180 µmol photons m^{-2} s^{-1} on a 12 h: 12 h light: dark cycle for 2 weeks of acclimation. Then, samples (n = 4) were allocated to 9 controlled aquariums set to three light regimes: 180 (control), 90 (50% shading) and 45 (75% shading) µmol photons m^{-2} s^{-1} and three temperatures (30˚C (control), 33˚C (RCP4.5 Scenario at year 2046-2065), and 36˚C (RCP8.5

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Intergovernmental Panel on Climate Change, 2013)) for 9 weeks (*Table 1*). Temperature and light for each treatment were controlled with water heater (Eheim, Germany) and LED lights (Chihiros, China, 400-700 nm), respectively. Water change (20%) to each aquarium were done weekly with deionized water: Hoagland solution (9:1). Nitrate and phosphate concentration were tested with Nitrate test kit (API, USA) and phosphate test kit (API, USA). Other water quality parameters such as dissolved oxygen and pH were measured weekly by YSI Pro Plus multiparameter meter (YSI Inc. / Xylem Inc, USA). Pigment concentration, growth and organic and carbon contents were weekly assessed at initial time (Week 0) and at the end of the experiment (Week 9). Photosynthetic performances were assessed every week from the start to the end of the experiment.

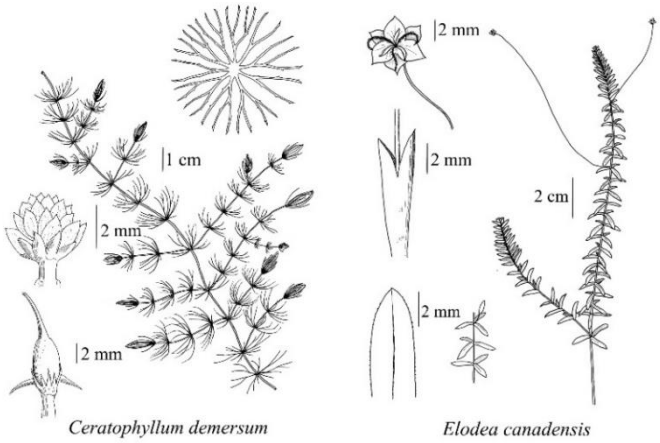

Figure 1. *Drawings of Ceratophyllum demersum (left) and Elodea canadensis (right)*

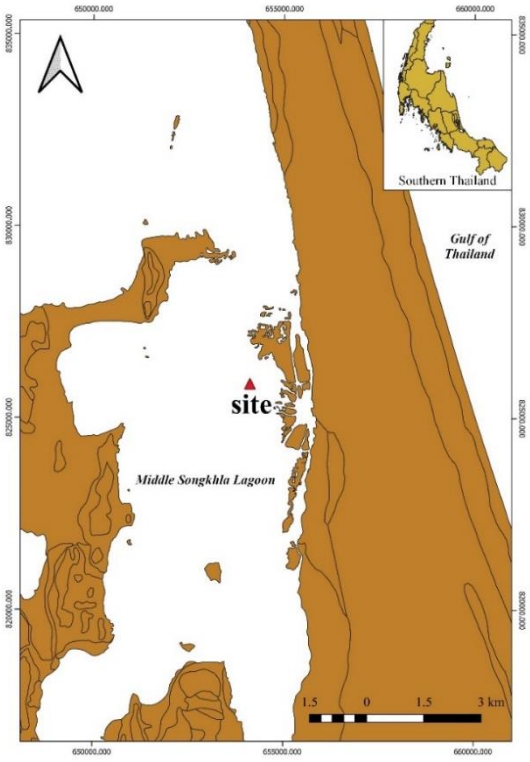

Figure 2. *The study site at the middle of Songkhla Lagoon in peninsular Thailand*

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Table 1. *Experimental design for each treatment and abbreviations*

		Light intensity (µmol photons m^{-2} s^{-1})		
		180	**90**	**45**
Temperature (˚C)	**30**	T30L180	T30L90	T30L45
	33	T33L180	T33L90	T33L45
	36	T36L180	T36L90	T36L45

Photosynthetic activity

Photosynthetic activities were observed weekly through a measure of chlorophyll *a* fluorescence using Junior Pulse Amplitude Modulated (Junior-PAM) fluorometer (Walz, Germany). Dark-adapted photosystem II (PSII) photochemical efficiency was measured as maximum quantum yield (MQY) before lights were on (n = 4). Rapid Light Curves (RLCs) were determined (n = 4) with 9 increasing actinic light intensities (0, 66, 90, 125, 190, 285, 420, 625 and 920 µmol photons m^{-2} s^{-1}), with 0.8 s saturating pulse (> 4500 µmol photons m^{-2} s^{-1}) between each actinic light intensity every 10 s. Effective quantum yield (EQY), maximum relative electron transport rate ($rETR_{max}$), minimum saturating irradiance (I_k) and initial slope (Alpha (α)) of each RLC were calculated using curve fitting protocols following Ralph and Gademann (2005).

Chlorophyll a and b concentrations

Photosynthetic pigment concentrations (chlorophyll (Chl) *a* and *b*) were determined (n = 4) using the standard spectrophotometric method of Ritchie (2006). Chlorophyll *a* and *b* (µg g^{-1} fw) were extracted by homogenizing samples in 4 ml of 90% acetone at 4˚C for 24 h. Samples were then centrifuged at 1500 g for 10 min and the supernatant was placed in a quartz cuvette for the spectrophotometer (Metertech, SP8001, 190-1100 nm), and absorbance was measured at 647, 664 and 750 nm.

Growth rate

Growth rates of *C. demersum* and *E. canadensis* were determined (n = 4) as changes in total length per day, using equation (*Eq.1*) that is modified from Knauer et al. (2006).

$$\text{Growth rate} = (\text{final length} - \text{initial length})/\text{day} \quad \text{(Eq.1)}$$

Organic and carbon contents

Macrophyte samples were oven dried at 105˚C and ground to less than 1 mm particle sizes. A 1.0 g ground sample was ashed in a muffle furnace (FHX, DAIHAN, China) at 550˚C for 8 h (Armecin and Gabon, 2008). Organic matter in the macrophytes was determined using data from the ashed samples (n = 4), and mineral matter (MM; *Eq.2*), organic matter (OM; *Eq.3*) and organic carbon (OC; *Eq.4*) contents were computed as follows:

$$\%\text{MM} = \left(\frac{\text{AW}}{\text{DW}}\right) \text{x } 100 \quad \text{(Eq.2)}$$

$$\%\text{OM} = 100 \text{ x } (\text{DW} - \text{AW})/\text{DW} \quad \text{(Eq.3)}$$

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$$\%OC = \%OM/1.724 \quad \text{(Eq.4)}$$

where AW and DW are ash weight and dry weight of the sample, respectively (Armecin and Gabon, 2008).

Statistical analyses

Three-way mixed ANOVA tests were used to test for significant differences among treatments over time, in the chlorophyll fluorescence parameters. Two-way ANOVA was used to test for significant differences among treatments and species in leaf pigments, growth rate, and organic content. All tests were performed with a significance level of 95%, and Tukey's honestly significant difference *post hoc* tests were used to verify statistical significances. If the data did not meet the assumptions of normality (Kolmogorov-Smirnov test) and constant variance (Levene's test), they were transformed using square root or $\log_{10}$. If the transformed data did not meet the assumptions, then non-parametric tests were used.

Results

Photosynthetic activity

Responses of photosynthetic activity were measured under ambient temperature (30˚C), or elevated temperature (33 and 36˚C) with ambient light (180 µmol photons m^{-2} s^{-1}) or low light (90 and 45 µmol photons m^{-2} s^{-1}). Results showed differences in photosynthetic activity for each macrophyte species.

Maximum quantum yield (MQY)

At week 0, MQY of *Ceratophyllum demersum* and *Elodea canadensis* was 0.77±0.01 and 0.76±0.03, respectively. A difference between the species showed up at elevated temperature (36˚C) where the lowest MQY of *C. demersum* (0.61) occurred in T36L180 (*Fig. 3a*). On the other hand, *E. canadensis* in T36L180 treatment significantly decreased at week 1 ($p<0.05$) then followed by T36L90 at week 3 and by T36L45 at week 4 (*Fig. 3b*). At the end of experiment, there were significant differences in MQY among the treatments in both species ($p<0.05$). The data indicate that only temperature affected MQY of *C. demersum* ($p<0.05$) while MQY of *E. canadensis* was affected by both light and temperature ($p<0.05$).

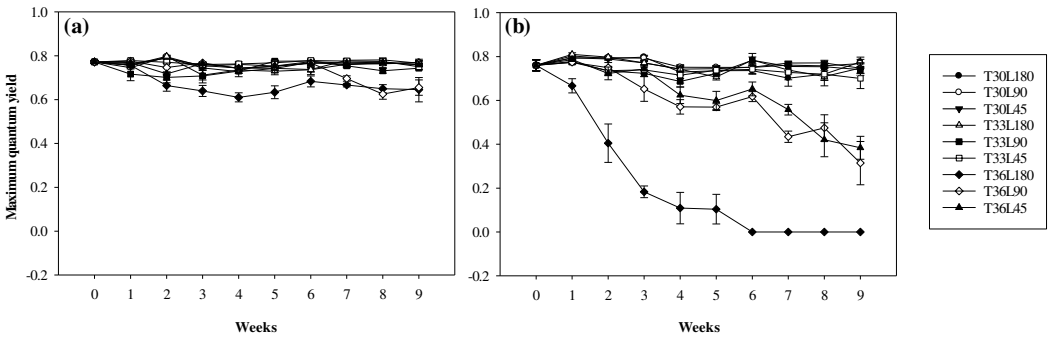

Figure 3. *Maximum quantum yields of C. demersum* (*a*)*, and E. canadensis* (*b*) *from Week 0 to Week 9 in each treatment. Data are shown as Mean±SE*

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Effective quantum yield (EQY)

At week 0, EQY of *C. demersum* and *E. canadensis* was 0.68±0.01 and 0.73±0.01, respectively. EQY showed similar trends to MQY for both species, and at the end of experiment there were significant differences in EQY among the treatments ($p<0.05$). T36L180 treatment gave the lowest of EQY in both species (*Figs. 4a and b*) but it instantly decreased in *E. canadensis*. EQY of *C. demersum* and *E. canadensis* depended on both light and temperature ($p<0.05$) and there was significant difference between the species ($p<0.05$).

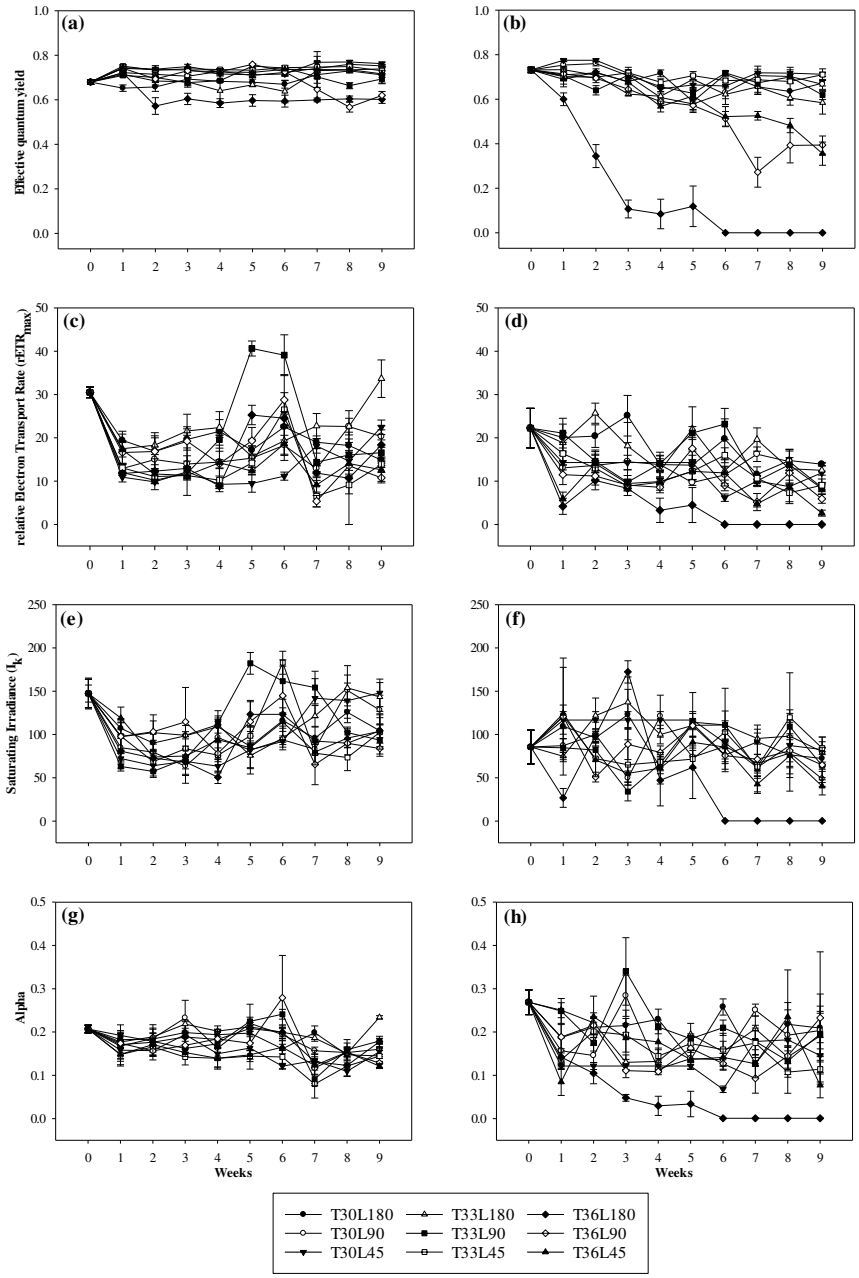

Figure 4. *Effective Quantum Yield (EQY; a,b), maximum relative Electron Transport Rate (rETRm; c,d), Saturating Irradiance (I_k; e,f) and Alpha (α; g,h) from Week 0 to Week 9 for C. demersum (a, c, d) and E. canadensis (b, d, f) in each treatment. Data are shown as Mean±SE*

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Maximum relative electron transport rate ($rETR_{max}$)

At week 0, $rETR_{max}$ of *C. demersum* and *E. canadensis* were 30.49±1.21 and 22.25±4.54 µmol electrons m^{-2} s^{-1}, respectively. $rETR_{max}$ of *C. demersum* did not significantly change from week 1 to week 9 (13.56 to 18.82 µmol electrons m^{-2} s^{-1}; *Fig. 4c*) while high $rETR_{max}$ was mostly found in T33L180 treatment. However, $rETR_{max}$ of *E. canadensis* slightly decreased from week 0 to week 9 and ranged between 8.07 to 15.22 µmol electrons m^{-2} s^{-1}(*Fig. 4d*), with high $rETR_{max}$ mostly found in T33L180 and T33L90 and low found in T36L180. At week 9, $rETR_{max}$ significantly differed by treatment in both species ($p<0.05$). $rETR_{max}$ of both species depended on light and temperature ($p<0.05$) and was significantly different by species ($p<0.05$).

Saturating irradiance (I_k)

At week 0, I_k of *C. demersum* and *E. canadensis* was 147.32±16.36 and 85.57±19.50 µmol photons m^{-2} s^{-1}, respectively. I_k in *C. demersum* significantly increased from week 2 to end of the experiment (*Fig. 4e*) continually. On the other hand, *E. canadensis* showed quite stable trend in the range from 59.70 to 96.18 µmol photons m^{-2} s^{-1} (*Fig. 4f*). I_k was not significantly different between treatments at the end of experiment for *C. demersum* ($p>0.05$) and there was no significant dependence on light or temperature ($p>0.05$). In contrast, *E. canadensis* showed significant differences between treatments at the end of experiment ($p<0.05$) and also by temperature ($p<0.05$), and there were significant differences by species ($p<0.05$). I_k for each treatment and species significantly differed by week ($p<0.05$). I_k of *C. demersum* showed acclimation in the last two weeks in 33 and 36˚C treatments, while *E. canadensis* showed acclimation in the last four weeks to 45 and 90 µmol photons m^{-2} s^{-1} treatments.

Alpha (α)

At week 0, α of *C. demersum* and *E. canadensis* was 0.21±0.01 and 0.27±0.03, respectively (*Figs. 4g and 4h*). At the end of experiment, there were significant differences in α by treatment for *C. demersum* ($p<0.05$) but not for *E. canadensis*. High α was mostly found in 180 and 90 µmol photons m^{-2} s^{-1} treatments for *C. demersum,* while *E. canadensis* showed various trends. It was found that only light affected α of *C. demersum* but *E. canadensis* was significantly affected by both light and temperature ($p<0.05$) and the difference between species was not significant ($p>0.05$).

Chlorophyll a and b concentrations

Chlorophyll a (Chl a)

Chl *a* concentration of *C. demersum* and *E. canadensis* at week 0 was 1,953.27±301.05 and 2,131.66±324.50 µg g^{-1} fw, respectively. Decreasing Chl *a* concentration with time was observed in both species and in all treatments (*Figs. 5a and 5b*). At week 8, Chl *a* concentration of both species was significantly different by treatment ($p<0.05$) and highest in T30L180 and T33L90 for *C. demersum* and *E. canadensis*. T30L180 gave the highest Chl *a* concentration for *C. demersum* while the lowest was observed with T33L180, T33L90 and T33L45 treatments of *E. canadensis*. It was found that Chl *a* significantly depended on temperature ($p<0.05$) and Chl *a* of *C. demersum* at 30˚C was higher than at other tested temperatures, while Chl *a* of *E. canadensis* in 36˚C treatment was lower than at other temperatures.

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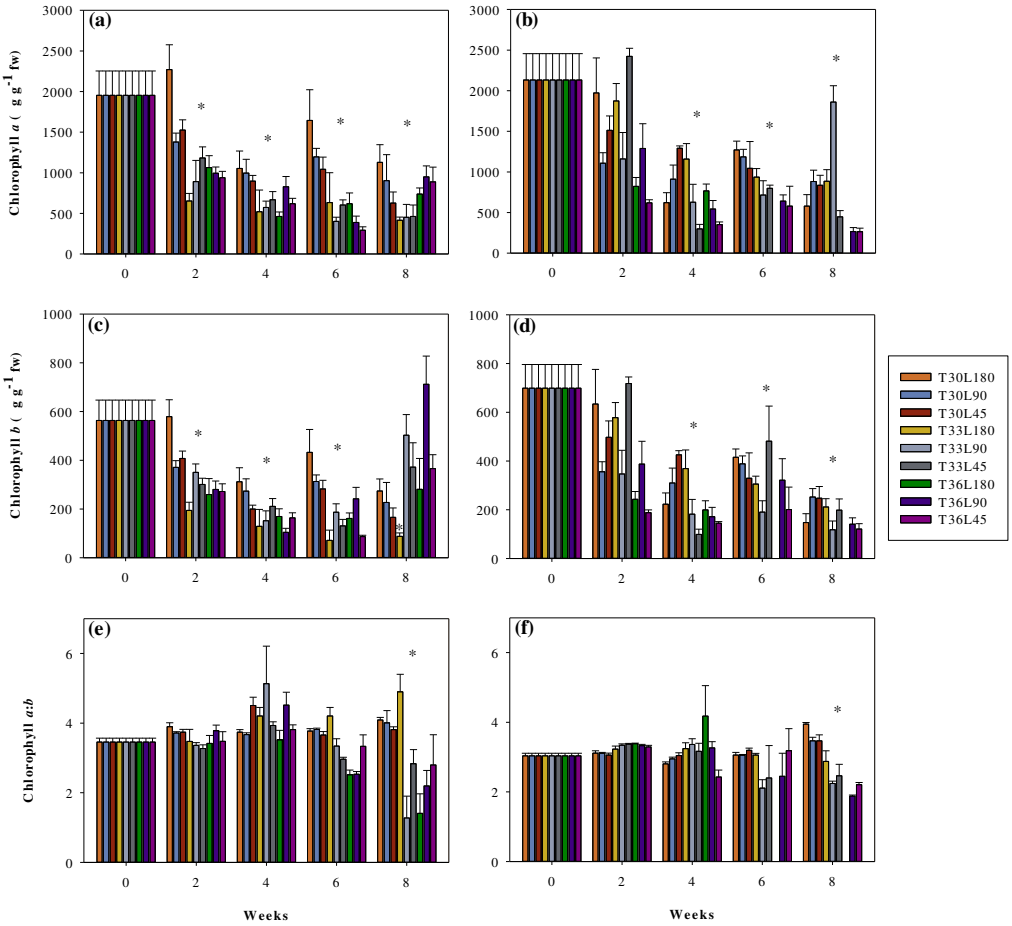

Figure 5. *Chlorophyll a* (*a,b*) *and b* (*c,d*) *concentrations and chlorophyll a*:*b ratio* (*e,f*) *at Week 0 and Week 9 for C. demersum* (*left*) *and E. canadensis* (*right*) *in each treatment. Data are shown as Mean±SE and* ******* *represent significant difference*

Chlorophyll b (Chl b)

Chl *b* concentration of *C. demersum* and *E. canadensis* at week 0 was 563.88±83.47 and 699.50±96.80 µg g^{-1} fw, respectively. Chl *b* of *C. demersum* decreased from week 2 to week 6, then increased at week 8 with an elevated temperature treatment (T33 or T36). Chl *b* of *E. canadensis* tended to dramatically decrease until the end of experiment (*Figs. 5c and 5d*). At week 8, Chl *b* was significantly different between the treatments ($p<0.05$) in both species being highest in T36L90 for *C. demersum* and in T33L90 for *E. canadensis*. Chl *b* of *C. demersum* did not significantly depend on temperature or light ($p>0.05$) but for *E. canadensis* it significantly depended on temperature ($p<0.05$) and was significantly different between the species ($p<0.05$).

Chlorophyll a:b ratio

Chl *a*:*b* ratio of *C. demersum* and *E. canadensis* at week 0 was 3.46±0.11 and 3.04±0.07, respectively. Response mostly showed in week 4 and was higher for *C. demersum*. 30˚C treatments showed more stable trends of Chl *a*:*b* ratio than the 33 and 36˚C treatments that gave slight decreases over time (*Figs. 5e and 5f*). There was a

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significant difference between the species and both species' responses significantly depended on temperature ($p<0.05$). At week 8, there were significant differences by treatment in both species ($p<0.05$).

Growth rates

Growth of *C. demersum* and *E. canadensis* was measured from week 0 to week 8. The highest growth rate of *C. demersum* and *E. canadensis* was in T30L180 treatment, namely 0.45±0.06 and 1.67±0.18 cm day^{-1}, respectively ($p<0.05$) (*Figs. 6a and 6b*); and the lowest was in T36L180 treatment at -0.04±0.01 and -0.05±0.00 cm day^{-1}, respectively ($p<0.05$). *E. canadensis* had significant faster growth than *C. demersum* in all treatments ($p<0.05$). Both species' growth significantly depended on temperature and light ($p<0.05$).

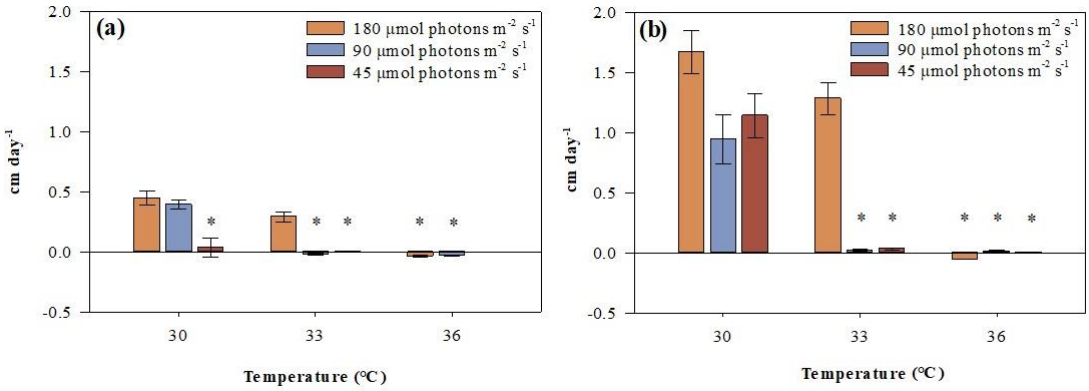

Figure 6. *Growth rate (cm day^{-1}) from Week 0 to Week 9 for C. demersum* (*a*) *and E. canadensis* (*b*) *in each treatment. Data are shown as Mean±SE and * represent significant difference*

Organic matter (OM) and organic carbon (OC) contents

OM of *C. demersum* and *E. canadensis* at week 0 was 89.58±1.24% and 89.94±0.92%, respectively. OM of *C. demersum* significantly decreased in week 8 in all treatments except for T33L180, but *E. canadensis* showed dramatic decrease from week 0 to week 8 (*Figs. 7a and 7b*). There was a significant difference between treatments, with OM of *C. demersum* the lowest in T36L90 and the highest in T30L180, while OM of *E. canadensis* was the lowest in T36L180 and the highest in T36L45 treatment. However, there was no significant difference between the species, but at week 8 the OM of both species significantly differed by treatment ($p<0.05$). Light and temperature did not significantly affect OM of *C. demersum* ($p>0.05$) but significantly affected OM of *E. canadensis* ($p<0.05$).

OC of *C. demersum* and *E. canadensis* at week 0 was 51.96±0.72% and 52.17±0.53%, respectively. OC of both species showed similar trends to OM (*Figs. 7c and 7d*) and there were significant differences by treatment. OC of *C. demersum* was the lowest in T36L90 and the highest in T30L180, while OC of *E. canadensis* was the lowest in T36L180 and the highest in T36L45 treatment. OC of both species significantly differed by treatment ($p<0.05$) at the end of experiment. Light and temperature did not significantly affect OC of *C. demersum* ($p>0.05$) but both significantly affected OC of *E. canadensis* ($p<0.05$).

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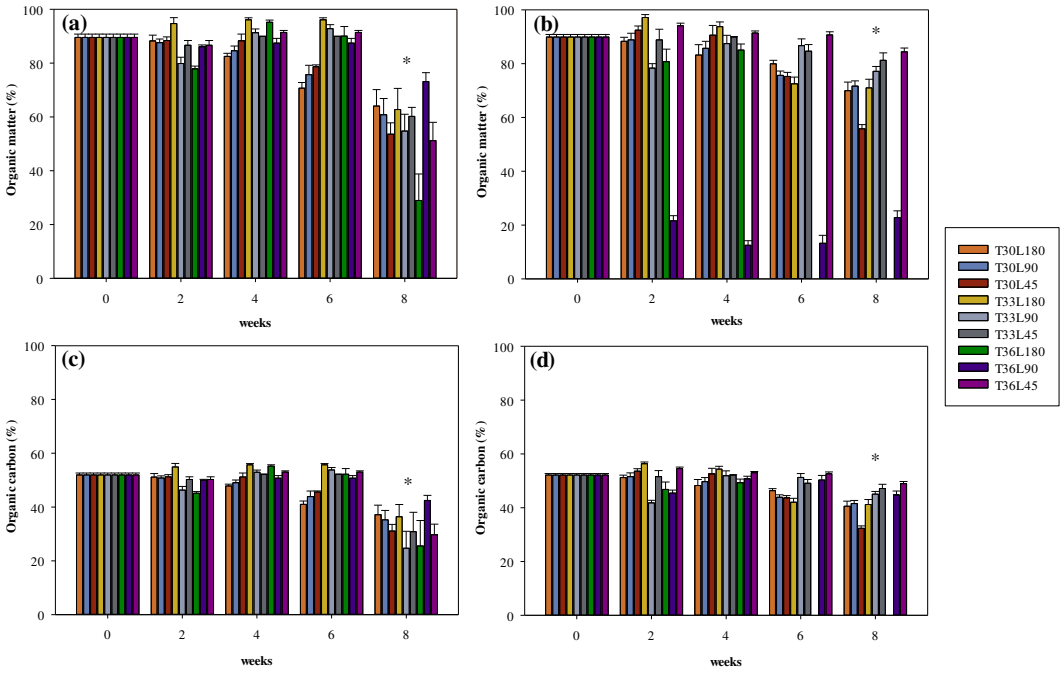

Figure 7. *Organic matter* (*a,b*) *and organic carbon contents* (*c,d*) *at Week 0 to Week 8 for C. demersum* (*a,c*) *and E. canadensis* (*b,d*) *in each treatment. Data are shown as Mean±SE and * represent significant difference*

Discussion

The combined effects of elevated temperature and low light were investigated in *C. demersum* and *E. Canadensis,* which are the dominant species in the middle of Songkhla lagoon, for 9 weeks. Photosynthetic performance of both species was affected by temperature and/or low light, as seen in the maximum quantum yield (MQY). Elevated temperature and ambient light (36˚C and 180 µmol photons m^{-2} s^{-1}) showed faster down-regulation of *C. demersum* photosynthesis at week 2, while elevated temperature with 50% shading (36˚C and 90 µmol photons m^{-2} s^{-1} treatment) caused down-regulation at weeks 2 and 7. On the other hand, other light shading at a lower temperature (30 or 33˚C) did not induce changes in MQY. This indicates that the temperature had stronger effects on photosynthesis performance of *C. demersum* than light shading. This result is consistent with Jiang et al. (2018) who found no difference in MQY of *C. demersum* between shade and no shade. On the other hand, *E. canadensis* was more sensitive to temperature and light stress, showing response to 36˚C treatment at weeks 1, 3 and 4 with 180, 90, and 45 µmol photons m^{-2} s^{-1}, respectively. This is consistent with Chalanika De Silva and Asaeda (2017) reporting that the PSII activity of *E. nuttallii* was significantly reduced under elevated temperatures. The present study showed that a combination of ambient light and elevated temperature induced the greatest impact in photosynthetic activities of both tested species, especially *E. canadensis*. Netten et al. (2013) showed that the effects of elevated temperature and low light on *E. canadensis* are most likely caused by increased metabolic activity and reduced photosynthesis, to respectively adjust photo-physiology and to maintain a net positive carbon balance (Chartrand et al., 2018).

Climate change scenario and human activities could change many environment factors (Gallardo et al., 2017) especially light and temperature, which are among the main factors

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for growth of aquatic plants (Carr et al., 1997). Thus, to maintain positive carbon balance and cope with light and temperature conditions, macrophytes had strategies, such as adjustments of light harvesting capacity and light use efficiency and modifying rates of growth (Chartrand et al., 2018). Moreover, light harvesting capacity (threshold) of each species can specify the species composition in the future, which faces climate and anthropogenic changes.

Light threshold for photosynthesis was shown by saturating irradiance (I_k). Our results indicate that *E. canadensis* was not affected by low light (high turbidity of water) and there was faster adaptation (I_k close to the light intensity) in the last 4 weeks in 90 and 45 µmol photons m^{-2} s^{-1} treatments, while *C. demersum* showed response in the last two weeks. *C. demersum* was more tolerant to ambient light (180 µmol photons m^{-2} s^{-1}), consistent with Jiang et al. (2018) stating that different light regimes did not show different photosynthetic activities in *C. demersum*; while the growth rate of *C. demersum* was lower in the light limited treatments than the rate of *E. canadensis* that was not affected.

E. canadensis was more sensitive to elevated temperature than *C. demersum* as shown by all indicators measured. *C. demersum* had more tolerance of a wide range of temperatures, and temperature did not much affect photosynthetic activities as *C. demersum* achieved homeostasis in photosynthesis and respiration rates, and the temperature optimum for photosynthesis changed according to its acclimation temperature (Hyldgaard et al., 2014). Elevated temperature, in contrast, did affect photosynthetic activities, chlorophyll concentration and carbon contents of *E. canadensis*.

Changes of environment by changing light intensity and temperature affect species composition (Li et al., 2017). Macrophytes capable of adaptation will survive and dominate in the lagoon. Decreasing light in a water column induced changes in photosynthetic activities, shown in the α for both tested species. Changes of α revealed the light responses clearly in *C. demersum*. Although *C. demersum* was able to adapt to a low light, due to the lower energy available the low light regime slowed down growth of *C. demersum*. *E. canadensis* showed various response in α, induced by combinations of light and temperature.

Only T33L90 treatment had increasing Chl *a* and *b,* revealing that only optimal temperature and light intensity allowed *E. canadensis* to thrive. Similar results were shown in *E. nuttallii* which Chl *a* and Chl *b* significantly increasing in a 30˚C heat shock treatment and decreasing in a 35˚C heat shock treatment (Chalanika De Silva and Asaeda, 2017). *C. demersum* showed increasing Chl *a* and *b* in T33L45, T36L90 and T36L45 to maintain photosynthetic activities. This reveals a wider range of temperature and light to which *C. demersum* can adapt, by more light harvesting in a low light regime, consistent with Dar et al. (2013) that found that the pigment in *C. demersum* was positively correlated with water temperature.

Macrophytes are fast growing plants that can on their death make a large amount of carbon settle to the bottom. Climate change and anthropogenic changes will affect growth (Zhang et al., 2018), species compositions (Li et al., 2017) and the carbon sink function as well. Carbon content of these 2 species did not differ, but only *E. canadensis* showed response to low light and elevated temperature that affected its photosynthetic activities. Degraded photosynthesis affected carbon capture by the macrophyte and reduced organic matter and organic carbon. Thus, reducing a macrophyte's carbon capture might reduce carbon sink efficiency in a lagoon, and might affect other species around the macrophyte.

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Mal et al. (2002) found that *E. canadensis* growth (in control) was about 1.4 cm per day. Comparing *C. demersum* and *E. canadensis* for growth in similar conditions, Pinowska (2002) found that *E. canadensis* (final weight increased by about 17% of initial fresh weight) grew faster than *C. demersum* (final weight increased by about 7% of initial fresh weight) which is consistent with this current study.

Growth rates showed that 30˚C was a near optimal temperature for growth of both species. At this temperature, *E. canadensis* had 2-3 times higher growth rates than *C. demersum* because the habitats of these species differ in position in the water column. *C. demersum* is a free-floating aquatic plant (Huxley et al., 1992) mostly found at the water surface where it can get full force of the sunlight, while *E. canadensis* is a submergent macrophyte with roots growing in the muddy bottom (Huxley et al., 1992) where it could be shaded by other plants and/or sediments. Thus, fast expansion is a strategy to get more sunlight. Previous research on competition of *E. nuttallii* with *Myriophyllum verticillatum*, *Vallisneria natans* and *C. demersum* found that *E. nuttallii* grew the fastest and its expansion ability was the strongest in autumn-winter and spring, but degenerated in the summer (Duan et al., 2011). In terms of species composition, there are spatial and temporal dynamics in species composition among these 2 species, dependent on environmental factors. However, climate change and anthropogenic activities that change the environment will affect species compositions in the future.

Anthropogenic activities that induce eutrophication (Nwankwegu et al., 2019) and algal blooms or high turbidity, and decrease light penetration in a water column, might affect growth of *C. demersum*. The IPCC Representative Concentration Pathways (RCPs) 4.5 and 8.5 scenarios predict that average temperatures would increase by about 1 to 3˚C in 2020-2050 (Science Framework Climate Working Group, 2016) and this might affect photosynthesis and carbon content of *E. Canadensis,* which can grow in narrow light intensity and temperature ranges.

Low light and elevated temperature will change species composition so that the scale of *E. canadensis* will decrease at an elevated temperature, while *C. demersum* might not decrease in scale but in growth. However, since *C. demersum* is a free-floating macrophyte on water surface, eutrophication might affect the light harvesting by *C. demersum* very little. Thus, in species composition *C. demersum* might increase and dominate in the lagoon. Due to different points in the water column for these 2 macrophytes species, the loss of *E. canadensis* might affect middle - bottom water column microhabitat and related ecosystem.

C. demersum can improve water quality, specifically turbidity and nutrients, but its overgrowth will have disadvantages, such as increased sediment accumulation, reduced light penetration, and dissolved oxygen. Dai et al. (2012) found that water quality measures, such as turbidity, chlorophyll *a,* and nutrient concentrations, improved significantly in the presence of *C. demersum,* but there was a negative correlation between these reductions and the coverage of *C. demersum*. Therefore, 20% is probably the optimal restoration coverage area for *C. demersum* in the lagoon.

These results indicate that *C. demersum* prefers ambient light intensity (180 and 90 μmol photons m^{-2} s^{-1}) while *E. canadensis* prefers ambient light with a comparatively low (30˚C) temperature. This study provided data related to species composition in a lagoon, associated to scenarios of climate change and expected anthropogenic activities in the future.

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Conclusions

To understand responses of macrophytes to climate change (RCP4.5 and RCP8.5) and to anthropogenic stresses, in terms of photosynthesis and carbon content, two dominant species from the middle of Songkhla lagoon in Thailand were subjected to experimental treatments in the lagoon. We found that photosynthetic activity indicators EQY, α and $rETR_{max}$ in both species were induced by light. Then, pigmentation in both species depended also on temperature, while the carbon content of *C. demersum* was unaffected by temperature or light, and *E. canadensis* was affected by both. *C. demersum* was more tolerant to temperature and light stresses but had slower growth of the two species. As regards species composition, the limiting factor for *C. demersum* vegetation is light shading, while for *E. canadensis* elevated temperature may be limiting. This study should study more about biochemical and physiological stress responses such as protein content, catalase activity, lipid peroxidation, and cellular membrane permeability as well as *in situ* measurement to confirm the results of this study.

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EFFECT OF *IN SITU* EXPERIMENTAL SHADING ON THE PHOTOSYNTHESIS OF CANADIAN WATERWEED (*ELODEA CANADENSIS*) FROM SONGKHLA LAGOON, THAILAND

CHOTIKARN, P.[1,2,3] – KAEWCHANA, P.[2,3] – PRATHEP, A.[4] – ROEKNGANDEE, P.[2,3] – SINUTOK, S.[2,3*]

[1]*Marine and Coastal Resources Institute, Faculty of Environmental Management, Prince of Songkla University, Hat Yai, Thailand*

[2]*Faculty of Environmental Management, Prince of Songkla University, Hat Yai, Thailand*

[3]*Coastal Oceanography and Climate Change Research Center, Prince of Songkla University, Hat Yai, Thailand*

[4]*Seaweed and Seagrass Research Unit, Excellence Center for Biodiversity of Peninsular Thailand, Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkhla, Thailand*

[*]*Corresponding author*
e-mail: ssutinee@gmail.com

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Abstract. Macrophytes play an important role in providing habitat structure, nutrient cycling and improvement of water quality in freshwater ecosystems. However, increasing human population and the industrial revolution during past several centuries have increased the utilization of natural resources and have caused strong changes in the structure and function of the environment, such as high sedimentation that decreases light penetration. This study investigated the effects of *in situ* experimental shading on photosynthetic performance of submerged *Elodea canadensis* macrophytes and identified the level of light that is critical for growth and photosynthesis of this species. Photosynthetic performance, chlorophyll *a* and *b* concentrations, organic and carbon contents, percentage cover, and morphology were estimated in *E. canadensis* from middle of Songkhla lagoon under 4 treatments (25, 50, 75, and 100% of natural light) for 10 weeks. The results show that there were no differences in growth and photosynthesis among the treatments, but low light led to changes in chlorophyll concentration, F_v/F_m, and I_k, suggesting adaptations to a low light regime. This study provides an understanding of physiological tolerance and response to shading and shows how species of aquatic macrophytes respond to future climatic and anthropogenic changes, thereby supporting development of sustainable lagoon management plans.
Keywords: *low light, submerged macrophyte, PAM fluorometry, ecophysiology, carbon content*

Introduction

Increasing human population and the industrial revolution during the past several centuries have led to increased utilization of natural resources of both aquatic and terrestrial types. Anthropogenic activities have direct and indirect effects on ecosystems, such as land transformation and altering hydrological processes. Agriculture causes soil erosion and large inputs of N and P into water bodies that can lead to eutrophication in a lake. Eutrophication is a condition of increased nutrient supply caused by human activities and/or natural processes, as lakes age and get filled with sediments, and this may become a major problem for aquatic systems globally (Wurtsbaugh et al., 2019). Sediment runoff and eutrophication have been the main causes of water quality management problems in lakes globally, including the Songkhla Lagoon in Southern part of Thailand (Sompongchaiyakul et al., 2004; Sompongchaiyakul and Sirinawin, 2007;

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Chesoh and Lim, 2008). Nutrient enrichment induces excessive growth of algae and aquatic plants and results in the disturbance of ecosystem functions, such as insufficient oxygen concentration for aquatic life, and a reduction in light penetration to the lake bottom (Wurtsbaugh et al., 2019). Sediment loads reduce light penetration and affect photosynthetic organisms.

Macrophytes play an important role in maintaining high physical and biological diversity and act as ecosystem engineers that provide nutrient cycling, habitat structure and refugia for aquatic organisms (Wigand et al., 2000; Qiu et al., 2001; Cronk and Fennessy, 2016). Macrophytes are potential biological tools to improve water quality in lakes and reservoirs due to their abilities in nutrient uptake, preventing phytoplankton blooms (Lone et al., 2014; Song et al., 2019) by producing allelochemicals to inhibit phytoplankton and epiphytic growth (Mohamed, 2017), and could prevent eutrophication in a lake.

E. canadensis is a submergent macrophyte with roots growing in mud at the bottom of the water (Huxley et al., 1992). This species originates from North America and has been introduced to Europe and nowadays *E. canadensis* are common in many water bodies and are invasive species in some areas. There are many factors that affect growth and photosynthesis of macrophytes, and consequently their species composition, such as temperature, light intensity, nutrients, and pH (Netten et al., 2013), and responses to these depend on the macrophyte species. Netten et al. (2013) showed that high levels of NH_x and temperature together with low pH and low light cause the strongest toxic effects to relative growth rate and leaf tissue mortality. Moreover, high temperature and low light had negative effects on *E. canadensis* via increased metabolic activity and reduced photosynthesis, respectively. Shading experiments were performed by Ellawala Kankanamge et al. (2019) revealing that *E. canadensis* at week 4 increased biomass under all shading conditions (35%, 63%, 79%, 90%, and 95%) except 35%, then after 8 weeks, *E. canadensis* had the highest biomass at lowest shade level.

Songkhla Lagoon is a tropical estuarine lagoon system located on the eastern side of the southern peninsular Thailand and consists of four interconnected water bodies: Thale Noi, upper, middle, and lower parts (Pongpiachan et al., 2019). Songkhla Lagoon not only supports biodiversity, but also a large number of people whose livelihoods depend on that biodiversity. Over 1.4 million people are living in Songkhla Lagoon Basin area (Community Development Department, 2009). *E. canadensis* is one of the dominant species in Songkhla Lagoon (Thongkao et al., 2001). Nowadays, Songkhla Lagoon is facing low water quality and sediment overloading due to human activities (Pradit et al., 2010; Somboonsuke et al., 2018). This could lead to loss of valuable ecosystem services and functions.

This study investigated the effects of *in situ* experimental shading (low light) on growth and photosynthesis of submerged *E. canadensis* macrophytes. An understanding of response to different light regimes is necessary for identifying which level of light is critical for growth, photosynthesis, and carbon capture potential of submerged macrophytes and for predicting their performance under shading. Furthermore, the findings could give a better understanding regarding how *E. canadensis* will respond to anthropogenic changes in light, and support the development of sustainable lagoon management plans.

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Materials and Methods

Study site and experimental design

This study was carried out from October to January 2018 (10 weeks) in the middle of Songkhla Lagoon (7° 28' 09.0'' N, 100° 23' 45.0" E), Thailand to investigate the effects of low light on growth and photosynthesis *in situ*. Shading net (XH-SNN, Hebei Tuosite Plastic Net) were used to manipulate light conditions to 25%, 50%, 75%, and 100% (no shade, control) of the natural irradiance in the experiment plots (n=3), which were randomly placed within a macrophytes patch. The experiment plots consist of shading net with PVC frame (1 m x 1 m) and 4 PVC poles (1.5 m height above ground) (*Fig. 1*). The experiment plots height was deployed at the median of tide level to stimulate shading at water surface. Light can penetrate through shading net approximately 25%, 50%, and 75% as experimental design and shading net was not change the wavelength composition (Kotilainen et al., 2018). Photosynthetic activity (maxiumum quantum yield (F_v/F_m), effective quantum yield ($\Delta F/F_{m'}$), relative Maximum Electron Transport Rate ($rETR_{max}$), initial slope (alpha, α), saturating irradiance (I_k)), pigment concentration, morphology, percentage cover, and organic matter and carbon contents of the macrophytes were estimated biweekly from the start to the end of the experiment.

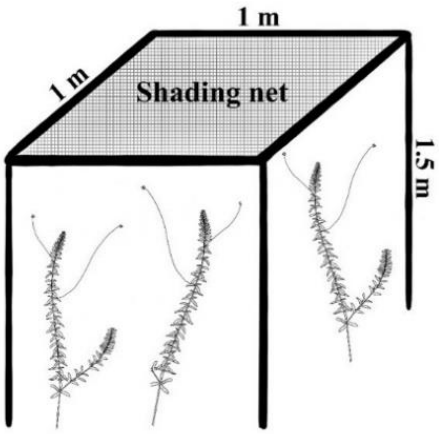

Figure 1. *The experiment plots*

Photosynthetic activity

Photosynthetic activities were observed biweekly by estimating chlorophyll *a* fluorescence using a Pulse Amplitude Modulated (Diving-PAM) fluorometer (Walz, Germany). Dark-adapted photosystem II (PSII) photochemical efficiency was measured as maximum quantum yield (F_v/F_m) after using dark-adapted leaf clip for 15 min (n=3). Rapid Light Curves (RLCs) were performed with 9 increasing actinic light intensities (0, 66, 90, 125, 190, 285, 420, 625 and 920 μmol photons m^{-2} s^{-1}), with 0.8 s saturating pulse (> 4500 μmol photons m^{-2} s^{-1}) between each actinic light intensity every 10 s. Effective quantum yield ($\Delta F/F_{m'}$), maximum relative electron transport rate ($rETR_{max}$), minimum saturating irradiance (I_k) and initial slope (alpha, α) of RLCs were calculated using the curve fitting protocols following Ralph and Gademann (2005).

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Chlorophyll a and b concentrations

Photosynthetic pigment concentrations (chlorophylls Chl *a* and Chl *b*) were determined using the standard spectrophotometric method of Ritchie (2006). Chl *a* and *b* ($\mu g\ g^{-1}$ fw) were extracted by homogenizing samples in 4 ml of 90% acetone at 4°C for 24 h then centrifuging at 1500 g for 10 min; and the supernatant was placed into a quartz cuvette in a spectrophotometer (Metertech, SP8001, 190-1100 nm), with absorbance measured at 647, 664 and 750 nm.

Organic matter (OM) and organic carbon (OC)

20 g *E. canadensis* samples for each time were collected and oven dried at 105°C and ground to particle sizes less than 1 mm. A 1.0 g subsample of a ground sample was ashed in a muffle furnace (FHX, DAIHAN, China) at 550°C for 8 h (Armecin and Gabon, 2008). Organic matters of macrophytes were determined using data obtained from the ashed samples, and mineral matter (MM; *Eq.1*), organic matter (OM; *Eq.2*) and organic carbon (OC; *Eq.3*) contents were computed using the following equations:

$$\%\mathrm{MM} = \left(\frac{\mathrm{AW}}{\mathrm{DW}}\right) \mathrm{x}\ 100 \qquad \text{(Eq.1)}$$

$$\%\mathrm{OM} = 100\ \mathrm{x}\ (\mathrm{DW} - \mathrm{AW})/\mathrm{DW} \qquad \text{(Eq.2)}$$

$$\%\mathrm{OC} = \%\mathrm{OM}/1.724 \qquad \text{(Eq.3)}$$

where AW and DW are ash weight of the sample and dry weight of the sample, respectively (Armecin and Gabon, 2008).

Statistical analyses

Two-way mixed ANOVA tests were used to test for significant differences among treatments over time in chlorophyll fluorescence parameters, pigment content, organic matter and carbon content, and percentage cover of *E. canadensis*. One-way ANOVA was used to test for significant differences among treatments in chlorophyll fluorescence parameters, pigment content, organic matter and carbon content, and percentage cover of *E. canadensis* at the end of experiment. All tests employed a significance level of 95%, and Tukey's honestly significant difference *post hoc* tests were used to confirm the statistical significances. If data did not meet the assumptions of normality (Kolmogorov-Smirnov test) and equal variance (Levene's test), they were transformed using square root or $\log_{10}$. If transformed data did not meet the assumptions, non-parametric tests were used.

Results

Macrophyte percentage cover

In the middle of Songkhla lagoon *E. canadensis* was the dominant species with 56.67±15.90%, 56.67±16.67%, 56.67±12.02%, and 53.33±18.56% (*Fig. 2a*), followed by *Cladophora* sp. with 28.33±15.90%, 40.00±15.28%, 43.33±12.02%, and 36.67±17.64% (*Fig. 2b*) percentage cover at the start of experiment (October) in control, 25%, 50%, and 75% treatments, respectively. There were significant increases with time ($p<0.05$) but no

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differences between the treatments (p>0.05). Percentage cover of *E. canadensis* decreased at week 2 in all treatments except for the 75% treatment, and tended to increase through time in all treatments (*Fig. 2*). At the end of the experiment, percentage cover of *E. canadensis* showed no differences among the treatments (p>0.05).

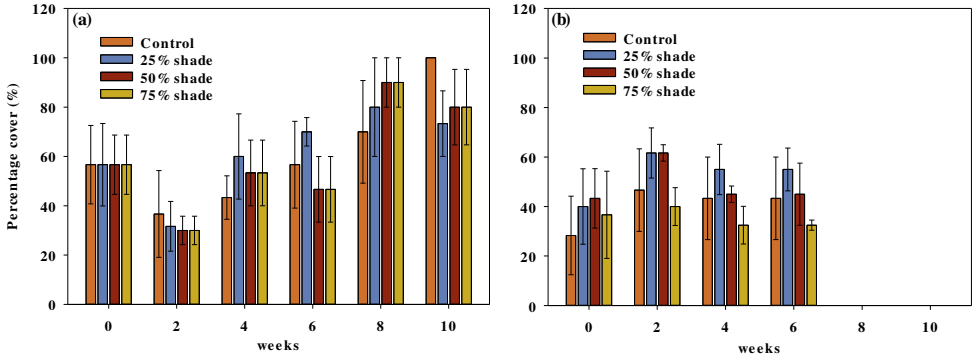

Figure 2. *Percentage cover of E. canadensis at Songkhla Lagoon from week 0 to week 10 for each treatment. Data are given as Mean±SE*

Morphology

Leaf length of *E. canadensis* at the start of the experiment was 0.78±0.07 cm. After 10 weeks, leaf lengths in control, 25%, 50%, and 75% were in the ranges 1.12±0.04, 0.96±0.03, 0.94±0.08, and 1.05±0.05 cm, respectively (*Fig. 3a*). Leaf width of *E. canadensis* at the start of experiment was 0.20±0.01 cm then after 10 weeks, leaf widths in control, 25%, 50%, and 75% were in the ranges 0.20±0.01, 0.19±0.01, 0.20±0.01 and 0.20±0.02 cm, respectively (*Fig. 3b*). At the end of the experiment, leaf length and width were not significantly different between the treatments (p>0.05).

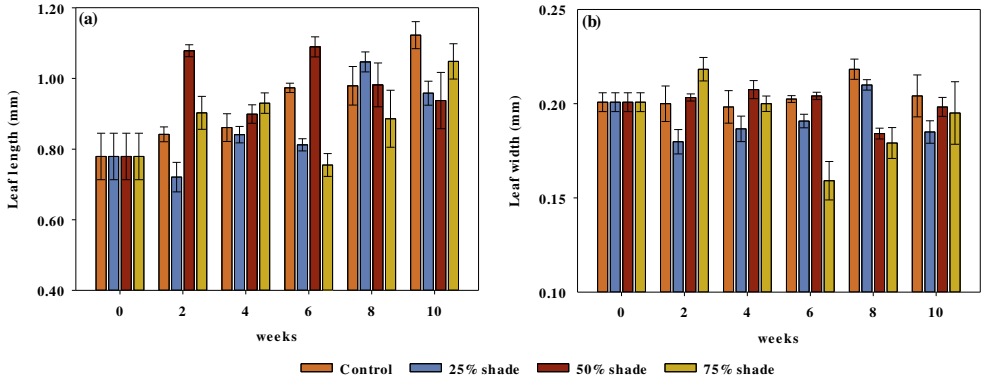

Figure 3. *Leaf length (a) and width (b) of E. canadensis at Songkhla Lagoon from week 0 to week 10 in each treatment. Data are given as Mean±SE*

Photosynthetic activities

Maximum quantum yield (F_v/F_m) and effective quantum yield ($\Delta F/F_m'$)

At the start of experiment, F_v/F_m of *E. canadensis* was 0.76±0.02. There was a significant increase through time in all treatments (p<0.05; *Fig. 4*). At the end of

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experiment (week 10), F_v/F_m of *E. canadensis* was not significantly different ($p>0.05$) among the treatments.

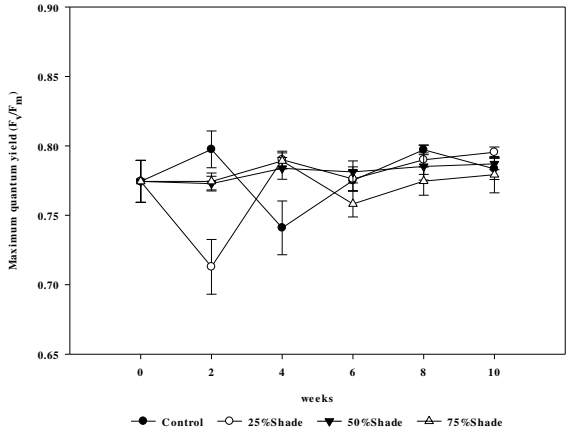

Figure 4. *F_v/F_m of E. canadensis at Songkhla Lagoon from week 0 to week 10 in each treatment. Data are given as Mean±SE*

At the start of experiment, $\Delta F/F_{m'}$ of *E. canadensis* was 0.74±0.01. There was significant increase through time in all treatments ($p<0.05$; *Fig. 5a*). At the end of experiment (week 10), $\Delta F/F_{m'}$ of *E. canadensis* was not significantly different ($p>0.05$) among the treatments.

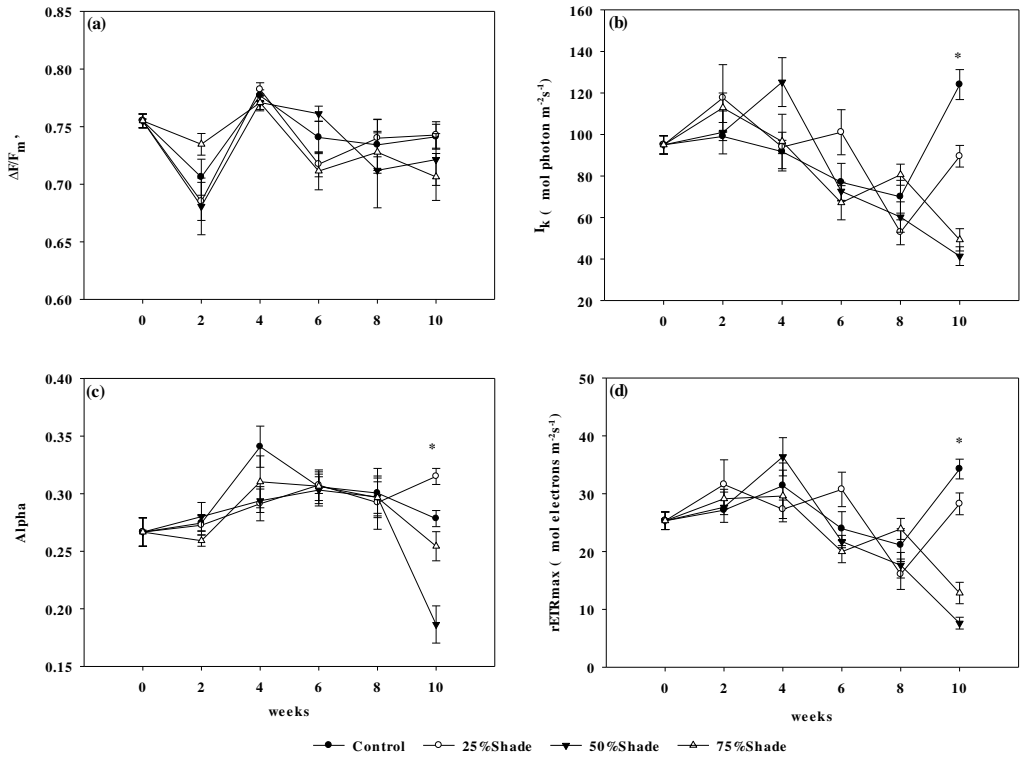

Figure 5. *$\Delta F/F_{m'}$(a), I_k (b), Alpha (c) and $rETR_{max}$ (d) of E. canadensis at Songkhla Lagoon from week 0 to week 10 in each treatment. Data are given as Mean±SE and * represents significant difference*

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Rapid light curves (RLCs)

Rapid light curves (RLCs) with 25%, 50% and 75% shading changed through duration of the experiment. At the end of the experiment (week 10), RLCs of 50 and 75% shading treatments were significantly lower than those of control and 25% treatments (*Fig. 6*).

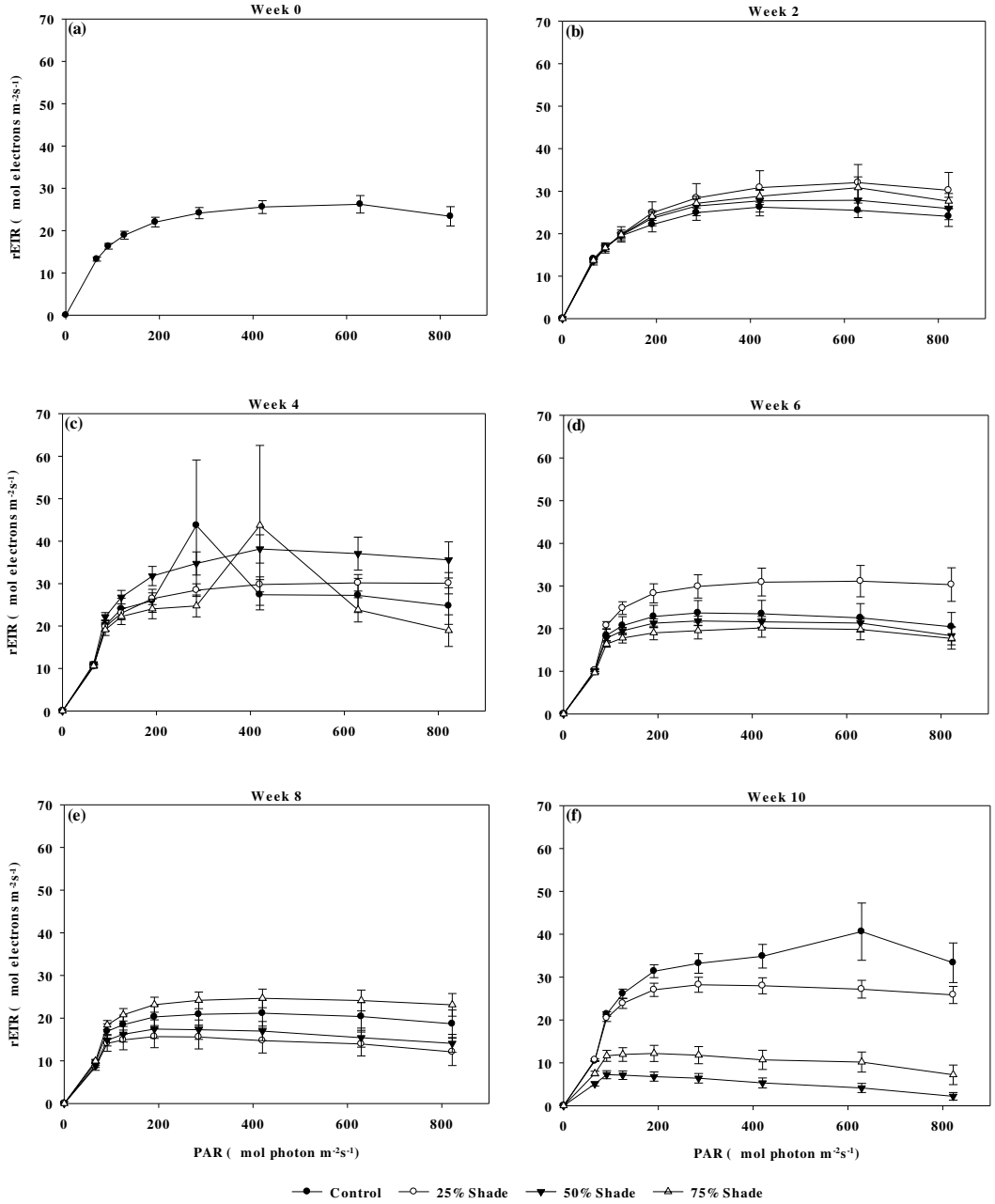

Figure 6. *rETR vs PAR of E. canadensis at Songkhla Lagoon from week 0 to week 10 (a,b,c,d, and f) in each treatment. Data are given as Mean±SE*

Saturating irradiance (I_k)

At the start of experiment, I_k of *E. canadensis* was 95.02±4.39 µmol photons m^{-2} s^{-1}. There was no significant difference among treatments or by time ($p>0.05$). At the end of

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experiment (week 10), I_k of *E. canadensis* was significantly different between the treatments ($p<0.05$). I_k at 50% treatment was significantly lower than those for 75%, 25%, and control, respectively (*Figure 5b*).

Initial slope (Alpha, α)

At the start of experiment, alpha of *E. canadensis* was 0.27±0.01. Increasing of alpha only occurred in 25% treatment. There was significant difference by time in all treatments ($p<0.05$; *Fig. 5c*). At the end of experiment (week 10), alpha of *E. canadensis* was different among the treatments ($p<0.05$) with 50% treatment significantly the lowest while the control had similar alpha as the 25% and 75% treatments.

Maximum relative electron transport rate ($rETR_{max}$)

At the start of experiment, $rETR_{max}$ of *E. canadensis* was 25.34±1.53 µmol electrons m^{-2} s^{-1}. $rETR_{max}$ of *E. canadensis* showed similar trend as the I_k (*Fig. 5d*). There was significant difference between treatments and by time ($p<0.05$). At the end of experiment (week 10), $rETR_{max}$ was different among the treatments ($p<0.05$). $rETR_{max}$ of 50% and 75% treatments were significantly lower than those of control and 25% treatments.

Chlorophyll a and b (Chl a and b) and chlorophyll a:b ratio (Chl a:b)

At the start of the experiment, Chl *a* and Chl *b* in control of *E. canadensis* were in the ranges 571.65±95.82 and 188.33±22.61 µg g^{-1} fresh weight (fw), respectively. Chl *a* and Chl *b* in all treatments tended to increase with time, except for the control treatment which decreased at week 10. The final Chl *a* and *b* concentrations in 25%, 50%, and 75% treatments were about 2.5 to 3-fold those from the start of experiment, in the ranges 1842.21±77.09, 1684.60±106.92, 1433.17±256.52, 734.85±102.05, 583.38±42.22, and 474.34±93.92 µg g^{-1} fw, respectively (*Fig. 7a,b*). There were significant changes with time ($p<0.05$). At week 10, there was a significant difference in Chl *a* and Chl *b* among the treatments ($p<0.05$) with 25% having the highest Chl *a* and *b* concentrations and control treatment the lowest concentrations.

At the start of experiment, Chl *a:b* ratio of *E. canadensis* was in the range 3.00±0.17. There was significant difference by time ($p<0.05$) but no differences among the treatments, even on week 10 ($p>0.05$).

Organic matter (OM) and organic carbon (OC)

At the start of experiment, OM of *E. canadensis* was in the range 91.37±0.79%. OM of *E. canadensis* was not significantly different among the treatments at week 10 ($p>0.05$) with control, 25%, 50% and 75% treatments in the ranges 82.37±0.27%, 86.68±1.02%, 80.84±2.65%, and 85.70±3.60%, respectively (*Fig. 8a*). However, there were significant decreases in OM with time ($p<0.05$).

OC of *E. canadensis* was in the range 53.00±0.46% at the start of experiment. OC showed similar trend as OM. The OC of *E. canadensis* was not significantly different among the treatments at week 10 ($p>0.05$) and the control, 25%, 50% and 75% treatments were in the ranges 47.78±0.15%, 50.28±0.59%, 46.89±1.54%, and 49.71±2.09%, respectively (*Fig. 8b*). However, there were significant decreases with time ($p<0.05$).

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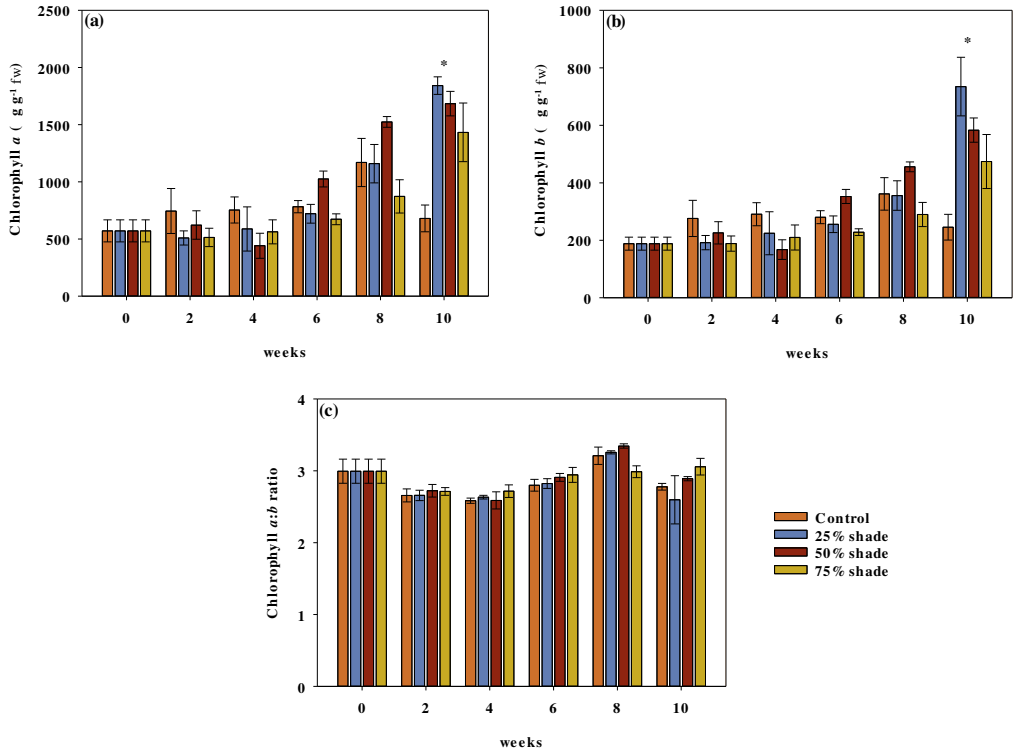

Figure 7. *Chlorophyll a (a) and b (b) concentrations and Chlorophyll a:b (c) ratio of E. canadensis at Songkhla Lagoon from week 0 to week 10 in each treatment. Data are given as Mean±SE and * represent significant difference*

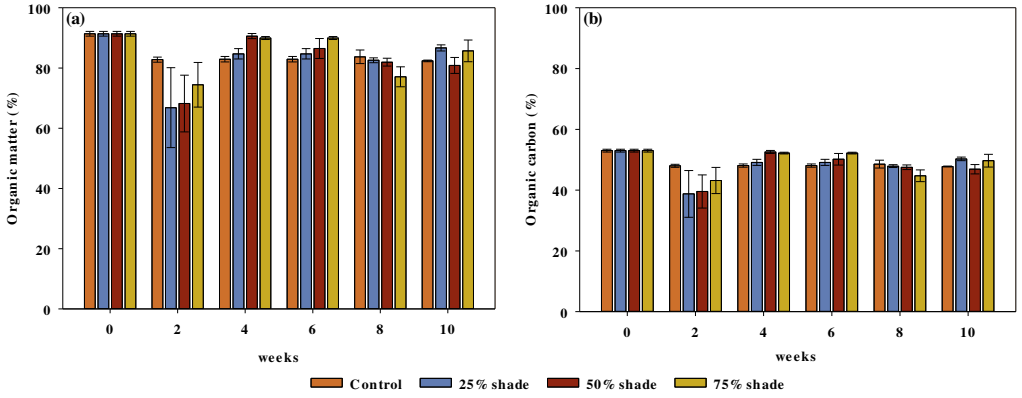

Figure 8. *Organic Matter (OM) (a) and Organic Carbon (OC) (b) of E. canadensis at Songkhla Lake from week 0 to week 10 in each treatment. Data are given as Mean±SE*

Discussion

Shading experiments (Control (100%), 25%, 50%, and 75% of ambient light) were set *in situ* to estimate effects of low light on photosynthetic activities and growth of *E. canadensis* in Songkhla lagoon, Thailand. This study found that the photosynthetic efficiency of *E. canadensis* significantly changed during the experimental period. Decrease of Effective Quantum Yield ($\Delta F/F_{m'}$) occurred on week 2 due to light reduction,

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which affected light harvesting. After week 2, *E. canadensis* showed adaptive capacity by increasing Chl *a* and *b* in order to maintain photosynthesis and a net positive carbon balance (Chartrand et al., 2018). Increasing chlorophyll led to improved efficiency in light harvesting to get more light in low light situation, which showed in increasing Maximum Quantum Yield (F_v/F_m) after week 2.

Saturating irradiant (I_k) revealed adaptation of *E. canadensis* to cope with light situation in this study. Light reduction led to decreased I_k (Ralph and Gademann, 2005) especially in 50% and 75% treatments in the final week, which was consistent with Chen et al. (2016) where *Potamogeton maackianus* and *Vallisneria natans* under low light (2.8%, 7.1%, 17.1%, and 39.5% ambient light) increased their initial slopes of RLCs (α) and decreased their minimum saturating irradiances (I_k). On the other hand, F_v/F_m increased revealing that *E. canadensis* was able to maintain its photosynthesis. The percentage cover of *E. canadensis* increased through duration of the experiment, which is consistent with F_v/F_m and chlorophyll concentrations, hence, no significant change in morphology of *E. canadensis* was seen in the experiment.

There are many factors that can negatively affect *E. Canadensis,* such as nutrient enrichment, low pH, elevated temperature, and low light (Netten et al., 2013) in combination, but low light by itself did not show clear effects on *E. canadensis* in this study. Different light intensities from shading did not affect photosynthesis and growth of *E. canadensis* due to its ability to adapt to a low light situation by chlorophyll adjustment. *E. canadensis* is a submergent macrophyte with roots in the mud at the bottom of the water (Huxley et al., 1992) where it could be shaded by other floating plants or sediment. Further, Ellawala Kankanamge et al. (2019) revealed that *Elodea* species are adapted to relatively low light conditions by gradual increasing in total chlorophyll content and act as pioneer macrophytes in eutrophic freshwater habitats in the transition from the phytoplankton-dominated to the macrophyte-dominated state, and they can tolerate moderate shading by periphyton and other submerged macrophytes.

Changes of environment as regards light intensity and temperature affect species composition (Li et al., 2017) especially in combination with other environmental factors. However, changes in light only did not affect population of this species in this study. At the end of the experiment, percentage cover by *E. canadensis* was not significantly different among the treatments, which is consistent with the photosynthetic activities, pigment content, and organic content that are factors supporting macrophyte growth (Kirschbaum, 2011) and percentage cover (Feng et al., 2008). Moreover, morphology (leaf length and width) of *E. canadensis* did not change by time or by treatment. However, it is suggested that the shoot length increased due to shading.

There are advantages that *E. canadensis* possesses in terms of materials cycling and energy flow and including phytoplankton and epiphytic growth inhibition with allelochemicals (Mohamed, 2017) that could prevent eutrophication in a lake. Exhibiting high growth rates with a high tolerance to wide ranges of environmental conditions, low vulnerability to grazing and other stress factors, high distribution, and reproduction potential of *E. canadensis* (Zehnsdorf et al., 2015) make it an invasive species that dominates in many lakes. This study found that the photosynthesis and growth of *E. canadensis* in Songkhla lagoon are affected by low light regime for a short period (2 weeks), while in the long run *E. canadensis* adapted by chlorophyll concentration adjustment to cope with a low light regime.

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Conclusions

E. canadensis was the dominant species and continuously increased during the experiment but there were no differences by shading treatment, while the leaf length and width did not show clear patterns in response to a low light. Photosynthetic activities showed changes due to shading in some parameters such as I_k and $rETR_{max}$ in 50% and 75% shading treatments, but there were no changes in photosynthetic efficiency of this species. This study suggests that this species is able to cope well with shading. Under future anthropogenic changes in turbidity, this species could be able to grow well and might flourish under eutrophic conditions (e.g. high nutrient loading), which might be a problem for Songkhla lagoon management. Therefore, management of this species should take an integrated approach recognizing these benefits and disadvantages. Future study should estimate about shading on other species for prediction the species composition of Songkhla lagoon which face to eutrophication in the future.

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